The Handbook of Environmental Chemistry 91
Series Editors: Damià Barceló · Andrey G. Kostianoy

Célia M. Manaia
Erica Donner
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Antibiotic Resistance in the Environment

A Worldwide Overview

Springer

The Handbook of Environmental Chemistry

Volume 91

Founding Editor: Otto Hutzinger

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Antibiotic Resistance in the Environment

A Worldwide Overview

Volume Editors: Célia M. Manaia · Erica Donner · Ivone Vaz-Moreira · Peiying Hong

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Series Preface

With remarkable vision, Prof. Otto Hutzinger initiated *The Handbook of Environmental Chemistry* in 1980 and became the founding Editor-in-Chief. At that time, environmental chemistry was an emerging field, aiming at a complete description of the Earth's environment, encompassing the physical, chemical, biological, and geological transformations of chemical substances occurring on a local as well as a global scale. Environmental chemistry was intended to provide an account of the impact of man's activities on the natural environment by describing observed changes.

While a considerable amount of knowledge has been accumulated over the last four decades, as reflected in the more than 150 volumes of *The Handbook of Environmental Chemistry*, there are still many scientific and policy challenges ahead due to the complexity and interdisciplinary nature of the field. The series will therefore continue to provide compilations of current knowledge. Contributions are written by leading experts with practical experience in their fields. *The Handbook of Environmental Chemistry* grows with the increases in our scientific understanding, and provides a valuable source not only for scientists but also for environmental managers and decision-makers. Today, the series covers a broad range of environmental topics from a chemical perspective, including methodological advances in environmental analytical chemistry.

In recent years, there has been a growing tendency to include subject matter of societal relevance in the broad view of environmental chemistry. Topics include life cycle analysis, environmental management, sustainable development, and socio-economic, legal and even political problems, among others. While these topics are of great importance for the development and acceptance of *The Handbook of Environmental Chemistry*, the publisher and Editors-in-Chief have decided to keep the handbook essentially a source of information on "hard sciences" with a particular emphasis on chemistry, but also covering biology, geology, hydrology and engineering as applied to environmental sciences.

The volumes of the series are written at an advanced level, addressing the needs of both researchers and graduate students, as well as of people outside the field of

"pure" chemistry, including those in industry, business, government, research establishments, and public interest groups. It would be very satisfying to see these volumes used as a basis for graduate courses in environmental chemistry. With its high standards of scientific quality and clarity, *The Handbook of Environmental Chemistry* provides a solid basis from which scientists can share their knowledge on the different aspects of environmental problems, presenting a wide spectrum of viewpoints and approaches.

The Handbook of Environmental Chemistry is available both in print and online via www.springerlink.com/content/110354/. Articles are published online as soon as they have been approved for publication. Authors, Volume Editors and Editors-in-Chief are rewarded by the broad acceptance of *The Handbook of Environmental Chemistry* by the scientific community, from whom suggestions for new topics to the Editors-in-Chief are always very welcome.

Damià Barceló
Andrey G. Kostianoy
Series Editors

Preface

Antibiotic resistance is a natural phenomenon that predates the human use of antibiotics. The emergence and acquisition of novel resistance genes and combinations of genes are driven by the natural process of microbial adaptation. Yet, decades of rampant use of antibiotics in human medicine and agricultural production systems have greatly increased the rates at which bacteria develop and acquire resistance. Artificially increased concentrations of antibiotics in humans, animals, and environments have enhanced horizontal gene transfer and led to the rapid evolution and spread of multidrug-resistant bacteria, including a growing number of multidrug-resistant pathogens. The World Health Organization has declared antibiotic resistance to be one of the greatest threats to global health, food security, and development. Economists have estimated that by 2050, antimicrobial-resistant infections could place a cumulative US$100 trillion of economic output at risk. It is thus crucial to mitigate the escalating spread and detrimental impacts arising from antibiotic resistance. Worldwide, experts agree that this highly complex, multifaceted problem requires a holistic, multi-sectoral "One Health" approach to management.

It is important to recognize that antimicrobial resistance does not only spread and have impacts within healthcare facilities such as hospitals where antibiotics are commonly prescribed. Problematic resistant bacteria and emerging antibiotic resistance genes are also widespread in other environments, from wastewater to food products and wildlife. This widespread occurrence stems from the multiple use of antibiotics, including in agriculture as growth promoters or for preventative medicine. These activities have resulted in the dissemination of (sub)therapeutic levels of antibiotics and their metabolites into the environment, where animal wastes may have also an important impact. Pharmaceutical companies produce large quantities of antibiotics to meet the still-growing demand. Many of these facilities are in countries with suboptimal waste management, and even in the most developed settings, it is apparent that standard wastewater treatment processes are not optimized to remove many contaminants of emerging concern, including antibiotics and antibiotic resistance genes.

The above scenarios describe just some of the potential routes by which emerging antibiotic resistance genes are disseminated to local environments. Mass gatherings and travel also contribute to the local and international dissemination of antibiotic-resistant bacteria. Although it is a global problem, the worldwide distribution of antibiotic resistance genes and impacts is asymmetrical. The most dramatic and concerning scenarios are observed in regions with poor or nonexistent sanitation, poverty, and weakened health systems or low healthcare expenditure. Nevertheless, bacteria do not recognize borders, and antibiotic-resistant bacteria are self-replicative biological contaminants that can rapidly be transported over long distances by humans, wildlife, and other transport vectors. Initial steps taken to understand antibiotic resistance biogeography have revealed the complex interplay of socioeconomic, climatic, and cultural factors that shape this stealthy but rapid wave of biological contamination. It is this understanding which provided the motivation for this book and our invitation for experts from a broad range of specialties and geographic regions to document and share the current state of knowledge about antibiotic resistance genes in the environment, the challenges this presents, and the measures we can take to mitigate this.

In our conversations with experts, most agreed that efficient wastewater collection and treatment systems are one of the most important control strategies for mitigating the environmental release and impact of anthropogenic use and production of antibiotics. This is especially evident in the context of developing countries and in those that practice water reclamation for irrigation and other purposes. Without adequate collection and treatment of industrial, animal, and human wastes, we are allowing the gradual release and accumulation of antibiotic-resistant bacteria and genes into water bodies, sediments, and soil, which may subsequently act as environmental reservoirs of antibiotic resistance.

This book collates a range of chapters detailing current knowledge on the topics above. First, we hear from leading researchers on their perspective of what needs to be done to tackle the environmental dimensions of antibiotic resistance. This is followed by chapters describing key routes by which antibiotic resistance is disseminated into the environment, both at the local and global scale. Control strategies to minimize antibiotic resistance dissemination are also documented in several chapters. We hope this book will provide a useful introduction and compendium for readers wishing to know more about antibiotic resistance in the environment and inspiration as to how we can act effectively across sectors and states to combat the still-growing threat of antibiotic resistance.

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Antibiotic Resistance in the Environment: *Expert Perspectives*

Célia M. Manaia, David Graham, Edward Topp, José Luis Martinez, Peter Collignon, and William H. Gaze

Contents

Abstract Antibiotic resistance is considered by different international organisations (e.g. World Health Organization, WHO; Food and Agriculture Organization of the United Nations, FAO-UN; Organisation for Economic Co-operation and Development, OECD) as not only a major threat to human life and wellbeing but also having tremendous economic impacts. Recent estimates indicate that globally at least 700,000 deaths per year are due to drug-resistant infections, with the largest and most important proportion of these attributable to antibiotic-resistant bacterial infections – and which are most often identified in hospitals. However, there are reasons to believe that antibiotic-resistant bacteria are common in the community,

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where they are acquired from other people, animals, foods, water and/or other environmental sources.

Over recent decades, the importance of the environment in the propagation and dissemination of antibiotic-resistant bacteria has been better evidenced, with human and animal sewage representing the most important emission nodes in a complex network of transmission routes. While the relevance of environmental sources and paths of transmission are nowadays considered pivotal in any One Health discussion about antibiotic resistance, some key topics are still under debate in the scientific community.

In this chapter, experts recognised in the field were invited to give their perspective on some commonly debated topics related to the risks and control of antibiotic resistance. Specifically, five invited experts gave their perspective on the relevance and control of the environmental dimensions of antibiotic resistance, based on six distinct thematic axes – transmission, critical control points, antibiotic-selective effects, interventions needed, authority's awareness and engagement and priorities for action.

Keywords Antibiotic-selective effects, Authority's awareness and engagement, Critical control points, Interventions needed, Priorities for action, Transmission

1 Introduction

The global dissemination of antibiotic resistance is an emblematic example of the impacts of human activities on microbial ecology and evolution, witnessed by healthcare and science professionals over the last eight decades. This is an interesting and scientifically challenging research problem, in need of much more investigation across sectoral scientific community, contributing to tackling this serious public health threat. More research is needed to elucidate the drivers and mechanisms behind the success of multidrug-resistant bacterial pathogens capable of endangering human lives and modern medicine. But any effective control measures will require interventions from multiple societal sectors. The natural, farmed and domestic environments are all now recognised as reservoirs of antibiotic resistance and locations for resistance evolution, with subsequent direct or indirect transmission to humans [1, 2]. This perspective meets the One Health concept, which recommends that collaborative efforts of clinical, veterinary and environmental science work locally, nationally and globally to attain optimal health for people, domestic animals, wildlife, plants and the environment [3].

Control of antibiotic resistance requires integrated interventions. The environment is likely to be a critical control point as both a receptor and then a source of transmission to humans. This perspective of the role of the environment has now been evidenced in key international reports and action plans (e.g. [1–4]). In general, these documents serve as orientation instruments used to implement measures that combat selection and dissemination of antibiotic resistance. However, actions based on these documents may be not easily implemented. Intervention and mitigation are difficult to design and implement due to the complexity of the processes underpinning antibiotic resistance and uncertainty about the costs and benefits of potential strategies.

To provide an expert perspective on specific aspects of the environmental dimension of antibiotic resistance, five experts, with distinct scientific backgrounds and research interests, although sharing an interest in the environmental dimension of antibiotic resistance, were invited to share their opinions based on their experience in the field by addressing six questions:

1. Transmission
   Antibiotic resistance is a serious human health threat. How important do you think the environmental dimension of antibiotic resistance is compared with other sources and transmission routes (e.g. person to person)?
2. Critical control points
   Are there key environments and/or critical control points between different environments that you think scientists and policy makers should be focusing more attention on? Why?
3. Antibiotic selection
   In terms of environmental contaminants, how important do you think antibiotics themselves are in driving the selection and spread of antibiotic resistance?
4. Interventions
   What interventions do you think are most likely to have a long-term positive benefit on lowering antibiotic resistance loads and risks in non-clinical environments?
5. Authorities
   What are the most important things you think government authorities, policy makers and regulators need to know about the environmental dimension of antibiotic resistance?
6. Priorities
   Given limited resources, which environments would you prioritise for standardised environmental antibiotic resistance surveillance, and what approach would you suggest?

2 Expert Perspectives

David W. Graham

David is a professor of ecosystems engineering at Newcastle University, performing research that straddles engineering, ecology, molecular microbiology and public health. He initially studied relationships between agrochemical use and water, soil and food quality, including AMR evolution and fate. He now studies root causes of globally increasing AMR, with projects in Ethiopia, India, Bangladesh and Malaysia and across Europe. His group specialises in sociotechnical AMR solutions, including sustainable wastewater treatment options for the developing world. Graham has advised the UK and US governments on waterborne AMR mitigation, working now with the WHO and other international agencies on identifying 'best buy' sanitation solutions to reduce AMR spread.

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1. Transmission

   The answer to this question is a function of context. The environmental dimension of AMR is most important to community health in places with inadequate waste management and poor water quality, especially places without at least improved safe sanitation. In more developed parts of the world, the influence of environmental factors is less direct and probably less important, although the environment still must be considered in developing holistic AMR mitigation solutions.

   Antibiotic resistance genes and bacteria spread in faecal matter (both human and animal); hence, exposure to faeces without adequate processing poses a transmission risk. Therefore, the more barriers you can place between faecal releases and the next exposure the better. Improving sanitation behaviour and wastewater management is universally critical, while the 'best buy' solutions vary from place to place, depending on the existing local water and wastewater infrastructure. In places with limited infrastructure, a safe toilet with a platform

barrier will reduce transmission; whereas, in other places, advanced wastewater treatment may be the best buy option (e.g. arid regions).

It should be noted that more nebulous environment pathways can be important, even in places with developed infrastructure, such as transmission in wildlife and through international travel. However, relative threats are less acute than in places without wastewater infrastructure because exposures are lower and more diffuse.

2. Critical control points

The key points of exposure are where less or untreated wastes come in direct contact with other people or animals. Therefore, the first critical control point is at the very beginning of the process, specifically reducing open defecation or exposure to raw sewage. If we can reduce open defecation on a global scale, the level of antibiotic resistance spread via environment pathways should drop dramatically. As such, policy should target reducing open defection globally by ensuring improved safe sanitation everywhere. How this is done will depend on existing sanitation infrastructure in each place; nevertheless, the key is reducing open defecation and providing barriers between people and faeces itself or sewage.

In places where sewers exist, the first critical control point is where wastewater treatment is provided. Policy should promote secondary treatment at a minimum. However, the optimal level and type of 'treatment' depends on existing infrastructure and available resources in that place. Therefore, policy should aim towards improving waste management in a locally sustainable manner (not by setting dogmatic standards). For example, improvements in sanitation capacity should match and incrementally improve existing infrastructure rather than mandating specific technologies or scales of treatment that will not be affordable in most places. Waste management solutions must fit the place and be progressively be more ambitious as infrastructure improves. Again, human use behaviour needs to be considered in the planning process.

The final point of exposure is via contaminated food, which is common in places where untreated or less-treated wastewater is used for irrigation. In many parts of the world, irrigation water is critical to the food supply; therefore, guidance is needed in wastewater reuse for irrigation to reduce food exposure to inadequately pretreated water. Israel and California are examples of places where barrier systems exist; they may serve as policy models for other parts of the world where treated wastewater is essential in food crop irrigation.

3. Antibiotic selection

Antibiotics at all concentrations can potentially select for resistance bacteria and genes; however, relative exposure concentrations and times are critical to whether they are important in the environment or not. In places with effective wastewater management, concentrations tend to very low and are probably less important to antibiotic resistance spread and selection. In contrast, in places without adequate treatment, especially related to pharmaceutical industry wastes, this may not be true.

Much recent research results imply that low-level antibiotics in the environment are less important to antibiotic resistance selection than other factors, such as faecal matter itself containing resistance genes and bacteria. This does not mean reducing antibiotic use and releases to the environment is not a key goal. However, in most of the world, antibiotics themselves do not strongly select for antibiotic resistance over shorter timeframes, especially compared to resistance enrichment due to inadequately treated faecal waste releases.

The exception is in places where pharmaceutical wastes are not or less treated. Strong evidence exist that such sources can hugely impact local-scale antibiotic resistance, because antibiotic concentrations are often very high, which is much different than in the effluents and vicinity of domestic wastewater treatment plants. Some minimal selection can occur in such places, but this selection appears minor compared with the influence of other factors, such as environmental spread via nebulous factors like travel and other migration.

4. Interventions
   Improved waste management must be provided whenever antibiotics are used (for whatever purpose), including all domestic, agricultural and industrial systems. In some places, 'improved' means providing and promoting the use of toilets. In other places, 'improved' might mean reducing antibiotic use and refining the quality of wastewater treatment, such as in agriculture. The same is true of domestic and industrial sources. Provision of treatment for industrial wastewater is overlooked sometimes, but it is critical because industrial wastes often can contain other pollutants, such as metals, which can influence antibiotic resistance selection or enrichment. Further, industrial operations often are international and include supply chains. One might mandate effective treatment along whole supply chains for an industry to obtain a safe seal of approval relative to reducing antibiotic resistance-related pollutants.
5. Authorities
   Two key things come to mind related to authorities. First, reducing antibiotic use alone will not solve the antibiotic resistance problem. The overuse and misuse of antibiotics are clearly driving the accelerated evolution of resistance, especially newly acquired resistance in human and veterinary medicine. However, the international spread of resistance is largely due to inadequate waste management in much of the world and poor water quality. Therefore, all AMR National Action Plans must include more prudent and targeted antibiotic use, while improving waste management and water quality mandates.

   Secondly, antibiotic resistance is a global problem that will be hugely expensive to fix. However, the greatest increases and problems with antibiotic resistance are in the poorest countries; therefore, a moral obligation exists for all governments to assist in solutions across world. As such, global community investment is needed to solve the problem, which is in everyone's best interest. Recent work in the Arctic has shown antibiotic resistance is global and knows no boundaries.

6. Priorities

   Surveillance should focus on critical sources of possible antibiotic resistance, such as raw faecal matter, community sewage, industrial waste sources, wastewater from agricultural operations, hospitals and effluents from whatever wastewater processing systems exist. This knowledge is key for all locations because it is often unclear what the main sources of resistance to the environment are in most of the world. We know dominant sources vary dramatically from place to place (e.g. human, agricultural, industrial, etc.), which is critical because each place will then have its own specific 'best' solutions based on local drivers. Researchers often make a priori assumptions about what is most important, which often results in inappropriate interventions because they do not fit the main local sources.

   Within this context, surveillance needs to be guided internationally, but informed and implemented locally. This means education is critical for sustainability. It is important that capacity for surveillance be developed locally; therefore, initial surveillance should target simple metrics (e.g. resistant coliforms), initially using standard culturing methods and 'simple' genetic techniques, such as targeted qPCR. As local capacity builds, progressively more expansive surveillance can follow with an emphasis on local education and training of standard methods.

Ed Topp

Ed is a principal research scientist with Agriculture and Agri-Food Canada in London Ontario. Ed obtained his PhD from the Department of Microbiology at the University of Minnesota in 1988. Ed's research concerns the interface between agriculture and human and environmental health. In the last decade, he has led several projects concerning the fate and management in agroecosystems of pharmaceuticals and pathogenic and antibiotic-resistant bacteria. He is the project coordinator for the Genomics Research and Development Initiative project on antimicrobial resistance, a key component of the innovation pillar of the Canadian Federal Framework for action on antimicrobial resistance. https://www.researchgate.net/profile/Edward_Topp.

Scopus ID #7006149139.

1. Transmission
   The relative importance of one to another can't be generalised. In some instances, notably in areas that have poor or no infrastructure for water sanitation and hygiene [WASH], waterborne transmission will be very important.
2. Critical Control Points
   Clearly reducing emissions of human or animal faecal waste streams into the environment needs to be an area of focus. These can contain antibiotic-resistant bacteria, antibiotic residues and other potential co-selective agents. Investments in WASH infrastructure in lower-income settings should be a top priority.
3. Antibiotic selection
   Potentially very important but more experimental data on the relationship between environmentally relevant concentrations and resistance selection [i.e. NOEL, no-observed-effect level] in terrestrial and aquatic systems is required to substantiate antibiotic PNECs (predicted no-effect concentration) and derived minimum selective concentrations.
4. Interventions
   Investments in WASH infrastructure in lower-income settings should be a top priority. In the agricultural realm, technologies and practices need to be deployed in commercial agriculture and aquaculture that will permit significant reduction in antimicrobial use. This will reduce the development and transmission of resistance to humans via both the environment and food products.
5. Authorities
   Non-technical stakeholders in antimicrobial resistance need to understand the One Health concept. They need to understand what a threat antimicrobial resistance is with respect to morbidity, mortality and healthcare costs. They need to understand the global dimension of the problem.
6. Priorities
   This is still at a 'definition of problem' stage at least in high-income settings, and we arguably don't have an adequate understanding of the cost-benefits of deploying conventional or molecular standardised surveillance. Gathering more data on aquatic systems in low-income settings may be helpful in building a compelling case for investments in WASH.

José Luis Martinez

José Luis is a research professor at the National Biotechnology Center, CSIC, in Madrid, Spain. He is currently the head of the Department of Microbial Biotechnology at CNB. He obtained his PhD in Biochemistry and Molecular Biology, by the Universidad Autonoma de Madrid. In the year 2015, Dr. Martínez received the Lilly Foundation distinguished career award. His major research topics/contributions in the field of antibiotic resistance include the study of basic mechanisms underlying the evolution and ecology of antibiotic resistance as well as the integration of the networks regulating resistance and virulence into the microbial metabolism.

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1. Transmission

   There are two aspects to be considered here; one is the emergence of resistance and the other is transmission. Antibiotic resistance can be acquired either by mutation or by the incorporation of antibiotic resistance genes in the genomes of bacterial pathogens. Antibiotic-resistant mutants in human pathogens are mainly selected along therapy, within the human host. However, it is generally accepted that antibiotic resistance genes are originated in environmental organisms. Consequently, the first step in the acquisition of antibiotic resistance genes by a bacterial pathogen occurs in natural (non-clinical) ecosystems. Concerning transmission, two aspects have to be considered. First is the One Health aspect; One Health applied to antibiotic resistance deals with the relevance of interconnected habitats in the spread of resistance. Here, while human-to-human contact stands as a relevant cause of epidemics, other elements have to be taken into consideration, and the relevance of each of these habitats depends on socioeconomic issues. For instance, the routes of dissemination of the NDM-1 beta-lactamase depend on water use; in some countries, water is a relevant transmission route,

whereas in others the main transmission occurs through human-to-human contacts. Contacts with animals-foods (and in general goods) are also a way of transmission of antibiotic resistance. Here it is important to distinguish antibiotic-resistant bacteria able of colonising/infecting both animals and humans, which can be selected as the consequence of the use of antibiotics in farming, from the contamination by bacteria that have a human origin, as the consequence of food manipulation. Global health concerns the dissemination of antibiotics at a global worldwide scale. For this transmission to happen, vectors are required, since several habitats are not geographically interconnected. Travellers are among these vectors but also trade of goods and animals, including migrating birds that have shown to be involved in the worldwide dissemination of resistance.

2. Critical Control Points
   The key points are those where human bacterial pathogens are found. Water is a relevant habitat because it is suitable for geographical transmission of pollutants (as antibiotics or antibiotic-resistant bacteria) and is the recipient of stools, containing antibiotic-resistant human pathogens and eventually antibiotics that can select for them. Potable water, but also water reutilization, as well as land application and release of sewage effluents are elements with relevance in the dissemination of resistance. As above stated, global (worldwide) transmission of antibiotic resistance requires transmission vectors. Among them, international trade of goods (including animals and food) can be involved in antibiotic resistance spread. There are already regulations to impede the entrance of products containing infective agents. Even more, when one country presents some endemic infections (as *Xylella fastidiosa* in the case of plants or African swine virus in the case of animals), this country is not allowed to export the plants/animals that can be eventually infected. Similarly, rules can be implemented to avoid the interchange of goods contaminated with antibiotic-resistant bacteria with relevance in human and animal health. Another key environment is that of farms (including fisheries), because a large percentage of antibiotics are currently used in animal production. Here, a key issue is the knowledge on the bacterial clones that can colonise/infect both humans and animals as well as the mobile genetic elements that can move among human adapted and animal adapted clones. The control here includes the development of specific tools for the fast detection of these risky bacterial clones/mobile genetic elements.
3. Antibiotic selection
   The first step in the acquisition of an antibiotic resistance gene by a bacterial pathogen from an environmental donor requires that both organisms are present in the same environment, most likely a non-clinical environment, and for this acquisition to occur, selection is needed. To note here that in some of the few cases in which the origin of an antibiotic resistance gene, now present in human pathogens, has been tracked, the original hosts of these genes were water-dwelling bacteria. This suggests that the use of antibiotics in fish farming might be an important force selecting the emergence of antibiotic resistance. Concerning transmission and selection of already resistant clones, the role of antibiotics as selectors is important mainly where they are more abundant: clinical

settings and farms. Nevertheless, a correlation between antibiotics concentrations and abundance of antibiotics resistance genes has been found. Although this correlation might be the consequence of the fact that human/animal residues may contain both antibiotic-resistant bacteria and antibiotics, which are released together in natural ecosystems via stools and urine, it also may support that antibiotics can be selectors in the natural ecosystems where they are released. The fact that antibiotic resistance can be selected even when antibiotics are present at subinhibitory concentrations (the most common situation in natural ecosystems) further supports that the presence of antibiotics in these ecosystems may help in the enrichment and spread of antibiotic resistance.

4. Interventions
   Main interventions have been made in terms of selection, reducing the amount of antibiotics in human therapy and in animal production. For this, nonantibiotic approaches to counteract infectious diseases are of utmost relevance. Among them, vaccination (humans and animals) and novel systems of management in animal production are relevant. In addition, sanitation in this case specifically targeting antibiotic-resistant bacteria and antibiotics should be the most efficient way of reducing the load of antibiotic resistance elements in natural ecosystems. Efforts as the 'Reinvent the toilet challenge' raised by the Bill and Melinda Gates Foundation (https://docs.gatesfoundation.org/Documents/Fact_Sheet_Reinvent_the_Toilet_Challenge.pdf) and aiming to 'bring sustainable sanitation solutions to the 2.5 billion people worldwide who don't have access to safe, affordable sanitation' may help in reducing the disposal in water bodies of non-treated stools containing antibiotic-resistant bacteria, particularly in low-medium-income countries.
5. Authorities
   There are two categories of elements involved in the dissemination of resistance where authorities may play a role. One is formed by the biological aspect of the process, which includes identification of the routes, the vectors, the bacteria, the mobile elements and the genes with relevance in the antibiotic resistance problem. Here authorities should be involved in studies aiming to define these elements by implementing the required epidemiological analysis. The other category is formed by the socioeconomic and cultural factors that impact antibiotic resistance. In addition, quantitative models analysing the specific contribution of each of these elements as well as the economic consequences of the actions to be taken for reducing antibiotic resistance burden are needed. Here, multidisciplinary approaches, including the biological, medical and ecological aspects together with socioeconomic and political issues, are needed in order to implement global plans for tackling antibiotic resistance. One example of this approach is the UK programme for fighting antibiotic resistance (https://www.gov.uk/government/publications/uk-5-year-action-plan-for-antimicrobial-resistance-2019-to-2024), which is based on a comprehensive analysis of the elements involved in the dissemination or resistance (https://amr-review.org) and the consequences of the actions for fighting such resistance.

6. Priorities
   Two actions can be foreseen: at the local level, water and manure because these are the environments where antibiotic-resistant bacteria relevant for human and animal health are released and; at international level, controls in the entrance of goods, similar to those already implemented for tracking presence of infectious agents, but in this case specifically focusing on antibiotic resistance. The methods must be fast, robust and cheap. The most suitable will be simple, well-implemented methodologies for detecting sentinel antibiotic-resistant clones or antibiotic-resistant genes. There are two aspects that still need to be defined: which sentinel genes must be chosen and the level of detection of these genes that will be considered as safe.

Peter Collignon

Peter is an infectious disease physician and clinical microbiologist at the Canberra Hospital. He is also a professor at the Australian National University Medical School.

Particular interests are antibiotic resistance, infection control and healthcare-acquired infections.

He is the patron of the Australasian College for Infection Prevention and Control. He is member of many national and international committees, including as an expert to the World Health Organization (WHO) on the issue of antibiotic resistance and the use of antibiotics in food animals. In 2009 he was made a member of the Order of Australia (AM) for services to medicine in infectious diseases, microbiology and infection control.

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1. Transmission
   It is very difficult to quantify what is the component that the environment contributes to antibiotic resistance levels seen, compared to other sources and transmission routes for antibiotic resistance (e.g. person to person directly). However I think it makes a considerable but underappreciated contribution. Its contribution will also vary in different regions of the world.

Some bacteria, such as the pneumococcus and gonococcus, will be almost entirely spread by person-to-person contact, and so any resistance in these bacteria will not be associated with the broader environment. However, other bacteria such as *Escherichia coli* (which are the commonest bacteria causing infections in people) is likely around the world to be predominantly not spread directly from person to person, but via the environment, and especially by contaminated water. This will also be the main way it gets from animals to people, as well as animals to animals and even people to animals.

2. Critical Control Points
   The key control points in the environment are where human and animal wastes are produced and then where this waste might enter waterways. We need to ensure that we minimise this risk of contamination occurring and so take appropriate steps such as having very good facilities in place to stop inadequately treated waste from entering the broader environment and also make water 'clean' before it enters waterways.
3. Antibiotic Selection
   Antibiotics themselves do help in the selection and spread of antibiotic resistance. This is because low levels of antibiotics will select out and then help these bacteria that are already carrying resistance genes to those antibiotics proliferate. Low levels of antibiotics in the environment may also facilitate the spread and interchange of plasmids and resistance genes between bacteria.
4. Interventions
   The most likely interventions with long-term benefits that will lower resistance loads are any procedures that target the key control points where human and animal wastes are produced and then where this waste might enter waterways. Local measures to contain waste and have it handled and stored safely are essential. For people, adequate and safe water and well-functioning sewage systems, treatment, etc. must be put in place. This means also doing all we can to stop animal as well as not only human waste but also animal waste from reaching waterways, particularly if it is untreated.
5. Authorities
   I think the most important thing is for government and policy makers to be aware that antibiotic resistance is a big issue also in the environment. They need to be aware that resistant bacteria carried by people and animals enter waterways and this helps spreads, i.e. 'contagion'. Therefore, we need policies that minimise risk from the environment. Both in homes and healthcare facilities, adequate physical structures need to be in place that minimise cross-contamination via people's hands after they touch contaminated surfaces. Even more emphasis needs to be given to ensure the water ingested by people is as clean/safe as and drug free as practical and to be as drug free (including form antibiotic residues). This principle needs be extended also to what happens with animals and for whenever what water is sprayed on crops; particularly for plants that those may be ingested uncooked.

   This is an important issue with the globalisation of food. Food may come to other countries but after it is produced and/or packaged in countries with much

poorer water management and water quality. I think it is important that we have good water quality measures and enforcement in place everywhere. Additionally however we need to ensure that any 'safe food production' rules or legislation in one country by having better water and sanitation regulations is not bypassed by importing products where such more stringent criteria are not enforced or followed.

6. Priorities
   In regions with limited resources, the waterways are the 'environments' where I think surveillance may be most important. I think the best bacteria to look at for resistance is *Escherichia coli*. A full range of known antibiotic resistance should be looked at. In addition I think we need some testing of drugs that might be present. This will include antibiotics, not only what is in waterways but also in the water that is ingested by people. Water that is sprayed on crops should also be tested for resistant bacteria and for drugs it might contain.

William Gaze

Will is professor at the European Centre for Environment and Human Health, University of Exeter Medical School. Will has >15 years of research experience of AMR research in natural environments. Current activity within his group (~25 researchers, >£4 mn in current and recent AMR funding) covers fundamental issues of AMR evolution in the environment using in situ and in vivo experiments, landscape scale dissemination of AMR and human exposure and transmission studies. He has been invited to speak on AMR on five continents in the last 2 years and has advised UK and overseas governments, UNEP/WHO, European Environment Agency, UK Environment Agency and Defra. His major research topics/contributions are in the field of antibiotic resistance evolution, ecology and transmission in natural and farmed environments. Selection for AMR in complex microbial communities or microbiomes is another major interest in the field.

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1. Transmission
   The environmental resistome is the source of all mobile resistance genes that emerge in clinical pathogens and that subsequently spread from person to person. So the fundamental importance of the environment is not in doubt. What is less certain is the risk of 'acute' transmission from environmental exposure, and this is likely to vary by environment, human exposure type, country and level and type of pollution. Even if environmental transmission is relatively rare, it is likely that this phenomenon facilitates emergence of the most important resistance mechanisms in epidemic human-associated and hospital-acquired strains.
2. Critical Control Points
   Wastewater treatment is a critical control point reducing environmental microbiological and pharmaceutical pollution. Even in high-income countries, wastewater treatment plants fundamentally affect the aquatic resistome, increasing levels of antimicrobial resistance in river catchments leading to increased probability of emergence of novel strains and environmental exposure and transmission to humans. Antibiotic usage in treatment of plant diseases and in aquaculture is also important to consider, as, although the total amounts are low, they are applied directly to the environment at effect concentrations which can be several orders of magnitude higher than environmental residues from human or animal usage.

   Pharmaceutical manufacturing sites have also been highlighted as key targets for mitigation as high levels of antibiotics are discharged to the natural environment.
3. Antibiotic selection
   Processes that lead to, or facilitate, emergence of resistance from the environmental resistome in human and animal pathogens are central to the problem of increasing antibiotic-resistant infections. The importance of antibiotic selection in the environment is a matter of debate; however, we know that environmental concentrations (excluding pharmaceutical manufacturing) of individual antibiotics can be in the same range of as minimal selective concentrations (MSCs) for some antibiotic classes. It is therefore not reasonable or logical to believe that these selective compounds do not play a role in emergence or maintenance of resistance, particularly when combined in complex mixtures which are likely to have additive or synergistic effects. Selection in the natural environment may not drive much of the quantitative variation in abundance of resistant organisms which can be attributed to faecal pollution in many cases, but it may alter the resistome qualitatively promoting emergence of novel resistance genes in human-associated bacteria. This low selective pressure is also likely to select for resistance mechanisms with low fitness cost which may persist within microbial populations in the absence of selection.
4. Interventions
   Interventions should address source control, improved wastewater and biosolid treatment, incentives to encourage extensive rather than intensive livestock and crop production. In general we should apply the same approach to infection control that is so valued within hospitals to the wider environment. As has been

demonstrated by others such as Peter Collignon et al., contagion or spread of resistance between different ecosystem compartments is strongly associated with resistant infections, particularly in low- and middle-income countries.

5. Authorities
   The authorities need to know that (1) the environment is the origin of much clinically important resistance and (2) strategies to reduce microbial and chemical pollution to mitigate antimicrobial resistance evolution and transmission will also have many other benefits in terms of protecting the environment and human health from emerging threats associated with chemical and microbial hazards.
6. Priorities
   Surveillance of antibiotic resistance in aquatic environments and farmed soil environments used for livestock and crop production, are priorities. I would suggest a nested approach to surveillance including a focus on priority pathogens or bacterial indicators such as *Escherichia coli*, e.g. Tricycle ESBL (extended spectrum beta-lactamases) producing *E. coli* (http://resistancecontrol.info/2017/the-esbl-tricycle-amr-surveillance-project-a-simple-one-health-approach-to-global-surveillance/) combined with a culture-independent resistance gene-focused metagenomic and/or a high-throughput qPCR method to investigate the entire resistome.

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Antibiotic-Resistant Bacteria in Wildlife

Monika Dolejska

Contents

Abstract The global dissemination of antibiotic-resistant bacteria (ARB) is one of the most important issues for current medicine, having serious implications for public health. Particular concern has been raised regarding the increasing occurrence of multidrug-resistant bacteria in the environment and wildlife. Wild animals

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inhabiting human-influenced environments can easily acquire ARB. Synanthropic animals that tend to live close to humans and seek food in cities, landfills or areas with intensive agriculture are more likely to carry ARB in their gut than those in places with limited human footprints. In the past years, wild animals were recognized as vectors and secondary sources of ARB for humans and animals. Moreover, wild birds are capable of long-range movements and may spread antibiotic resistance (AR) across borders or continents. This chapter provides a summary of various aspects of AR in wildlife which is presented with respect to the One Health concept. It highlights the most important sources of AR for wildlife and outlines transmission routes of AR into the environment. Ecological and biological factors of various groups of wild animals driving the occurrence of AR and the role of wild animals as spreaders of resistant bacteria are addressed. An overview of selected resistant pathogens carrying epidemiologically and clinically relevant AR found in wildlife is presented and linked to the situation in humans and livestock. Current gaps in our understanding of AR in wildlife and suggestions for future actions and research activities are also highlighted.

Keywords Antibiotics, *Escherichia coli*, Environmental source, One Health, Resistance, Transmission, Wildlife

Abbreviations

AR	Antibiotic resistance
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance genes
CC	Clonal complex (defined by multilocus sequence typing)
CPE	Carbapenemase-producing *Enterobacteriaceae*
ESBL	Extended-spectrum beta-lactamase
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
MDR	Multidrug resistance
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PMQR	Plasmid-mediated quinolone resistance
qPCR	Quantitative PCR
rep-PCR	Repetitive element sequence-based PCR
SSCmec	Staphylococcal cassette chromosome *mec*
ST	Sequence type
VRE	Vancomycin-resistant enterococci
WGS	Whole-genome sequencing
WWTP	Wastewater treatment plant

1 Introduction

Increasing trends of antibiotic resistance (AR) among bacteria causing infections in humans and animals have been reported worldwide. Since the beginning of the new millennium, the prevalence of multidrug-resistant (MDR) bacteria and emerging resistance mechanisms to drugs of last resort have increased, resulting in limited therapeutic options for the treatment of hospital-acquired infections [1].

Antibiotic-resistant bacteria (ARB) have been also widely reported in nonclinical samples including food-producing animals, food and companion animals and in the environment [2]. Bacteria with clinically important resistance mechanisms have been repeatedly isolated all over the globe from urban and hospital wastewaters, surface waters, livestock-associated manure, sewage and agricultural soils [3]. Current data indicate that MDR bacteria can spill over from their anthropogenic sources into natural ecosystems, possibly creating secondary reservoirs in the environment where clinically important resistance can be maintained, subjected to further evolution and from where it can spread to other niches. As most antibiotics are produced by fungi and bacteria, ARB naturally resistant to antibiotics are commonly present in environmental microbial communities. In human-influenced environments, this naturally occurring resistance along with ARB and antibiotics of human and animal origin can mix together, providing ideal conditions for new resistant strains of clinical importance to arise [4].

Although wild animals are not directly exposed to antibiotics, they are affected by their extensive use in human and veterinary medicine. Human-influenced habitats create important sources of ARB for the environment and wildlife. Natural preservation state, densities of livestock and human population and remoteness of the area have been suggested as important criteria for the occurrence of AR in wildlife [5]. Wildlife living and getting food in polluted environments can easily acquire ARB. Animals that tend to live close to humans and seek food in cities, landfills or areas with intensive agriculture are more likely to carry ARB than those in areas with limited human footprints [5, 6]. The occurrence of AR in wildlife is influenced by various factors which are not yet fully understood. Biology and ecology of the host as well as the level of anthropogenic impact in the area are among the key factors [5].

Wildlife is generally overlooked as part of the environment which is highly influenced by human activities but plays an important role in relation to AR and the overall transmission scenarios of resistant pathogens (Fig. 1). Wild animals represent useful sentinels mirroring the presence of the AR in human-influenced environments as ARB and antibiotic resistance genes (ARG) found in wildlife resemble those spreading in humans and livestock. They have also been recognized as vectors and secondary sources of MDR bacteria for humans and animals. Many wild animal species, especially synanthropic wild birds living in close proximity to humans and their activities, are ubiquitous. Their faeces are freely dispersed into the environment, leading to the contamination of surface waters and soils by AR and consequent risks for public health [5, 7].

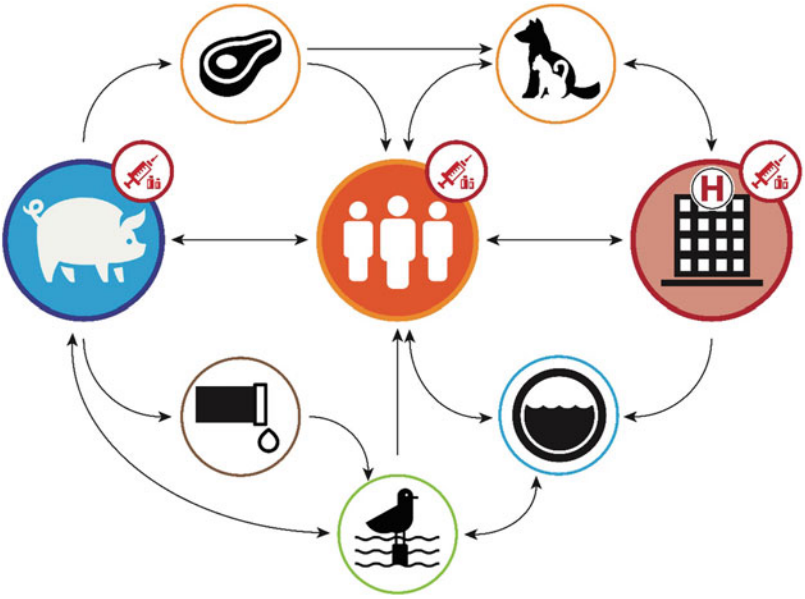

Fig. 1 A simplified schematic of complex transmission pathways of ARB between humans, animals and the environment

In the following sections, various aspects of AR in wildlife with respect to the One Health concept are introduced. The text is focused on the most important sources of human and livestock AR for wildlife along with the transmission routes mediated by the environment to wildlife populations. Attention is also paid to the role of wild animals as spreaders of AR. The final sections present an overview of selected resistant pathogens carrying epidemiologically and clinically relevant AR.

2 Environmental Sources of ARB for Wildlife

With growing pressure from expanding human populations and their increased impact in some areas, wild animals are increasingly forced to feed in contaminated environment. Such human-influenced habitats thus become important sources of antibiotic, ARB and other anthropogenic pollutants for the environment and wildlife. Although assigning the source and directionality of AR dissemination is challenging, several transmission routes of AR of human and livestock origin for wildlife have been suggested. Livestock manure, agricultural run-off, hospital and urban wastewaters, raw meat or other animal products, contaminated soil and pet faeces are among the most important anthropogenic routes of AR contaminating the environment (Fig. 2).

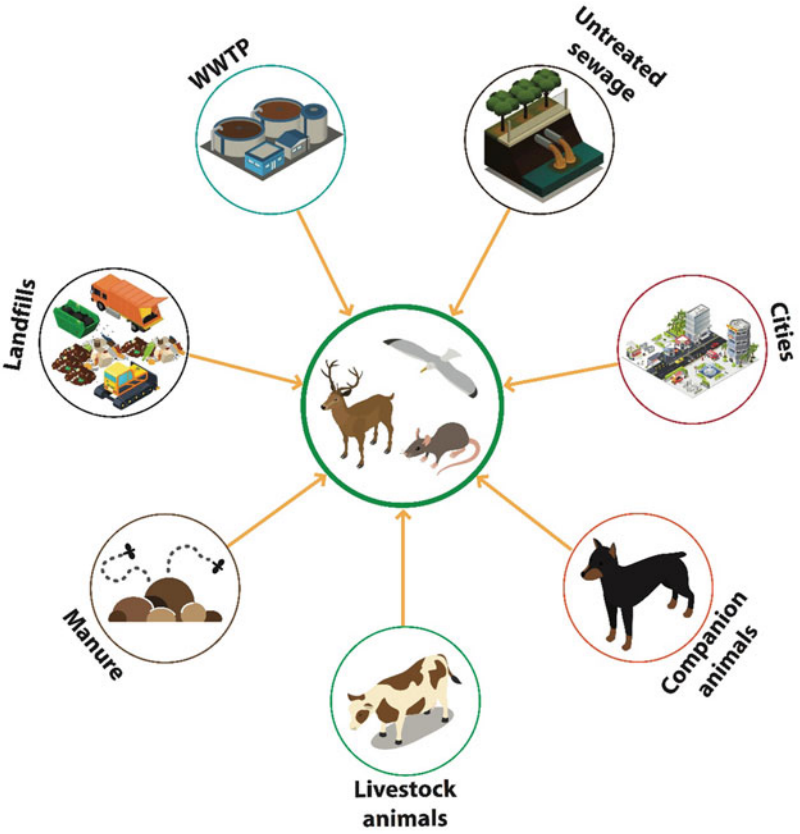

Fig. 2 Environmental sources of ARB for wild animals

2.1 Sewage Contaminating Surface Waters

Water plays an important role in the dissemination of bacterial pathogens including ARB. Resistant bacteria were isolated from rivers, lakes and sea water [8]. ARB found in water originate either from human or farm animal populations, and sewage is considered the major source of ARB for aquatic environment. Wastewater treatment plant (WWTP) play a vital role in the treatment of human sewage as they reduce the concentration of bacteria in final effluent by at least 10-fold to 100-folds. However, substantial amount of ARB, antibiotics, heavy metals and ARG that are not removed by water treatment are released into downstream ecosystems [9]. Moreover, by processing waste from a variety of sources including urban areas, hospitals or slaughterhouses, WWTP may offer good conditions for horizontal gene transfer between bacteria of diverse origins [10, 11].

Water contaminated by ARB represent an important route by which AR can reach wildlife. Increased risk of MDR bacterial carriage in water-associated species has been recently demonstrated. A study performed in Sweden showed that up to 47% of faecal samples from mallards were positive for extended-spectrum beta-lactamases (ESBL)-producing *E. coli* [12]. Mallards are species that often forage in water downstream of WWTP, where they can be exposed to antibiotics and ARB from wastewater effluents [13]. Detection of AR *E. coli* isolates with highly similar genomic profiles as those from hospitalized patients, wastewater and wild birds [14, 15] provides the indirect evidence for the transmission of ARB from humans to wildlife and vice versa. It has been demonstrated that waterfowls nesting near waste and agricultural water harbour more AR *E. coli* than birds associated with unpolluted waters [16]. Marcelino et al. observed that wild birds foraging at the partially treated lagoons of a WWTP (containing the waste from the last stage of the treatment process) had significantly higher diversity and abundance of ARG than those from other locations [17]. The occurrence of specific *Salmonella enterica* serotypes in gulls was linked to their feeding at WWTP [18]. ARB acquired by waterfowls can be reintroduced into the environment during the bird defecation [19] and might infect humans via contact with contaminated drinking or recreational water.

However much severe situation of water pollution exists in many low- and middle-income countries. These areas are generally characterized by higher use or misuse of antimicrobials, extremely high human density, generally poor hygiene standards and sanitation, insufficient wastewater treatment and waste management, generating higher levels of environmental pollution [20]. In these regions, animals and humans usually live close together, and clinically important MDR bacteria are widely present in the environment, increasing the probability of exchange of AR between humans and animals. We may assume that ARB prevalence in water-associated wildlife species from these countries are much higher compared to places where WWTP are routinely used to treat raw sewage. However, data on the occurrence of the ARB in wildlife are largely missing from these areas.

Interestingly, wild rodents nearer to a river receiving treated sewage effluent excreted more resistant *E. coli* than inland animals [21]. Another example are rodents living and getting food in the sewage system containing untreated waste in urban areas that can become colonized by bacterial pathogens producing ESBL [22].

2.2 *Landfills and Garbage in Urban Areas*

Human activities have transformed a large proportion of the Earth's land surface, and food and habitat availability for wildlife has been significantly impacted. Several species of wild animals have adapted to anthropogenic environments and learned to take advantage of waste disposal, especially during periods of low natural food availability (e.g., winter) and high demand (e.g., breeding season) [23]. Landfills provide accessible, consistent and nearly limitless food sources for scavenging animals.

Food wasted by humans is often accessible to wildlife in these sites, affecting their ecology and behaviour. The use of organic waste as a food resource has resulted in expansion of some wildlife populations [23]. For instance, over the last 50 years, the number of gulls has increased dramatically in many parts of the world. It is believed that this rise is related to the increased quantity of artificial food from human fishery activities and the concentration of garbage in landfills [24]. Gulls feed commonly on artificial diets as they tend to seek highly nutritious food in cities, fisheries and especially on landfills. The quality and quantity of food are among the crucial factors for gulls, as early nutritional conditions strongly affect both survival and development of their chicks. A similar shift in feeding behaviour has been observed in other wild bird species such as corvids, herons, storks and ibises and some birds of prey (black kites, vultures) in various parts of the world [23], for example, black kites in urban areas of Mediterranean frequent rubbish dumps where they have established large breeding colonies [25].

Food resources from landfills are also an important factor influencing animal movement. These food substitutes alter animal space use by reducing their home ranges and modifying migration behaviour [23]. For example, the presence of landfills determines the roost selection of some vulture populations since close food resources reduce energetic cost of movement [26]. Crows and ravens reduce their home ranges near human settlement where they exploit organic waste [27]. Similarly, island foxes have smaller home ranges in urban landscapes than in rural populations due to food availability [28]. Some white stork populations in Europe stopped migrating to Africa and established resident populations that are substantially dependent on the food sources from local landfills during winter [29]. This change in movement patterns can have different ecological consequences including changes in pathogen distribution.

Several examples from the current literature suggest that the accumulation of human refuse on landfills is one of the major anthropogenic-induced drivers in the transmission of pathogens and MDR bacteria into wildlife [30, 31]. The occurrence of *Campylobacter jejuni* in juvenile gulls was found to be associated with refuse consumption [32]. However, direct evidence of AR transmission between the anthropogenic waste and wildlife is largely missing. One of the few examples published so far is a study by Nelson et al. demonstrating identical genotypes of *E. coli* isolates from landfills and gulls [14]. Gulls foraging at landfills often roost on nearby fields and pastures and wash in local water bodies, and in this way bacteria ingested at feeding sites may enter other food chains once excreted by the birds [33].

The management of landfills is therefore an important step to minimize the numbers of birds feeding there and disseminating AR. For instance, reducing the active area where waste is dumped from the trucks and covering of the refuse should limit access of wild birds to garbage. Avoiding water accumulation in shallow depressions may also prevent birds from using these sites [23]. Active scaring programmes using falcons that rely upon escape behaviour are effective in deterring gulls from landfills [34].

2.3 Livestock and Manure Application

Veterinary antibiotics applied to farm animals result in the selection of ARB in livestock [35]. These bacteria and antibiotic residues may then reach the environment via animal wastes (manure or wastewater) routinely applied to farm land as fertilizer. Manure has become a reservoir of ARB and can significantly increase the abundance of ARG in soil [36]. In soil, the intestinal bacteria and ARG mix with environmental bacteria, which also harbour various resistance determinants, providing additional genetic material for evolutionary processes [36]. The abundance of ARG in farmland treated with faeces can be up to 100 times higher than that in unfertilized soil [37]. Moreover, manure and manure-amended soils can be flushed by heavy rainfall or run-offs into nearby water bodies used by humans and for domestic purposes or utilized by various wild animals.

Farm environments (water, soil, feeds, wastewater, sewage, lagoon, manure and treated sludge) serve as AR pollution sources. These sites allow important interactions between wild animals and anthropogenic waste, resulting in the transmission of ARB and ARG of livestock origin into wildlife. Wild animals can easily pick up bacterial pathogens and ARB when moving and feeding in the farm environment or surrounding contaminated areas including fertilized fields. ARB have been reported in various groups of farm-associated animals including insects, birds or mammals. For example, wild small mammals living on farms or in their vicinity were found to be several times more likely to carry *E. coli* isolates with tetracycline resistance determinants and MDR strains than animals living in natural areas [38]. In another study Rogers et al. investigated the occurrence of ARB and zoonotic pathogens in the faeces of white-tailed deer in relation to their proximity to land that received livestock manure [39]. They observed that AR abundance in the faeces of deer was spatially correlated with these activities. Organic waste in and around animal production facilities also provides an excellent habitat for the development of insects and transmission and further dissemination of AR [40].

3 Dissemination of ARB by Wildlife

Wildlife hosting zoonotic agents is a potential hazard to human health and food safety. It is estimated that approximately 40% of human diseases likely have their origin in wildlife [41]. Wild animals can disseminate resistant pathogens not only in the environment, but, at some extent, they can transfer them back to humans and animals via diverse routes (Fig. 3). Wild animals can contaminate animal feed, pasture, food for human consumption, urban environments, drinking water reservoirs and recreational waters. Species with different movement patterns, resource requirements and foraging behaviours have different roles in the dispersal of AR [42]. Environmental factors such as location and diet are among the major

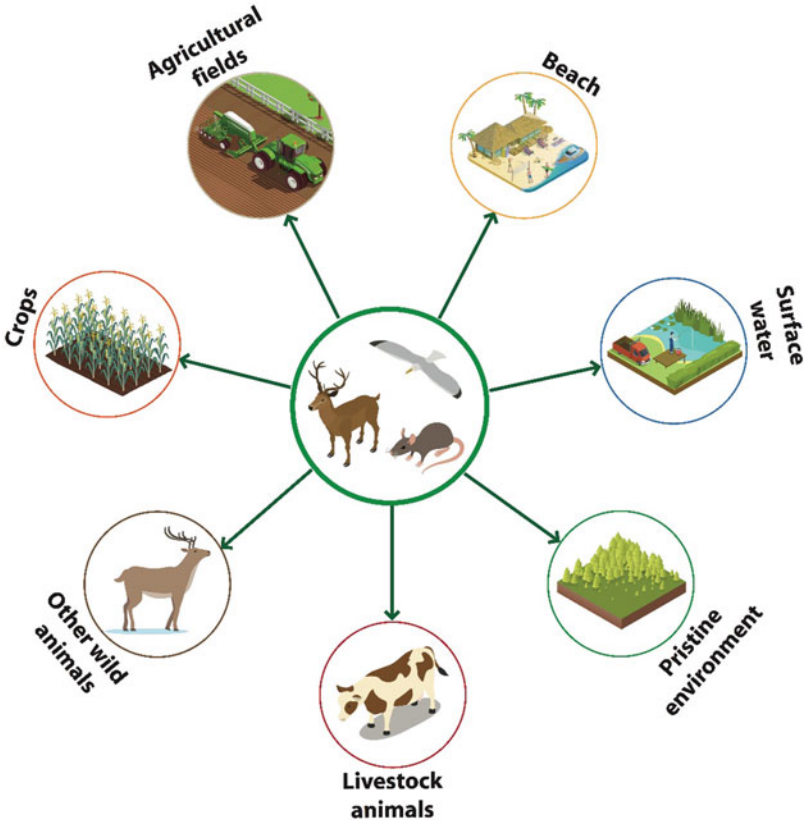

Fig. 3 Dissemination of ARB by wildlife

elements influencing the composition of gut microbiota and the carriage of bacterial pathogens [43]. Understanding the dynamics of wild animal microbiota including the shedding of ARB provides important insights into their interaction with the environment and their role in the transmission of AR. However, as the ARB abundance and the duration of their shedding in wildlife is largely unknown, it is currently difficult to quantify the level of health risks that wild animals pose to humans and domestic animals.

3.1 Gut Microbiota and Shedding of AR

Gastrointestinal microbiota plays a crucial role in maintaining animal health. Its composition and dynamics are determined by diverse factors that include mainly genetics, age, diet, social interactions and the environment [43]. Although many studies focused on microbiota of humans and domestic animals have been

published during the last decade, microbial gut communities of wild animals have received little attention [44, 45]. Unlike domestic animals, wildlife varies widely in environmental preferences, physiology and spatial-temporal movements. Wild animals are exposed to different microbes through diverse environmental conditions in preferred habitats [43].

Feeding ecology appears to be the major factor influencing the exposure of many wild animals to bacteria present in the environment, including enteric pathogens. Foraging in urbanized and agricultural areas, primarily scavenging on human refuse or sewage, not only influences the composition of gut microbiota but also increases the probability of acquiring ARB [33]. Animal population density is another important factor that plays a significant role. For example, large aggregations of birds typical for colonial breeding species could also result in easier exchange of gut microbiota. Birds congregating at high-density communal roosts are more vulnerable to the spread of disease, both through direct contact and through the contamination of food and water sources by bacterial pathogens [33]. Exposure to different microbial environments during migration as well as the formation of mixed-species flocks and avian aggregation at stopover sites could facilitate the transfer of microorganisms. It has been suggested that environmental exposure to bacterial pathogens and AR in migratory species may be greater and more diverse than in resident birds, depending on their site fidelity and space use [43].

Gut microbiota also varies depending on the age of the individual. Young or immature birds were found to display higher bacterial carriage rates of *Salmonella* than mature birds [46]. This trend was observed mainly in species breeding in colonies where birds occur at high density which probably results in increased likelihood of disease transmission until they fledge [33]. Faecal matter in nests likely increase exposure levels of nestlings to faecal bacteria. Moreover, differences in microbiota composition and presence of enteropathogens could be associated with the fact that immature birds tend to feed at untreated sewage outflows or landfills more often than adults [33]. Studies in food-producing animals suggested that the dynamics of shedding of AR and pathogenic bacteria is likely associated with physiological and microbiological factors [47]. This can be attributed to an unstable microbiome in young animals and changes in the epithelial structure of the gastrointestinal tract [48, 49]. Such age-related changes may exist also in wild animals and could explain different observations of carriage rate of pathogens or ARB observed across wildlife studies.

The duration of shedding of resistant bacteria by wild animals is largely unknown although it has strong implications for the transmission of AR especially in highly mobile animals such as wild birds. Currently, there are little data available on how long an individual *E. coli* strain can colonize wild birds and mammals. Sandegren et al. performed an experimental study investigating carriage time and interindividual transmission of ESBL-producing *E. coli* in mallards [13]. They aimed to understand if the birds can carry the bacteria long enough to transfer them geographically during migration. Laboratory experiments showed the presence of resistant bacteria in the bird gut the entire 29-day period along with the extensive and rapid transmission between individuals in the flock. However, experiments

that are reflecting the real situation present in the environment are difficult to conduct in the case of wildlife. The shedding time of *Salmonella enterica* in gulls seems to be short (several days), while this pathogen may survive in the soil of gull nesting colonies between breeding seasons [50, 51]. However, the isolation of ARB in migratory birds in remote areas with little human impact may indicate long shedding time (see Sect. 3.3).

3.2 Wildlife as Vectors of ARB

Wildlife can participate on human-animal transmission of infectious agents including ARB in different ways. Wild animals associated with farming or agricultural areas may transmit AR between herds, farms or agricultural environments. Rodents and flies in food processing facilities with poor sanitary conditions can disseminate bacterial pathogens. Mice and rat droppings can contaminate food by pathogens such as *Salmonella*, increasing risks for human infections [52]. Interactions of wildlife with farmland growing crops for human consumption have been linked to outbreaks of *E. coli* O157:H7 [53]. Flies have been recognized to spread ESBL-producing *E. coli* in broiler farms and contaminate food for human consumption by AR enterococci [54]. Handling of dead wild animals, for example, during hunting has been also recognized as a risk factor for wildlife-human transmission of infectious agents [55].

Waterfowl tend to congregate in large numbers and roost on water reservoirs which can possibly lead to the contamination of drinking water supplies for humans or domestic animals [56]. Waterborne outbreaks of *C. jejuni* infections in humans have been attributed to the contamination of drinking water by the droppings of geese [57]. Several studies revealed wild birds, especially gulls, as an important source of pollution in coastal environments. Birds that feed at landfills ingest pathogenic microorganisms present in the waste, and when visiting beaches, they may serve as vectors of these bacteria and contaminate sand and water. It has been shown that bacteria persist longer in sand than in water because of the adherence to sediment particles [58].

Contamination of recreational beaches by gull droppings poses significant risks to public health. In a recent study by Alm et al., radio-telemetry devices were used to follow gulls that visited two different recreational areas located nearby a wastewater lagoon [59]. Samples were collected from landfills, treated wastewater storage lagoons and public beaches and examined for the presence and abundance of gull- and human-associated faecal markers and potential pathogens using qPCR assays. A spatial and temporal overlap of markers for gull- and human-associated microorganisms was demonstrated. This study highlighted the potential for gulls that visit waste sites to disperse human-associated microorganisms in the beach landscape. As gulls are recognized as important contributors of faecal contamination to surface waters, some recreational beaches have used gull control measures to improve microbial water quality. In one study, gulls were chased from a Lake

Michigan beach using specially trained dogs, and water quality improvements were quantified [19]. The results indicated that a 50% reduction in gulls was associated with a 38% and 29% decrease in *Enterococcus* spp. and *E. coli* densities, respectively.

3.3 Bird Migration in ARB Transmission

The seasonal migration of wild birds is an important factor in the transmission of pathogenic microorganisms and establishment of endemic foci in new areas. Billions of birds migrate between continents annually. It is estimated that 5 billion birds of over 300 species migrate from North America to Central and South America each autumn, and similar numbers travel from Eastern Europe to Africa [60]. Patterns of migration for wild birds tend to be highly complex and variable between species and can even be different for distinct populations within the same species. Short-distance migrants travel only few hundred kilometres or less from their breeding sites to wintering locations, while long-distance migrants travel hundreds to thousands of kilometres [61].

As birds are capable of long-range movements, they can spread any bacterium, virus, parasite or drug-resistant organism they harbour along their migration routes. Bird migration has been linked with the spread of many infectious agents [61]. Intercontinental exchange of spirochete-infected ticks by seabirds highlights the capacity for wild birds to carry infected ticks for long distances [61]. The avian influenza viruses have been shown to be transmitted over long distances during the seasonal migration of birds. Wild waterfowl, in particular, is considered the reservoir of low-pathogenic avian influenza viruses and has been shown to spread these pathogens along migratory flyways in Asia, Africa and the Americas. Spread of West Nile virus has been also linked to bird migration [62]. Interestingly, differences in the gut microbiomes of migratory and resident birds have been reported, suggesting that bird migration as a significant physiological challenge can also affect the host microbiota [63]. Although these changes may be only temporary and animal species-related, they may have an impact on the transmission of important pathogens. Most long-distance migrants make a series of shorter flights between stopover sites. These sites are important from the viewpoint of infectious diseases because they provide the opportunity for close interaction of species that are normally separated during the majority of the year. Therefore, the opportunity for exchange of pathogens is increased among avian species in these sites [61].

The capacity of wild birds for long-range movements across borders or continents is a particular concern for the dispersal of AR. Bird migration could contribute to the dissemination of AR across the globe in a similar way as is observed for human travellers. Resistant bacteria can spread from regions with high levels of AR contamination to less affected areas. For example, a study focused on arctic birds in three geographical areas in the Arctic including Siberia, Alaska and Greenland showed the presence of MDR *E. coli* [64]. It has been also demonstrated that

over the past years, the frequency of human-associated ARB in Arctic birds has increased [65]. ARB could be imported into these regions either by migratory birds or through human refuse from fishermen and prospectors in the area, highlighting the complexity of dissemination of AR to pristine environments.

The role of globally moving species in disseminating AR over long distances remains unclear as this aspect of AR in wildlife is not well covered by the scientific literature and also provides contradictory results. ARB have been described in migratory birds in remote areas with low human exposure [66], suggesting the importance of long-distance animal movement in the transmission of AR. Bird migration in regard to the occurrence of AR has been addressed by a study on wild birds in Austria [67]. Two rook populations, one resident and one migratory, that were approx. 70 km apart were examined for ARB. The sampled resident population of rooks wintering in Austria has their breeding sites in Russia, Belarus and Poland. The study showed that birds from the migratory population have significantly higher occurrence of ESBL- and AmpC beta-lactamase-producing *E. coli* compared to the resident population.

In contrast to the studies discussed above, different results were obtained by research on migratory and non-migratory wild songbirds populations associated with dairy farms in the USA [68]. The authors did not identify any difference in the prevalence of ESBL- or AmpC beta-lactamase-producing bacteria between migratory and resident birds. Similarly, a study on Franklin's gull migrating between Canada and Chile suggested that the ARB were likely obtained locally rather than via significant transhemispheric exchange [69, 70]. By comparing *E. coli* genotypes and ARGs, they did not find any strong indications of long-distance dissemination between the continents.

4 Wildlife as Reservoirs, Sentinels and Spreaders of Clinically Relevant AR

Concerns have been raised regarding the role of wildlife in the dissemination of clinically relevant resistance mechanisms to diverse ecosystems. Plasmid-mediated resistance to broad-spectrum beta-lactams such as ESBL- and AmpC-type beta-lactamases are of special interest as they are widely disseminated in bacteria of human and livestock origin and a key focus in many national AR surveillance programmes [2]. *Enterobacteriaceae*, especially *E. coli,* with the above-mentioned resistance mechanisms have received attention in many wildlife studies, especially from wild birds. The occurrence of other clinically and epidemiologically relevant AR mechanisms associated with resistance to last-line antibiotics such as carbapenems and colistin is also being studied in wildlife. ARB isolated from wildlife are of the same types of those identified from human and livestock, including pathogens such as vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) and MDR *Salmonella* spp. In the

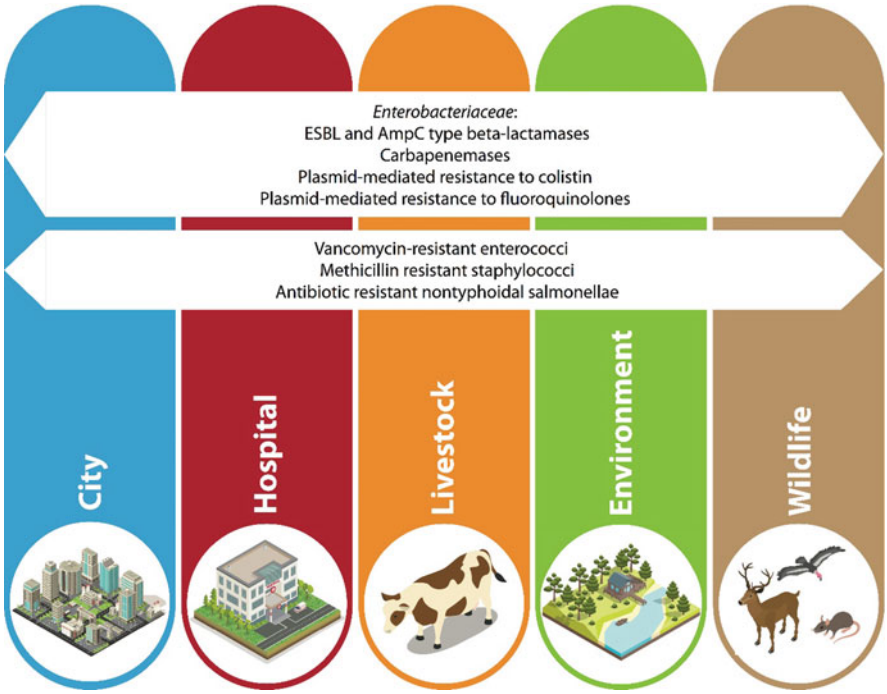

Fig. 4 ARB and clinically relevant resistance mechanisms are present in diverse niches

following sections, the above-mentioned AR pathogens and indicator bacteria with emerging resistance mechanisms and their occurrence in wildlife are presented using selected examples from the current literature (Fig. 4). The role of wild animals as sentinels and vectors of these ARB of human and livestock origin is discussed in the context of One Health.

4.1 ESBL- and AmpC-Type Beta-Lactamase-Producing Enterobacteriaceae

Beta-lactam antibiotics with broad-spectrum activity, in particular third- and fourth-generation cephalosporins, are among the most important groups of antimicrobial agents in human and veterinary medicine. However, resistance to these antibiotics has been rapidly increasing in Gram-negative bacteria over the last decade as a result of dissemination of ESBL- and AmpC-type beta-lactamases. ESBL dissemination caused a global change in the epidemiology of beta-lactamases. Among various ESBL types identified, enzymes of the TEM, SHV, and CTX-M families have been increasingly reported from clinical isolates. Since about 2000, the CTX-M enzymes have formed a rapidly growing family of ESBLs, whereas the prevalence of classical

ESBL enzymes like TEM or SHV has been decreasing [71]. From the diverse group of AmpC beta-lactamases, enzymes of CMY-2 family are among the most widely disseminated in *Enterobacteriaceae* of both human and animal origin [72].

Until the end of the 2000s, most infections caused by ESBL-producing *E. coli* or *Klebsiella pneumoniae* were hospital-acquired. During the last decade, several reports described an impressive increase in the number of ESBL-producing *E. coli* isolated from nonhospitalized patients [73], livestock and companion animals [74]. Furthermore, many recent studies have reported bacteria with these resistance mechanisms in wild animals [75]. As documented below, the level of resistance and reported ESBL and AmpC genes in bacteria from wildlife seems to correlate well with the epidemiological situation reported in humans and domestic animals.

The first ESBL-producing *E. coli* isolates of wildlife origin were reported in species sampled between 2003 and 2004 in Portugal [76]. In the following years, many reports describing ESBL producers in various wild birds and mammals across all continents followed [75]. AmpC beta-lactamases, mainly CMY-2, have also been reported in *Enterobacteriaceae* isolates from various groups of wild animals [75, 77, 78]. In parallel to the current epidemiological situation in human and veterinary medicine and agriculture, enzymes of the CTX-M family also dominate among ESBL-producing *Enterobacteriaceae* from wildlife, reflecting their transmission to the environment. The most widely reported variants of CTX-M in bacteria from wildlife include CTX-M-1, CTX-M-14 and CTX-M-15 which are similar to the situation in the human and veterinary health sectors. It is important to note that CTX-M-15 and CTX-M-14 are predominant ESBL types reported in human clinical isolates, while CTX-M-1 enzymes are in general more common in farm animals [74, 79].

Most research to date has focused on gulls, with studies showing that up to 68% of these birds can carry ESBL- or AmpC-producing *Enterobacteriaceae* in their gut (Table 1). Some of these studies have pointed to possible anthropogenic sources of these bacteria. For example, Atterby et al. aimed to characterize ESBL-producing *E. coli* in Swedish wild gulls and compared them to isolates from humans, livestock and surface water collected in the same country and similar time period [80]. Interestingly, the occurrence of ESBL-producing *E. coli* in gulls was three times higher compared to the carriers in healthy human populations (17% versus 5%). Moreover, the genotypes of ESBL-producing *E. coli* isolates from gulls, surface waters and humans were similar, highlighting surface waters as potential dissemination routes between wildlife and the human population. In another study, gulls from two locations in Alaska differing in the level of anthropogenic impact were examined for ESBL and plasmid-mediated AmpC beta-lactamases [81]. A total of 16% of gull samples collected in the urban setting carried ESBL- or AmpC-positive isolates in contrast to the more remote location where no such bacteria were detected. Widespread occurrence of ESBL producers in gulls utilizing urban and agricultural areas suggests that AR may also be spread through birds. ESBL-producing *E. coli* isolates were identified also in other water-associated birds such as great cormorants and mallards in Central Europe [82], wild geese in Belgium [83] and various birds in wetland habitats in Pakistan [84].

Table 1 List of studies reporting ESBL-producing bacteria in gulls

Reference	Gull[a]	Country	Year of sampling	No. of samples (% positive)	No. of isolates	ESBL	Bacteria[b]
[102]	nd	Portugal	2007	57 (19.3%)	11	CTX-M-1, CTX-M-14, CTX-M-32, TEM-52 (8)	EC
[103]	Yellow-legged g.	France	nd	96 (16.7%)	16	CTX-M-1 (8), CTX-M-15, SHV + TEM (2), TEM (5)	EC
[104]	Black-headed g.	Czech Republic	2006	216 (3.2%)	7	CTX-M-1 (1), CTX-M-15, SHV-2 (1), SHV-12 (2)	EC
[105]	Black-headed g.	Sweden	2008	100 (3%)	3	CTX-M-14 (2), CTX-M-15	EC
[106]	Glaucous winged g.	Russia	2007	532 (0.8%)	4	CTX-M-1, CTX-M-14, CTX-M-15 (2)	EC
[107]	Herring g.	Poland	2008–2009	27 (11.1%)	3	CTX-M-1 (2), CTX-M-9 (1)	EC
[108]	Lesser black-backed g., Caspian g.	Portugal	2007–2008	139 (32.4%)	45	CTX-M-1 (8), CTX-M-9 (4), CTX-M-15 (17), CTX-M-32 (15)	EC
[109]	Black-headed g., common g., herring g., lesser black-headed g.	Sweden	2010	283 (6.4%)	18	CTX-M-1 (16), CTX-M-14, SHV-12	EC
[110]	Ring-billed g.	USA (Florida)	2010	53 (15.1%)	8	CTX-M-15 (5), CTX-M-32, CTX-M-124	EC
[111]	Franklin's g.	Chile	2009	370 (30.3%)[c]	129	CTX-M-1 (39), CTX-M-3 (2), CTX-M-15 (8), CTX-M-14[d]	EC
[112]	European herring g., lesser black-headed g., common g.	Netherlands	2011–2012	150 (13.3%)	20	CTX-M-1 (4), CTX-M-3 (2), CTX-M-14 (3), CTX-M-15 (9), SHV-12 (1), SHV-12 + TEM-52 (1)	EC

[113]	nd	USA (Alaska)	2010	150 (36.7%)[e]	68	CTX-M-14 (46), CTX-M-27 (3), CTX-M-15 (2), CTX-M-15 + SHV-1 (4), CTX-M-15 + SHV-2 (4), TEM-19 (6), TEM-52, SHV-2, SHV-12 (7), SHV-102 (8), SHV-2 + SHV-19 (2)	EC
[114]	Brown-headed g.	Bengal[f]	2010	150 (17.3%)	29	CTX-M-14, CTX-M-15 (29), CTX-M-55 + CTX-M-79	EC (27); KP (1); EA (1)
[115]	Franklin's g.	Chile	2011	124 (54%)	67	CTX-M-2 (12), CTX-M-3 (1), CTX-M-15 (38), CTX-M-32 (11), TEM-40 (5), TEM-198	EC
[69]	Franklin's g.	Canada	2010	354 (17.5%)	62	CTX-M-1 (2), CTX-M-3, CTX-M-14 (24), CTX-M-15 (19), CTX-M-55, CTX-M-14 + TEM-52, SHV-2 (2), SHV-11, SHV-12, SHV-14	EC (52); KP (7)
[116]	Herring g., lesser black-headed g., yellow-headed g.	Europe[g]	2009	3,158 (28.7%)	948	CTX-M1 (182), CTX-M-2 (28), CTX-M-3 (4), CTX-M-8 (1), CTX-M-9 (1), CTX-M-14 (181), CTX-M-15 (84), CTX-M-24 (3), CTX-M-27 (1), CTX-M-32 (28), CTX-M-55 (1),CTX-M-65 (1), SHV, TEM[h]	EC, KP, KO,CS, ES
[81]	Glaucous-winged g., herring g.	USA (Alaska)	2014	50 (6%)	3	CTX-M-15 (3)	EC
[70]	Kelp g.	Argentina	2012	50 (68%)	34	CTX-M-2, CTX-M-14 (4)	EC
[80]	Great black-headed g., European herring g., mew g., black-headed g.	Sweden	2013	170 (17.1%)	29	CTX-M-1 (2), CTX-M-14 (5), CTX-M-15 (13), CTX-M-27 (1), CTX-M-32 (1), CTX-M-55 (4), SHV-12 (3)	EC
[117]	Yellow-legged g.	Spain	2014	132 (54.5%)	72	CTX-M-1 (7), CTX-M-14 (10), CTX-M-15 (13), SHV-2 (1), SHV-12 (37)	EC

(continued)

Table 1 (continued)

Reference	Gull[a]	Country	Year of sampling	No. of samples (% positive)	No. of isolates	ESBL	Bacteria[b]
[118]	Silver g.	Australia	2015–2017	562 (24.3%)[i]	137	CTX-M-1 (1), CTX-M-3 (1), CTX-M-14 (17), CTX-M-15 (82), CTX-M-24 (1), CTX-M-27 (34), CTX-M-55 (1)	EC

[a]g., gull; nd, not determined

[b]*CS Citrobacter* spp., *EA Enterobacter aerogenes*, *EC E. coli, ES Enterobacter* spp., *KP K. pneumonia*e, *KS Klebsiella oxytoca*

[c]Prevalence is calculated as the ratio of birds carrying at least one ESBL-positive isolate per the total number of samples examined. Several birds carried more than one isolate. A total of 112 were positive for ESBL isolates

[d]Only 50 isolates were subjected to ESBL typing

[e]Sixty-eight isolates were obtained from 55 birds

[f]Bengal, Bay of Bengal divided between Bangladesh and India

[g]Samples (no. of samples shown in brackets) were taken from the following countries: Denmark (158), England (133), Ireland (266), Latvia (424), the Netherlands (560), Poland (280), Portugal (425), Spain (595), Sweden (217)

[h]Only the number of ESBL types from CTX-M group is shown

[i]Only ESBL isolates positive for CTX-M are included

Several studies have explored ESBL-producing *E. coli* in various species of terrestrial birds [75]. CTX-M-1 producers dominating among farm animals in Europe were also frequently isolated in birds of prey in Portugal [85] and Germany [66]. Corvids are another group of synanthropic terrestrial birds commonly found to carry ESBL- and AmpC-producing bacteria. Prevalence, genetic characteristics and geographical aspects of cefotaxime-resistant *E. coli* isolates from wintering rooks in nine countries in Europe have been investigated [86]. In this study, a total of 14% samples of bird faeces were positive for either ESBL- or AmpC-producing *E. coli* with significant differences between the sampling sites. The most dominant ESBL gene was $bla_{\text{CTX-M-1}}$ (48%) followed by $bla_{\text{CTX-M-15}}$ (30%), suggesting links to both human and animal sources. The highest prevalence of rooks carrying ESBL producers (25%) was observed in a location in the Czech Republic which was surrounded by fields, agricultural production areas and urban agglomerations and which had a WWTP and garbage dumps nearby. The distribution of ESBL genes closely corresponded to that previously described in hospital facilities, domestic animals and the environment, including wild birds and WWTP effluent, within that country. In Germany, the highest distribution of ESBL-producing *E. coli* strains was found at two sampling sites. In a rural area with high level of agriculture, the $bla_{\text{CTX-M-1}}$ gene was predominant (60%), while the second location, an urban area near a hospital clinic, showed high proportions of isolates carrying $bla_{\text{CTX-M-15}}$ (50%), suggesting exposure to human sources.

Synanthropic corvids discussed in the previous paragraph were also examined in Asia and North America, bringing some interesting insights into transmission of AR in the environment. High (59%, $n = 238$) occurrence of ESBL-producing *Enterobacteriaceae* was found in house crows foraging on hospital wastes in Bangladesh [87]. ESBL-producing crow isolates were characterized and compared with 31 patient isolates. The ESBL phenotype in crow isolates was associated predominantly with CTX-M-15 (95%), and this beta-lactamase was also present in all human isolates. This study also showed that crows and patients shared *E. coli* sequence types, including the epidemic *E. coli* O25b-ST131 clone. The scavenging behaviour of crows at poorly managed hospital waste dumps made them potential reservoirs of AR. In contrast to the epidemiological situation in Europe and Asia where ESBLs are dominating, CMY-2 beta-lactamase is the main resistance mechanisms among isolates resistant to cephalosporins of higher generation in North America, disseminating in food-producing animals, food, companion animals and human clinical samples. Recently, two studies documenting high prevalence of CMY-2-producing *E. coli* in faeces of corvids (American crow, common raven) in several roosting sites across the USA [77] and Canada [78] have been published, highlighting their wide dissemination in the environment in North America (Fig. 5).

Less attention has been paid to other animal groups including mammals. Several studies documented ESBL producers in wild boars [88–90], deers and foxes [76, 91]. Small rodents such as brown and black rats seem to be commonly carrying ESBL-positive *E. coli* [89, 92–94]. These synanthropic species often interact with human faeces in the sewage system in urban environments and can easily acquire ARB. A territory-wide study investigating the faecal carriage of ESBL-producing *E. coli*

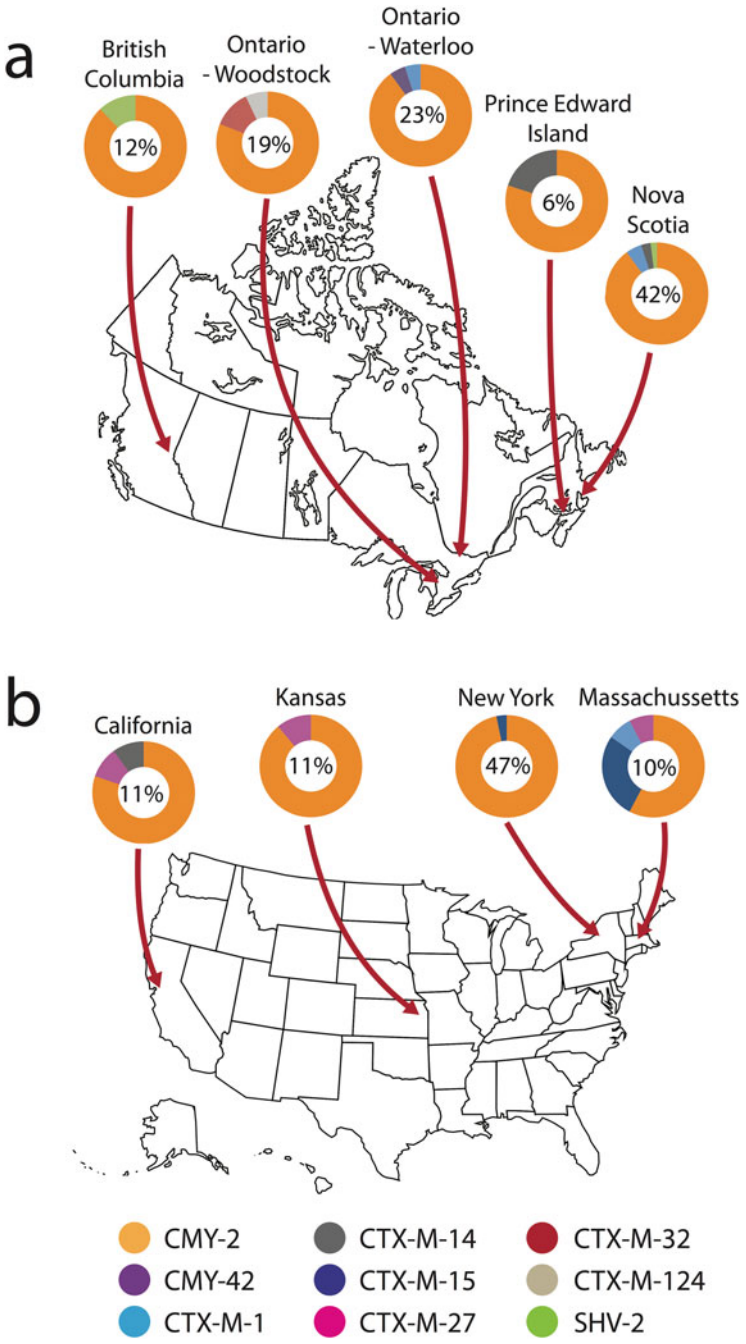

Fig. 5 Occurrence of cefotaxime-resistant *E. coli* in American crows and common ravens in Canada (**a**) and the USA (**b**). Prepared based on the data in Jamborova et al. [77, 78]. Numbers inside the circles represent the occurrence of cefotaxime-resistant *E. coli* found in faecal samples in each location. The type of ESBL or AmpC beta-lactamase is indicated by different colours

among almost 1,000 wild rodents from 18 districts in Hong Kong examined animals from four different species; the highest ESBL prevalence was found among black (14%) and brown (8%) rats. Among brown rats, the prevalence of ESBL carriage differed markedly by geographical location ranging from 0% up to 50%. However, no correlation between the prevalence of ESBL in brown rats and human population density was observed. Insects associated with livestock including cattle, poultry and pig farms [95–98], food markets [99] or hospital environment [100] have also been identified as common carriers and possible vectors of ESBL-positive bacteria. Moreover, bacteria-producing ESBLs have been isolated from marine wildlife although studies are scarce. For example, 21% of wild fish from a polluted area of the Mediterranean Sea in Algeria carried ESBL-producing isolates of *Enterobacteriaceae* [101].

4.2 Carbapenemase-Producing Bacteria

Carbapenems are among the most important beta-lactam antibiotics and are regarded as drugs of last resort to treat life-threatening infections in humans caused by MDR bacteria [119]. Because of their clinical importance, carbapenems are not approved for veterinary medicine. Unfortunately, clinical efficiency of carbapenems is threatened by carbapenemases, broad-spectrum beta-lactamases capable of rapid degradation of these reserved antibiotics. Acquired carbapenem resistance is frequently encoded by genes associated with mobile genetic elements (plasmids, transposons, and integrons) that often carry additional genes conferring resistance to various antimicrobials. MDR, which is common among carbapenemase producers, limits therapeutical options for infections caused by these bacteria, and the emergence and global spread of carbapenemase-producing microorganisms is of great concern to human health.

Although resistance to carbapenems is associated mainly with hospital-acquired infections and humans are currently considered to be the primary reservoir, there is growing evidence of carbapenemase-producing *Enterobacteriaceae* (CPE) also occurring outside hospitals. To date, only sporadic studies have documented CPE in food-producing animals, the farm environment, food or companion animals [120], but carbapenemase-producing bacteria have been reported in aquatic environments [121–123], highlighting their role as a transmission route of CPE to the environment. High abundance of CPE and carbapenemase genes has been detected in hospital effluents as one of the most important sources for the environment [124]. Resistant bacteria from raw hospital sewage enter the main wastewater stream and may subsequently spread further into the environment via wastewater treatment effluents or (in regions lacking sanitation) via untreated wastewaters.

Compared to other resistance mechanisms such as ESBL, generally low prevalence of CPE (with a few exceptions that will be highlighted below) has been documented in wildlife until now (Table 2). The first study of carbapenemase-producing bacteria in wildlife was published in 2013 when

Table 2 List of studies documenting CPE in wildlife

Reference	Animal	Country	Year of sampling	No. samples (% positive)	No. isolates	CP[a]	Other ARGs	Bacterial species/ST[b]
[125]	Black kite	Germany	nd	nd	1	NDM-1	bla_{CMY-16}, *sul1, sul2, dfrA17, aacA4, aadA5, aph(3′)-Via, aph(6)-Id, aph(3″)-Ib, fosA3, erm(B), mph(E), msr(E), mph(A), tet*(A), *floR*	*Salmonella* Corvallis
[127]	Yellow-legged gull	France	2013	93 (1.1%)	1	VIM-1, -4	*sul1, aac(6′)-Iic*	*Vibrio cholerae*
[31]	Silver gull	Australia	2012	504 (16%)[c]	120	IMP-4 (116)	*aacA4, catB3*	*E. coli* ST48, ST58 (14), ST167 (8), ST189 (3), ST216 (27), ST224 (5), ST345, ST354 (9), ST541, ST542, ST744, ST746 (2), ST1139 (2),ST1114 (4), ST1178, ST1421, ST2178, ST4567, ST4658 (2); EF ($n = 10$); KP (8); KG (2); EA (5); ECl (2); CB; PM (4); PP
						IMP-26 (2)	*aacA4, catB3*	KP (2)
						IMP-38 (2)	*aacA4, catB3*	CF (2)
[100]	German cockroach	Algeria	2015	10 (10%)	1	OXA-48	$bla_{CTX-M-15}$, bla_{TEM}	ECl
[126]	Yellow-legged gull	France	2012	93 (23.7%)	22	VIM-1	nd	*E. coli* ST22
[130]	Swallow	China	2014–2015	10 (40%)	4	NDM-1, -5	*mcr-1*	*E. coli* ST10, ST156, ST6417

[130]	Fly	China	2014–2015	120 (26.7%)	32	NDM-1, -5, -9	*mcr-1*	*E. coli* ST10 (2), ST48 (2), ST156 (3), ST167 (5), ST226, ST227, ST648, ST746 (5), ST1518, ST1638, ST4408, ST6388 (2), ST6390 (3), ST6393, ST6395 (2), ST6396
[131]	Fly	Germany	2011–2012	4 (25%)	1	VIM-1	nd	*E. coli*
[129]	Eurasian eagle-owl, lesser kestrel, and common buzzard, egret	Spain	2015–2016	668 (0.7%)	5	OXA-48		*E. coli* ST23, KP (3), ES
[128]	Wild boar	Algeria	2014–2016	168 (1.8%)	3	OXA-48	nd	*E. coli* ST635 (2); KP
[133]	Kelp gull	Alaska	2016	939 (0.7%)	7	KPC-2 (3)	*aadA5, aph(6′)-Id, aphd(3″)-Ib, aph(3′)-Ia, aph(3′)-Iic, aadA2, blaSHV-12, bla*$_{\text{TEM-1}}$*, floR, ere(A), erm(24), erm*(B)*, mdf*(A)*, mph*(A)*, mph*(E)*, msr*(E)*,sul1, sul2, qnrB2, dfA17, dfA19*	*E. coli* ST410
						OXA-48 (4)	*aac(3)-Iid, aadA5, bla*$_{\text{CTX-M-14}}$*, bla*$_{\text{TEM}}$*, mdf*(A)*, mph*(A)*, sul1, dfA17*	*E. coli* ST38
[118]	Silver gull	Australia	2015–2017	562 (0.2%)	1	OXA-48	nd	*E. coli* ST410

nd not determined, *ST* sequence type

[a]*CP* carbapenemase

[b]Abbreviations are used for the following species: *CB Citrobacter braakii*, *CF Citrobacter freundii*, *EA Enterobacter aerogenes*, *ECl Enterobacter cloacae*, *EF Escherichia fergusonii*, *KG Kluyvera georgiana*, *KP K. pneumoniae*, *PM Proteus mirabilis*, *PP Proteus penneri*. The affiliation to ST is shown only for *E. coli*

[c]Prevalence is calculated as the ration of birds carrying at least one CPE isolate per the total number of samples examined. CPE isolates ($n = 120$) were obtained from 80 birds, meaning that several birds carried >1 isolate

Fischer et al. found an NDM-1-producing *Salmonella* Corvalis in a black kite in Germany [125]. Subsequent studies from Europe described VIM metallo-beta-lactamase in *Vibrio cholerae* and *E. coli* isolates from a single colony of yellow-legged gulls in France [126, 127]. Interestingly, VIM-producing *E. coli* isolates were found in 19% birds, and all belonged to the same sequence type, suggesting a common source in the environment. OXA-48 producers were isolated from 13% wild boars [128] in Algeria or wild birds in Spain [129]. Insects carrying bacteria resistant to carbapenems, as OXA-48 and NDM producers, were reported in cockroaches in Algeria [100] and in flies in China [130], respectively. Interestingly, surveillance of carbapenem resistance in German livestock identified VIM-1-producing *Enterobacteriaceae* not only in pigs, farm environments and manure but also in flies [131].

Striking results have been reported from Australia of silver gulls carrying CPE in their intestines [31]. Silver gulls were originally considered to be coastal birds, but over the last century, a massive expansion of their population was observed in Australia [24] as they increasingly exploited habitat changes and artificially enhanced food supplies introduced by the increasing human population. In Australia, the highest densities of gulls accumulate around major urban areas, particularly Newcastle-Sydney-Wollongong. In the above-mentioned study, metallo-beta-lactamase IMP-4 was found in different members of the *Enterobacteriaceae* in as many as 40% of young birds from one nesting colony in the Five Islands Nature Reserve. Moreover, in several birds two to three different bacterial species or genotypes were isolated. In that study birds from two other locations were sampled but no CPE were isolated. One population was sampled in Sydney harbour where birds feed mainly on local fish markets and another one in Montague Island where the birds maintain a natural diet. As Five Islands is a nature reserve area, the presence of such a high prevalence of CRE was unexpected, raising questions about the possible sources of these bacteria. Longitudinal observation of the gull population on Five Islands brought a possible explanation. The feeding habits of silver gulls nesting on the islands demonstrated that during the breeding season, up to 6,000 birds per hour visited the refuse dump on the mainland near the city of Wollongong (12 km away from the nesting colony) for food [24]. Moreover, 85% of regurgitations from silver gull young trapped on nests contained only human refuse.

The carriage of CPE has also been investigated in gulls in Alaska. Ahlstrom et al. examined faeces from almost 1,000 birds from 7 locations near solid waste sites for CPE and obtained 7 *E. coli* isolates positive for carbapenemase genes $bla_{\text{KPC-2}}$ or $bla_{\text{OXA-48}}$ [30]. Since the surveillance of carbapenem resistance in Alaska initiated in 2013 has so far revealed only four imported cases of human CPE infections, this result was quite surprising, notwithstanding the low overall prevalence of CPE found in gulls (<1%). Three KPC-2-positive *E. coli* isolates from gulls were assigned to sequence type (ST) 410, a successful clone with reported interspecies transmission between wildlife, humans, companion animals and the environment. Four highly related (only two SNPs) *E. coli* ST38 with chromosomally-integrated $bla_{\text{OXA-48}}$ were found. Of note, ST38 is considered

a globally dispersed MDR clonal group of extra-intestinal pathogenic *E. coli* commonly associated with urinary tract infections and bacteraemia in humans. This *E. coli* clone has been also recently reported in Mongolian birds [132], highlighting its dissemination potential.

4.3 Plasmid-Mediated Resistance to Colistin

Colistin belongs to polymyxin group of antimicrobials that has been used in both human and veterinary medicine for more than 50 years [134]. Due to its side effects, which include nephrotoxicity and neurotoxicity when administered systematically, the use of polymyxins in humans stopped in most countries ~20 years ago. However, colistin has been used extensively over the decades for treatment and prevention of infectious diseases in animals, especially for gastrointestinal infections caused by *E. coli* in pigs and less frequently in poultry. It was also used as a growth promoter in livestock [135, 136]. More recently, the growing worldwide incidence of MDR infections has forced physicians to reintroduce polymyxins as critically important systemic antibiotics of last resort [137]. In many cases, particularly in infections caused by carbapenemase-producing *K. pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, colistin is the only effective antibiotic agent [134]. However, colistin resistance among CPE is also now increasingly reported [138, 139].

Until recently, resistance to colistin was only described as related to chromosomal alterations, which affected lipid A and reduced the binding of colistin to the cell wall [140]. However, in 2015, transferable colistin resistance mechanisms linked to the *mcr-1* gene encoding a phosphoethanolamine transferase were found in *E. coli* isolates from animals, food and bloodstream infections from human patients in China [135, 136], resulting in re-evaluation of colistin's use in veterinary medicine and as growth promoter [141]. Current literature suggests that the *mcr-1* gene is the most widespread, but other variants have been recently described [142–144]. Currently, plasmid-mediated resistance to colistin represents one of the top issues in the epidemiology of AR.

The greater abundance of *mcr-1* genes observed in veterinary isolates together with the high use of colistin in livestock raised concerns that this emerging resistance mechanism may have originated in the veterinary sector [134, 142]. Exchange of drug-resistant bacteria between humans and animals is a subject of major importance to public health worldwide, particularly when the activity of last-line antibiotics is compromised. Since its discovery in China in late 2015 [136], bacteria carrying *mcr* genes have been detected in a wide range of geographic locations and sampling sources including humans, livestock, food and companion animals. Environmental reservoirs reported include wastewaters, livestock areas [134, 142] and wild animals [6].

Only a few reports have been published so far describing the dissemination of plasmid-mediated colistin resistance to wildlife (Table 3). Bacteria carrying this emerging resistance mechanism have been isolated in wild birds, mammals and insects in Europe, South America and Asia [75]. The first known occurrence of *mcr-1* in *E. coli* isolated from a European herring gull in Lithuania was published in 2016 [145]. Faeces of the *mcr-1*-positive bird were collected on a city dump, suggesting the birds might have become colonized by colistin-resistant bacteria when collecting food there. This study highlights how inappropriate management of waste may influence the spread of bacterial pathogens, including resistant ones, through wild birds. Analysis of migration routes which the authors performed within this study showed that juvenile European herring gulls ringed in the Baltic States can be later observed in almost all European countries, pointing at their possible role in spreading clinically relevant bacteria across Europe. Gulls from other continents, particularly in South America, were also found to carry colistin-resistant bacteria. Liakopoulos et al. recovered CTX-M-producing *E. coli* isolates that were also positive for the *mcr-1* gene from kelp gulls in Argentina [146]. In South America, *Enterobacteriaceae* isolates carrying *mcr-1* gene have been described in various human clinical materials as well as in poultry, pigs and chicken meat [147], suggesting the existence of potential sources of such bacteria for wildlife.

Apart from waterfowls, which were discussed above, colistin-resistant bacteria were reported in other groups of wildlife. The only report of *mcr-1*-positive isolates from wild mammals was published in 2018, describing a single isolate of CTX-M-15-producing *E. coli* ST405 from 86 fresh stool samples of Barbary macaques in Algeria [148]. Interestingly, the authors noted that the very same *E. coli* ST with *mcr-1* and $bla_{\text{CTX-M-15}}$ has also been obtained from human clinical samples in Algeria which may suggest the existence of an emerging clone with high AR and virulence circulating in diverse sources in the country. Plasmid-mediated colistin resistance was also reported in birds of prey in Pakistan. Mohsin et al. obtained a single isolate of CTX-M-15-producing *E. coli* in a migratory Eurasian coot that was also positive for *mcr-1* [149]. The study was conducted in a wetland habitat that hosts thousands of migratory birds coming from Siberia and Central Asia in winter. A single *E. coli* isolate with *mcr-1* has been recently reported from a black kite in Russia, and possible link to a local landfill used by these birds for feeding has been suggested [150].

Very interesting results highlighting environmental contamination by bacteria with emerging resistance mechanisms were obtained in a comprehensive study in a Chinese poultry production system [130]. Samples from hatcheries, commercial poultry farms, a slaughterhouse and supermarkets as well as samples from dogs, sewage, wild birds (swallows) and flies were collected. An alarming association of resistance to drugs of last resort, carbapenems and colistin, was found. Carbapenem-resistant *Enterobacteriaceae* isolates producing NDM enzymes were recovered from 33% of samples, and interestingly, 23% of these isolates also carried *mcr-1* gene. Positive samples included also those from flies and wild birds, suggesting the frequent dissemination of resistant isolates to the farm environment and the importance of wildlife in further dissemination from the farm. As mentioned earlier,

Table 3 The list of studies documenting *mcr*-positive isolates in wildlife

Reference	Animal	Country	Year of sampling	No. of samples (% positive)	No. of isolates	Other ARGs	Bacterial species/ST
[145]	European herring gull	Lithuania	nd	160 (0.6%)	1	nd	*E. coli*
[146]	Kelp gull	Argentina	2012	50 (8%)	1	$bla_{\text{CTX-M}}$	*E. coli* ST101
					3	$bla_{\text{CTX-M-14}}$	*E. coli* ST744 (4)
[149]	Eurasian coot	Pakistan	2014	nd	1	$bla_{\text{CTX-M-15}}$	*E. coli* ST354
[154]	Magellanic penguins	Brazil	2013	13 (7.6%)	1	$bla_{\text{CTX-M-1}}$	*E. coli* ST10
[130]	Swallow	China	2014–2015	10 (30%)	3	nd	*E. coli*
[129]	Black vulture	Spain	2015–2016	668 (0.1%)	1	bla_{CIT}	*E. coli* ST162
[148]	Barbary macaque	Algeria	2016	86 (1.1. %)	1	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *qnrB19*	*E. coli* ST405
[155]	Egret	China	2015	5 (25%)	1	nd	*E. coli*
[150]	Black kite	Russia	2018	16 (17%)	1	–	*E. coli*
[156]	Barn swallow	Thailand	2016–2017	178 (0.6%)	1	$bla_{\text{TEM-1B}}$, *mef*(A), *mef*(B), *qnrS1*, *sul3*, *tet*(A), *tet*(B)	*E. coli* ST101
[118]	Silver gull	Australia	2015–2017	562 (0.2%)	1	nd	*E. coli* ST345
[152]	Common blowfly	Thailand	nd	300 (16%)	48	nd	*E. coli* ST10 (7), ST58 (3), ST162, ST181 (2), ST201, ST218, ST457, ST549 (4), ST648 (5), ST1244, ST2345, ST2705, ST5487; KP (17)

(continued)

Table 3 (continued)

Reference	Animal	Country	Year of sampling	No. of samples (% positive)	No. of isolates	Other ARGs	Bacterial species/ST
[157]	Gull	Spain	2009	695 (0.6%)	4	*aac(3), aadA, aph (3″), aph (3′), aph(6),* $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{SHV-10}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1}}$, *catA1, catA2, catB, cmlA1, dfrA1, dfrA12, dfrA15, erm (B), mdf*(A), *mph*(A), *oqxA, oqxB, sul1, sul2, sul3, tet*(A), *fosA*	*E. coli* ST57, ST448, 746; KP
		Portugal	2009	425 (0.2%)	1	*aadA,* $bla_{\text{SHV-12}}$, *cmlA1, mdf* (A), *sul3*	*E. coli* ST665

nd not determined, – no ARGs detected, *ST* sequence type. Abbreviation is used for *K. pneumoniae* (KP), and the affiliation to ST is presented only for *E. coli*

China is the country where the first isolate with plasmid-mediated colistin resistance was described and the use of colistin was historically high. Interestingly, retrospective studies were able to trace this resistance mechanism in chicken *E. coli* isolates back as early as the 1980s. A significant increase of *mcr-1* among poultry isolates between 2009 and 2014 was also observed [151]. These observations are consistent with the fact that colistin was highly used in China from the early 2000s [136] until its ban as a growth promoter in 2017. It has been suggested that the use of colistin in animal feed has probably accelerated the dissemination of *mcr-1* in animals and, subsequently, in humans.

Recently, insects have been recognized as important carriers and possibly significant vectors of colistin-resistant bacteria. In Thailand, 16.0% of flies originating from local markets in the urban community as well as from several suburbs and rural areas were found positive for *E. coli* and *K. pneumoniae* with *mcr-1* genes [152]. Other reports from China showed the presence of several *mcr* variants in housefly and blow fly [153]. Using quantitative PCR assays up to 37%, 1% and 11% of flies were found positive for *mcr-1*, *mcr-2* and *mcr-3*, respectively.

4.4 Bacteria with Plasmid-Mediated Resistance to Fluoroquinolones

Fluoroquinolones are widely used in veterinary and human medicine. Quinolone resistance is mostly caused by point mutations in certain positions in DNA gyrase or topoisomerase within the bacterial chromosome. However, *Enterobacteriaceae* isolates with plasmid-mediated quinolone resistance (PMQR) have been increasingly reported worldwide in the last decade [158]. PMQR genes provide low-level resistance to quinolones that may not reach clinical breakpoints. However, PMQR genes facilitate the selection of mutants with higher resistance to quinolones, and the combination of both chromosomal- and plasmid-mediated mechanisms in one bacteria is quite common.

PMQR genes have been described mainly in *E. coli*, *Salmonella enterica* and *Klebsiella* spp. not only in human clinical samples but also in farm animals and food [158]. Of the various PMQR variants described so far, some of them seem to be more prevalent than others, and these are often linked to particular sources. A large European survey of *S. enterica* and *E. coli* isolates with reduced susceptibility to fluoroquinolones showed their widespread occurrence especially among human and poultry isolates and the predominance of *qnrS1* and *qnrB* variants [159]. Another prevalent PMQR determinant is *aac(6′)-Ib-cr* which is usually found in a cassette as part of an integron on MDR plasmids along with ESBL, AmpC or carbapenemase genes [158], highlighting the importance of co-selection in the epidemiology of PMQR determinants.

In contrast to the large amount of research describing the dissemination of ESBL- and AmpC-type beta-lactamase-producing bacteria into wildlife, fewer studies have focused on isolates with PMQR determinants [82, 86, 107, 112, 160–163]. Various PMQR genes have been identified in bacteria from wildlife, mainly *qnrS1*, *qnrB* variants and *aac(6′)-Ib-cr*; the later one usually present along with ESBL genes. Other PMQR variants such as *qnrA*, *qnrC*, *qnrD*, *qepA* or *oqxAB* are not that widespread in general [158] and also rarely found in wildlife.

A large-scale screening of more than 1,000 samples of European rook faeces from nine roosting sites across Europe [162] showed that the *qnrS1* gene was common in faecal microbiota of birds from five countries, especially from the sites examined in the Czech Republic and Poland. Similarly, studies in water bird species such as mallards, great cormorants and herring gulls from Central Europe showed the predominance of *qnrS1* in *E. coli* isolates with reduced susceptibility to fluoroquinolones [82, 107]. Such isolates have also been described in wild boars in Poland [164]. PMQR genes, especially *qnrS1* and *qnrB19*, have been frequently reported in chickens and turkeys in Poland [165] and the Czech Republic [166, 167] as well as in wastewaters from slaughterhouses that process poultry meat [166], suggesting the poultry farming as an important source of these resistance mechanisms for the environment. In contrast to data on European wildlife, predominance of various *qnrB* variants and low prevalence of *qnrS1* gene have been reported in American crows in the USA [163].

4.5 Vancomycin-Resistant Enterococci

Enterococci, mainly *Enterococcus faecalis* and *Enterococcus faecium*, are opportunistic pathogens associated with serious hospital-acquired infections. Resistance to glycopeptides (e.g., vancomycin) is among the most medically relevant AR in this genus. VRE are etiological agents of bacteraemia, endocarditis and infections of surgical wounds and the urinary tract [168]. In general, resistance to vancomycin is more common in *Ent. faecium* compared to *Ent. faecalis*. The main mechanism of glycopeptide resistance in enterococci involves the alteration of the peptidoglycan synthesis pathway. Up to now, several genes associated with resistance to glycopeptides have been identified in enterococci including *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN*. Among these resistance mechanisms, those mediated by plasmids and associated with the genes *vanA*, *vanB* and *vanM* are the most important as they can spread via horizontal gene transfer to other enterococci and to other Gram-positive cocci, especially to *S. aureus* [168, 169].

Livestock have been implicated as a reservoir for the transmission of vancomycin resistance to zoonotic pathogens [170]. In 1975, avoparcin was introduced for growth promotion in livestock. It was used extensively in most countries in Europe as well as in the rest of the world mainly in broilers and pigs but to some extent also in other food-producing animals. As avoparcin confers cross-resistance to vancomycin, the use of this agent is selected for VRE. Later on, the connection between avoparcin use and the increasing occurrence of VRE in farm animals was confirmed. In Europe, the use of avoparcin was discontinued as a precautionary measure to avoid further spread of VRE to the community and into hospital settings. This action was followed by declining VRE colonization not only in farm animals and food but also in human infections [171]. Similar strains of VRE have been isolated from both livestock and humans, supporting the zoonotic potential of VRE. However, VRE isolates from hospitals generally show unique genotypes distinct from the isolates found in animals [172]. Several high-risk clones have been identified to play an important role in the epidemiology of VRE dissemination. For example, hospital-adapted *Ent. faecium* clonal complex 17 (CC17) or *Ent. faecalis* sequence type 6 (ST6) are frequently isolated from patients [173].

Recent studies have shown that VRE are also present in wild animals. In Europe, VRE carrying vancomycin resistance genes were found in various wild bird and mammal species. *Ent. faecalis* and *Ent. faecium* isolates with *vanA* have been isolated from red foxes, seagulls, buzzards and other wild birds in Azores [174–176] and from migrating gulls in Sweden [177]. In another study, faecal samples of red-legged partridge from the north of Portugal were tested for VRE, and 6 (2%, $n = 305$) *vanA*-positive *Ent. faecium* have been recovered [178]. In a large study conducted in several countries across Europe, VRE were screened in faeces of European rooks, and only 8 (<1%) isolates of *Ent. faecalis* and *Ent. faecium* harboured *vanA* [179].

VRE have occasionally been reported in wild birds in North America. VRE are rarely found in healthy community and livestock on this continent [180], likely reflecting the fact that avoparcin has never been used as a growth promoter in the USA and Canada. Enterococci carrying *vanA* were isolated from wood frogs in Michigan, USA [181]. Recent research on American crows revealed the occurrence of VRE with *van* genes in 15 (2.5%) of 590 faecal samples collected in four states [182]. Twenty-two isolates of *Ent. faecalis* and *Ent. faecium* carrying *vanA* were obtained. Isolates were assigned to different ST and were predominantly *Ent. faecium* ST18 and *Ent. faecalis* ST16 which are double-locus variants of hospital-associated high-risk CC17 and CC2, respectively. In the USA, the occurrence of VRE outside hospital settings is rare [183]. Crows often live close to humans and obtain food from anthropogenically impacted environments and may thereby be colonized with ARB of human origin. The prevalence of VRE in US hospitals is rapidly increasing from 0.3% in 1989 to 7.9% in 1993 up to 12% in 2006, and the most rapid increase is seen in intensive care units [184]. In the study conducted on American crows, the prevalence of VRE varied among states with the highest (6%) prevalence found in samples collected in Massachusetts. Several hospitals reporting relative high occurrence of VRE [185] were located close to the sampling site in Massachusetts, suggesting the possible human origin of the VRE in crows. Another study focusing on American crows compared bird isolates with those obtained from WWTP, dairy farms and environmental samples from the roosting site of the crows [186]. Four (2.5%, $n = 156$) *vanA*-positive *Ent. faecium* were identified from crows isolated from three examined sites. They observed that crows were more likely to carry VRE than either the cow faeces or environmental samples from the roosting sites.

Reports of VRE are not strictly limited to areas with strong human impact. Interestingly, one of the first reports of VRE in wildlife originates from Alaska [187]. During a polar research expedition to the Beringia region in 2005, the researchers collected faecal samples from birds at sites with no or low human population. Two *Ent. faecium* isolates with *vanA* were recovered from 33 sampled glaucous gulls. A clinically important clonal *Ent. faecium* CC17 lineage characterized by significant AR and virulence was found, thus strongly supporting a human origin.

Studies on VRE in mammals are less common. One example is a study on healthy wild boars recovered in Portugal [188]. *Ent. faecium* isolates with *vanA* were recovered from two of 67 faecal samples (3%) collected. In Spain, wild rats were found to carry *vanB*-positive *Ent. faecalis* and *vanA*-carrying *Ent. faecium* [189].

4.6 Methicillin-Resistant Staphylococci

S. aureus is an important human pathogen causing skin and soft tissue infections as well as severe life-threatening infections such as pneumonia and sepsis. Methicillin-resistant staphylococci and especially MRSA have become of special

concern [190, 191]. MRSA was initially isolated from humans as hospital- or community-acquired bacteria and later began to be detected in pets and livestock.

Methicillin resistance in *S. aureus* is encoded by the gene *mecA* which is located within a mobile genetic element called staphylococcal cassette chromosome *mec* (SSCmec). The *mec* genes encode penicillin-binding protein 2a (PBP2a), an enzyme responsible for cross-linking the peptidoglycan in the bacterial cell wall [192]. PBP2a has lower affinity for beta-lactam antibiotics, resulting in resistance to these antimicrobials including methicillin. In 2011, another *mec* gene, *mecC*, encoding an alternative PBP protein on a novel SSCmec was discovered [193]. Methicillin-resistant *S. aureus* harbouring either *mecA* or *mecC* have been reported from humans, livestock, companion and wild animals. Livestock-associated MRSA (LA-MRSA) have emerged, especially in countries with high-density livestock [191]. There is no significant pandemic strain, although several dominant MRSA clonal complexes have been described in humans and animals. In human patients MRSA CC1, CC8, CC22 and CC45 are currently isolated. LA-MRSA usually belong to CC9, CC30 and most importantly CC398. They have spread in several countries and have been also occasionally isolated from human infections [190, 191].

MRSA have been reported in various wildlife species in several countries, mainly in wild mammals [67, 194–199]. A single strain belonging to CC8, a human-associated lineage, was found in one out of 76 faecal samples from wild birds in Poland [194]. Migratory European rooks from Austria were found to carry MRSA belonging to diverse CC [67]. Foxes from rural and semi-rural areas in the UK frequently carried *mecA*-positive coagulase-negative staphylococci [195]. Most isolates were identified as *S. sciuri* group followed by *S. equorum* and *S. capitis*. The *mecA* gene was found in 33 (89%) isolates obtained from 38 examined foxes, suggesting that these animals are potential reservoirs of *mecA*-positive staphylococci. Three MRSA strains with the *mecA* gene were isolated in faeces of 61 small mammals in Slovakia [197]. Another example is a Portuguese study in which samples from the mouth and nose of 45 wild boars collected during hunting were examined for MRSA; only one isolate of CC398 was obtained [198].

Staphylococci isolates carrying the novel *mecC* gene have been also found in wildlife [196, 200–205]. The gene *mecC* is situated on an SCCmec XI element and was found in several CC of MRSA. It has been suggested that CC130 is a zoonotic lineage of *S. aureus* and that *mecC* likely originates from animal pathogens. It has been proposed that some wild animal species such as European hedgehogs could serve as reservoirs of *mecC*-positive isolates. A first study describing two isolates of CC130 *mecC*-MRSA in hedgehogs was published in 2013 in Sweden [203]. The first strain originated from an animal found dead in 2003 in central Sweden, suffering from *S. aureus* septicaemia. The second strain was obtained from a hedgehog with severe dermatitis on the island of Gotland in the Baltic Sea in 2011. ST130-MRSA-XI isolates with SCCmec XI element were obtained from lesions from both hedgehogs and were essentially identical to previously described isolates from humans. This primary observation was followed by another study documenting the occurrence of *mecC*-MRSA in wild hedgehogs

in Sweden [206]. *mecC*-MRSA was confirmed in 35 (64%, $n = 55$) animals from three geographically separated areas, and most of them belonged to CC130.

MRSA belonging to CC130 and carrying *mecC* were also found in a wild rodent [204]. Other studies from Spain described the frequent occurrence of *mecC*-MRSA CC130 (16.9%) in red deer and small mammals and also suggested that wildlife could be a source of *mecC*-MRSA which could potentially be transmitted to other animals, or even to humans [200, 201]. Moreover, indistinguishable *mecC*-carrying MRSA isolates were detected in brown hares, domestic cattle and sheep [202] sharing the same habitat, suggesting the frequent transmission between livestock and wildlife.

4.7 Antibiotic-Resistant Nontyphoidal Salmonellae

Nontyphoidal *Salmonella enterica* is an important pathogen and one of the most common bacterial agents of foodborne gastrointestinal disease in humans worldwide. It can also colonize the intestinal tract of various animals, including pigs, cattle, poultry, companion animals and wildlife. In animals, salmonellae can either cause disease, or they can be carried asymptomatically [207]. *S. enterica* have been isolated from several wild animal species including lizards, snakes and amphibians [208], birds, wild raptors [209] and mammals [210]. Wild animals carrying *Salmonella* spp. can develop disease such as enteritis, septicaemia and abortion, or they can work as healthy asymptomatic carriers. *Salmonella* infection has been recognized in wild birds of prey [211, 212] or is known to cause outbreaks during winter months resulting in death especially in small passerines [213, 214]. Fatal infections associated with serovar Choleraesuis or Saintpaul have been also reported in wild boars [215].

It has been shown that bird species living close to humans and feeding on human refuse or sewage are more likely to carry *Salmonella*, while the carriage rate in the wild bird population in more remote areas with less anthropogenic influence is low [46]. Additionally, genetically closely related strains have been isolated from humans, food-producing and companion animals and wildlife, suggesting that wildlife species can act as reservoirs for *Salmonella*. Large-scale surveillance of *Salmonella* serotypes from humans, food, domestic animals and wildlife in Australia revealed some overlap between serotypes in companion animals and wildlife, with cats in particular having a large number of serotypes in common with wild birds [216]. Several cases of human outbreaks due to *Salmonella* from wild animals have been reported [217, 218]. For example, in Tasmania, Australian wildlife species are the likely reservoir for *S.* Mississippi, contaminating land and water environments [219]. The role of wild animals in the epidemiology of *Salmonella* in food-producing animals has been investigated in several studies. Wild birds and rodents may influence the dynamics of subclinical pig salmonellosis, either by introducing the bacteria onto the farm or as receptors of an infection already established at the farm [220]. Similarly, wild roof rats were found to persistently

carry *Salmonella* and contaminate commercial poultry facilities through faecal shedding [221].

Previous studies demonstrated that some groups of wild birds, especially gulls, are among the most important environmental *Salmonella* reservoir in Europe. It has been reported that gulls near sewage outflows are more likely to carry *Salmonella* [222]. In one silver gull colony in Australia, gulls predominantly feeding on a landfill were frequent (28%) carriers of *Salmonella* of 16 different serotypes [31]. However, AR among salmonellae from this study was low. AR *Salmonella enterica* has been also investigated in free-living gulls in Spain [223]. Of the 39 isolates, 17 showed MDR profile and 8 of these belonged to serovar Typhimurium. Other clinically relevant MDR *S. enterica* belonged to serovars Hadar, Bredeney and Virchow. In another study from the Czech Republic, 38 *Salmonella* from 284 young black-headed gulls were obtained [224]. Ten different serotypes were detected, and 37% isolates were resistant to antimicrobials. *Salmonella* of serovars Agona, Enteritidis, Infantis and Senftenberg with identical genomic profiles as those isolated from the discharged treated water from a WWTP located 35 km upstream from the sampled gull colony were found, thus indicating the possibility of resistant isolates spreading over long distances in the environment. Similarly, genetically related isolates of *S.* Typhimurium DT195 have been described in black-headed gulls, domestic animals and humans in Sweden, suggesting that gulls might play an important role in the spread of salmonellae in the country [225]. High (24%) prevalence of salmonellae among seagulls has been also reported in Spain [226].

AR salmonellae have been isolated from birds of prey [212, 227]. Interestingly, high carriage rates of *S. enterica* in Egyptian vultures and other avian scavengers associated with the consumption of pig carcasses intentionally discarded as supplementary food for these species have been reported [227]. In this study, *Salmonella* strains isolated from Egyptian vulture faeces were all resistant to tetracyclines, and they also frequently showed resistance to ampicillin, amoxicillin, streptomycin and neomycin. An MDR monophasic variant of *S.* Typhimurium predominantly found in these wild raptors is also the most frequently reported serovar in fattening pigs in Spain. This study showed that MDR strains of zoonotic *Salmonella* can spread from medicated livestock to wildlife, thus creating new reservoirs that can also act as amplifiers and long-distance dispersers. MDR salmonellae of various serovars have also been isolated from mammals such as otters, wild boars, rodents or foxes [228–230].

5 Studying AR in Wildlife: Indicator Bacteria and Methodological Approaches

E. coli is one of the predominant enteric species in the normal intestinal microbiota of vertebrates, and it is also the most widely used indicator bacterium for addressing AR dissemination in different niches and host species [231]. It is ubiquitous and

found globally in human and animal guts and is also used as an indicator of faecal contamination in the environment. However, some serotypes or sequence types of *E. coli* are more pathogenic; they are equipped with various virulence factors and can cause severe diseases in humans and animals [232]. Since *E. coli* is broadly used to monitor AR development in the medical and veterinary sectors, it is also a suitable target for studying AR in the environment and in wildlife. The use of the same indicator bacterium and methodological approaches makes it feasible to compare the prevalence of resistance and to detect transfer of ARB and ARG across diverse hosts and environments.

Apart from studying the occurrence and characteristics of strains resistant to diverse groups of antibiotics within *E. coli* populations in wildlife, many studies focus on specific clinically important resistance mechanisms. For this purpose, antibiotic-supplemented media are usually used to selectively isolate the target resistant bacteria from rectal or cloacal swabs and fresh animal faeces. *E. coli* producing ESBL enzymes has been the common target bacteria with the resistance mechanism of epidemiological and clinical relevance. Apart from *E. coli* or the order *Enterobacteriaceae* in general, other indicator and pathogenic bacteria have been investigated in wildlife including VRE, MRSA and MDR *Salmonella* spp. as described in Sects. 4.5–4.7. The occurrence and dissemination of these bacterial pathogens and clinically important resistance mechanisms in wild animals are discussed in Sect. 6.

Wildlife species have been used as sentinels to measure the occurrence of AR in the environment and as a reflection of the situation in humans and livestock. Most studies have focused on wild animals living close to human populations, livestock or in human-influenced settings, thus addressing the health risks at the human-animal-environmental interface. For example, a study from Uganda showed that gorilla populations that overlapped in their use of habitat with people and livestock carried *E. coli* isolates that were genetically similar to *E. coli* from those people and livestock. On the contrary, isolates from gorillas that did not overlap in their use of habitats with people and livestock were more genetically distinct from human or livestock bacteria [233]. Most studies describing the transmission of AR into wildlife focused on wild birds as they are good indicators reflecting the presence of resistant microorganisms in different ecosystems. Many bird species are abundant, they frequently use anthropogenic habitats with high antibiotic pressure, and their movement over long distances enables easy dispersal of ARB [80, 81].

Detailed characterization of the ARB isolated from wildlife using molecular typing methods allows to analyse their genetic similarities to human and livestock isolates, providing the evidence of cross-species transmission. For this purpose, rep-PCR or other methods with better resolution and reproducibility such as multilocus sequence typing (MLST), pulse-field gel electrophoresis (PFGE) or whole-genome sequencing (WGS) are used [234]. MLST scheme is available for many bacterial species including *E. coli* and broadly used in molecular epidemiology along with PFGE to reveal clonal relatedness of epidemiologically unrelated strains. In the MLST protocol, internal regions of selected housekeeping genes are sequenced; the strain is subsequently characterized by its unique allelic profile and assigned

to a specific ST. It allows to identify strains of identical ST isolated from different hosts and sources (Fig. 6). It has been widely used to type ARB in wildlife, especially ESBL-producing *E. coli* from wild birds, and to compare the results to others sectors [86, 132, 133, 235–238]. These studies pointed out that identical STs or clonal groups are present in humans, food-producing animals, the environment and wildlife, suggesting the interspecies transmission of phylogenetically related AR strains. However, genotypes of bacteria in many studies rarely overlap between wildlife, human and livestock, which indicate that the exchange of genes and mobile genetic elements might also play an important role in the spread of AR to wildlife [77, 84, 239]. Identical or highly related plasmids disseminating clinically relevant resistance mechanisms among human and animal pathogens have been also identified in bacteria from wildlife [6], highlighting the role of horizontal gene transfer.

Currently, WGS is becoming an important tool in surveillance, clinical diagnostics and infection control of AR in both the human medical and veterinary sectors. WGS is highly reproducible for determining genetic relatedness, based on MLST or broader, and also provides a complete genetic profile including the content of ARGs, virulence factors and mobile genetic elements [240]. The genetic relatedness

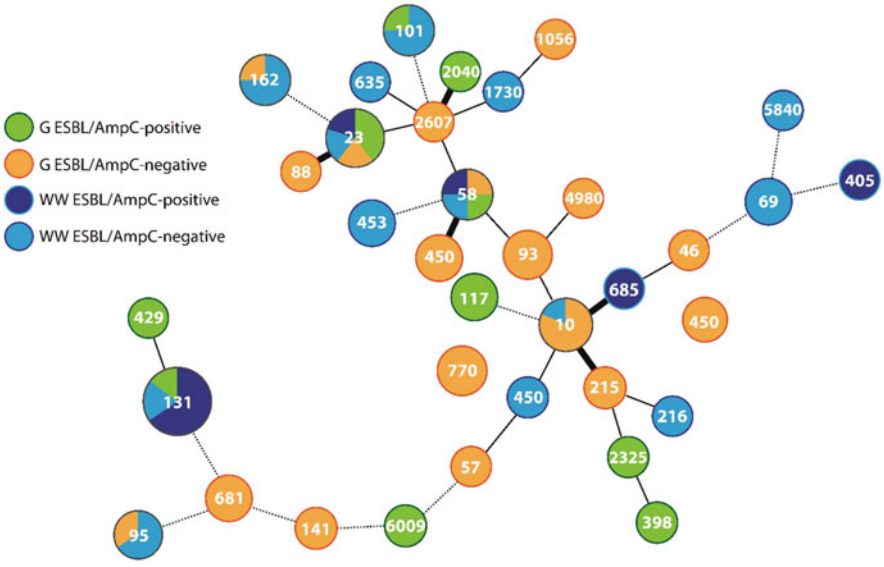

Fig. 6 A minimum spanning tree of *E. coli* isolates from black-headed gulls and wastewaters in the Czech Republic using MLST scheme [242]. The figure is based on unpublished data by Masarikova and Dolejska as a follow-up of a previous study [224]. Each circle represents a particular ST based on MLST data. The number inside the circle stands for a particular ST type; e.g. 10 stands for ST10. The size of the circles is proportional to the number of isolates. The number of allelic differences between different STs is indicated by the different styles of the connecting lines: thick solid lines (1–2 differences), narrow solid lines (3–4 differences) and dashed lines (5–6 differences). Colouring is based on the source of isolation (G, gull; WW, wastewater) and ESBL/AmpC gene content

by using WGS-based data can be then determined by MLST schemes or more specifically by the phylogenetic analysis of single-nucleotide variants (SNVs). In comparison to PCR-based techniques, it allows more accurate and high-resolution comparison of isolates from different hosts. It has been recently utilized in several studies focused on wildlife [84, 132, 150, 238] or helped to identify epidemic clones shared across humans, food-producing and companion animals and wildlife [238, 241].

6 Conclusions

Over the past decade, ARB have been widely described in various wild animal species around the globe. The spread of bacterial pathogens resistant to critically important and last-line antibiotics into wildlife is especially concerning. Most studies assumed that AR found in wild animals is a consequence of a spill over from domestic animals and humans. Current data indicate that biology and ecology of the host along with the level of anthropogenic impact in wildlife habitats are among the key factors driving the occurrence of AR. The possible transmission routes to wild animals through waste, sewage and manure contaminating the aquatic and terrestrial environments have been suggested. Interventions to minimize transmission of AR from intensive livestock and human populations into the environment (e.g. better waste management) are therefore crucial. Moreover, wild animals have been not only used as sentinels of AR in the environment, but their role as vectors further disseminating resistant bacteria in the environment and to humans and animals has been highlighted in scientific literature.

Although various bacteria and resistance mechanisms have been reported in wildlife, most studies conducted over the last decade have focused on ESBL-producing *E. coli* as a suitable indicator that makes the comparison to human and veterinary sector feasible. As nearly all environments and wildlife around the globe are influenced by anthropogenic activities, a worldwide One Health collaborative approach is needed to address all essential aspects of AR. Unfortunately, current national and international programmes remain biased towards human and domestic animal AR surveillance, and the environment is often overlooked. Harmonized and globally comparable One Health surveillance that also includes the environment and wildlife should be our goal in the coming years.

Although it is not questionable that wild animals represent important vehicles in dissemination of the AR, the role of globally moving species such as migratory birds in AR transmission over long distances remains unclear and needs further clarification. To resolve this, it is crucial to understand whether wild animals are just temporary carriers of the AR or if the resistant bacteria obtained from the contaminated environment can be maintained in their gut for a long time, giving more opportunities for further transmission to other individuals in the population or through the environment to other species. Studies with longitudinal observations may provide useful information on dynamics of the ARB in the gut of wildlife

species. The mechanisms that allow resistant bacteria to persist in wild populations despite the energetic burden of ARGs and the absence of antibiotic-selective pressure should be addressed in future studies. Another aspect of AR in regard to selective pressure that is yet not covered by the scientific literature is the potential role of antibiotics and other selective agents ingested via water or food influencing the gut microbiota of wildlife and selecting for ARB.

Spread of AR in wildlife deserves considerable attention. More research focusing on human-animal-environmental interface and using novel or combined approaches is required to understand the role of wildlife in the transmission of AR and to estimate the risks for public health. Technologies such as whole-genome sequencing of ARB isolates carrying resistance mechanisms of interest are highly useful for identifying shared bacterial strains across different sectors. However, other methods broadly used to evaluate the abundance of AR in water and soil such as metagenomics could also provide useful information. Although WGS-based comparative analysis was utilized in wildlife studies in past 5 years, the use of metagenomics is still scarce. Our better understanding of the abundance and duration of bacterial shedding in wildlife will clearly provide essential insights into their relative importance as reservoirs and vectors of AR.

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Genomic Surveillance for One Health Antimicrobial Resistance: Understanding Human, Animal, and Environmental Reservoirs and Transmission

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Abstract Whole-genome sequencing (WGS) has significantly improved our ability to understand how, through gene acquisition, bacteria can become resistant to antibiotic therapies and cause an increasingly substantial burden of disease. In this chapter, we take the well-known indicator bacteria and opportunistic pathogen *Escherichia coli*, predicted to be one of the leading causes of antimicrobial resistance (AMR) infections in the next decades, and demonstrate the potential insights that can be gained using WGS and genomic epidemiology. Specifically, we discuss the mechanisms by which these bacteria acquire, retain, propagate, and disperse gene combinations with a focus on key mobile genetic elements, notably ColV/BM plasmids. Efforts are underway to further standardise and streamline WGS and resistome screening from multiple environments to support the rapidly increasing user base and facilitate regional and global public health monitoring, outbreak tracking, and AMR evolutionary prediction and preparedness. The ability of *E. coli* to exist in multiple environments as both a pathogen and commensal organism are central to its value for establishing meaningful One Health systems-based AMR monitoring, mitigation, and management.

Keywords APEC, *Escherichia coli*, ExPEC, One Health, Whole-genome sequencing, Zooanthroponoses, Zoonoses

1 Genomic Surveillance for One Health AMR

The development of rapid and affordable whole-genome sequencing (WGS) technology has given scientists a bacterial subtyping tool of unprecedented precision and discrimination that has revolutionised diagnostic microbiology and microbial surveillance [1]. WGS is progressively replacing/supplementing traditional phenotyping and genotyping workflows [2], and as it becomes increasingly accessible and affordable, its utility in revealing the evolutionary dynamics of bacteria is also proving central to state-of-the-art antimicrobial resistance (AMR) surveillance [3]. There are now numerous real-world examples showing how WGS and genomic epidemiology can be used to understand and track the way AMR variants can emerge, be maintained, and spread at different spatiotemporal scales within human populations (e.g. within hospital wards, healthcare networks, and internationally [4–9]). Advances in WGS have not only revolutionised molecular epidemiology for the diagnosis and treatment of infectious disease in clinical settings but also for public health surveillance and the epidemiology of food-borne pathogen outbreaks

[10]. WGS can be used to provide more reliable microbial identification, definitive phylogenetic relationships, and comprehensive catalogues of resistance and virulence traits that are of central relevance in epidemiological investigations. These advances have been greatly facilitated by the open sharing of free bioinformatics resources, including dozens of resources designed to detect AMR determinants in sequence data [11]. WGS-based pathogen surveillance and AMR tracking will become even more powerful once critical genomics pipelines and databases become standardised [2].

WGS allows scientists to track the transmission and diversification of specific bacterial populations as well as the genetic determinants of AMR present in those populations. It can thus reveal the detailed temporal and spatial dynamics of AMR evolution as well as the impact of AMR selection on pathogen populations [4]. WGS also opens a window on the complex gene transmission dynamics affecting multispecies, multi-environment host-webs [12–14]. The genetic basis for resistance can be either intrinsic, mutation associated, or acquired via horizontal gene transfer (HGT) (also known as lateral gene transfer (LGT)) [15–17]. This is one of the most important factors influencing AMR maintenance and transmission within bacterial populations/communities as this will determine whether resistance genes are passed vertically to descendants or horizontally to a broader host range both within and across bacterial genera [18]. When the fitness cost is minimal and ongoing selective pressure is maintained, local clonal expansion may occur [4, 19]. WGS has shown that clonal expansion and subsequent geographical spread of virulent/resistant bacteria can often be traced to the acquisition of specific AMR genes, indicating that mobile AMR elements can play an important role in determining which clones dominate locally, regionally, and globally [4]. An example is the $bla_{\text{CTX-M-15}}$ gene, which has reportedly contributed to the global success of the widespread pathogenic *E. coli* clone named sequence type (ST) 131 that is annually responsible for millions of AMR infections globally [20, 21]. The $bla_{\text{CTX-M-15}}$ gene is also implicated in the successful expansion of important *Klebsiella pneumoniae* clones such as CG14/15 and ST101 [22]. Successful AMR clones such as these can contribute significantly to the further spread of resistance through subsequent gene transfer, especially where those genes are located on broad host range plasmid vectors (e.g. IncF plasmids) [4].

Humans are exposed to AMR bacteria and genes through the environment, food chain, and numerous complex host interactions [23–25]. This has been highlighted by reports describing high AMR gene carriage rates in bacterial populations in the gut of healthy humans [12, 26–28]. They in turn impact on the spread of AMR genes and bacteria via multiple pathways, including waste streams, human-human, and human-animal contact [29]. Growing recognition of this complexity has prompted calls for a 'One Health' multisector-based approach to AMR management. It is increasingly evident that WGS has an important role to play in translating global, national, and local One Health AMR strategies from high-level policy documents into active surveillance to inform interventions [3, 25]. By staying abreast of current and emerging AMR trends across a diversity of AMR hotspots and transmission points, it may be feasible to slow the spread of key AMR determinants and/or take actions to minimise their impact. WGS provides important information about

combinations of multidrug resistance (MDR) and the genetic context of resistance genes that can be of great significance for predicting co-selection potential (e.g. from other antimicrobial agents such as metals and disinfectants) and the possibility for HGT [2, 3, 30]. Importantly, it can also shed light on the distribution of MDR bacteria and HGT vectors across a variety of different hosts (humans, animals, sentinel species, etc.) as well as suspected environmental hotspots that may function as persistent AMR reservoirs and transmission sources [13, 14, 23, 31]. An important knowledge gap that can only properly be explored through a One Health approach is the potential role of these different environments and reservoirs in the evolving ecology of resistance [25, 29, 32]. Microbial communities are far more complex than the pathogen centric view that has prevailed in public health surveillance to date, and many significant AMR genes reside in commensal gut bacteria of healthy humans and animals or in common/previously considered insignificant environmental microbes [2, 12, 23, 26, 28, 33]. Moreover, infections often considered 'food-borne' are not always actually transmitted by food-borne pathogens, but acquired through contact with non-food sources such as animals, humans, or environmental exposure [34]. As such, they can only be thoroughly explored and addressed in a One Health context.

2 Genomic Workflow for the Detection of Antimicrobial Resistance Genes (ARGs)

Whereas culture-based antibiotic susceptibility and nucleic acid amplification tests are often limited by the finite number of phenotypes and genes being targeted, WGS provides high-resolution data on complete ARG carriage across the entire genome. WGS also has the added advantage of concurrently identifying mobile genetic elements (MGEs), such as insertion sequences (IS), transposons, phages, genomic islands, integrative and conjugative elements (ICE), and plasmids, which can help reveal evolutionary HGT events and the potential of ARGs to spread [4, 35, 36]. Furthermore, WGS simultaneously identifies any metal or biocide resistance genes within ARG-carrying MGEs which could lead to AMR co-selection in the presence of relevant selective pressures [37, 38], as well as any single-nucleotide polymorphisms (SNPs) in genes that can give rise to AMR [39].

Performing WGS and then analysing the data for ARGs is a multistep process (Fig. 1). The key stages are DNA extraction, shotgun sequencing, genome assembly, and then ARG screening and data visualisation. Each stage presents options as there are multiple sequencing platforms now available and a myriad of bioinformatic tools for assembling and analysing WGS data (a small selection are provided in Table 1), most of which require some aptitude in command line. Here we provide a brief and broad overview of a genomic workflow for the detection of ARGs in bacterial genomes.

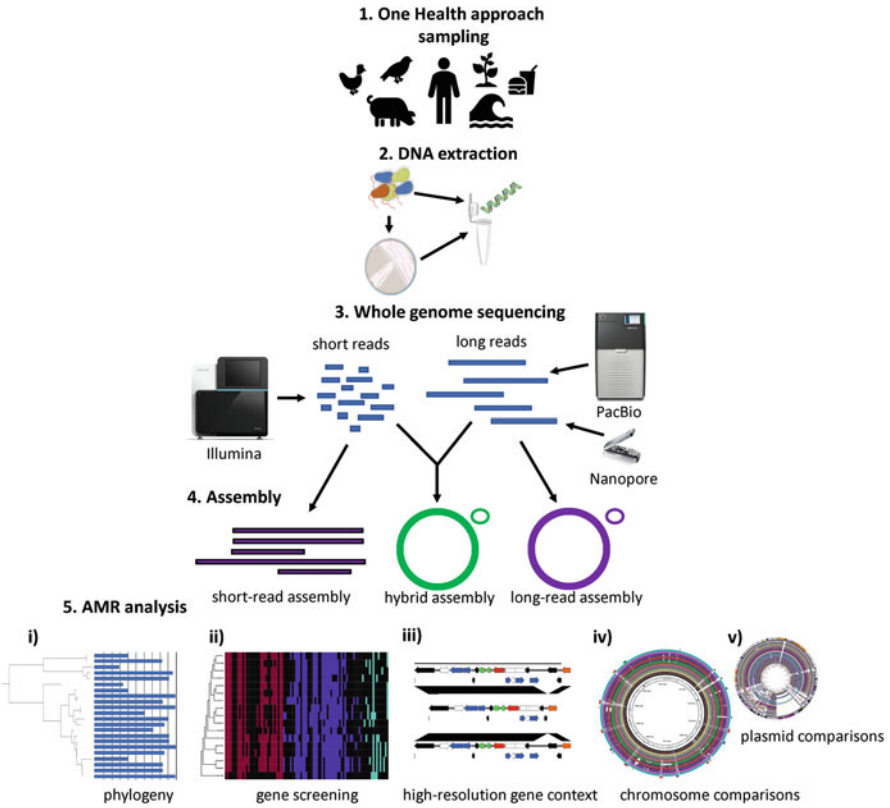

Fig. 1 Example of a whole-genome sequencing workflow. (1) Samples sourced from humans, animals, and the environment. (2) DNA is extracted either directly from sample (metagenomics) or from pure culture. (3) Shotgun sequencing can be performed on short-read platforms, such as Illumina, or long-read platforms, such as PacBio and Nanopore. (4) Genomes are assembled using various software. Long reads and short reads can be used together to produce hybrid assemblies. (5) Examples of WGS outputs: (i) phylogenetic analysis, (ii) gene screening, (iii) comparisons of AMR regions, (iv) comparisons of complete chromosomes and plasmids, (v) comparisons of complete plasmids

Once DNA has been extracted from samples, choosing which sequencing platform to use comes with trade-offs regarding accuracy, efficiency, and cost [65, 66]. The advantages of next-generation Illumina sequencing are the low costs and production of low error (0.1%) reads; however these reads are short (<300 bp) meaning subsequent assembly typically results in genomes fragmented into multiple contigs and collapsed repeat regions [67]. Repetitive elements are abundant in MGEs and AMR regions [16]. Consequently, complex AMR regions and plasmids are rarely resolved using Illumina sequencing. On the other hand, third-generation sequencing technologies, such as Pacific Biosciences (PacBio) and Oxford

Nanopore, come with increased costs and error rates but produce long DNA reads (typically 10–100 kbp) often spanning lengthy repeat regions, which allow for the resolution of complex AMR regions, plasmids, and complete chromosomes [67].

Regardless of the chosen method, sequencing produces FASTQ files as outputs – large text files containing sequence data and quality scores for each base – which can be screened immediately for ARGs using tools such as ARIBA [51]. However, to utilise most ARG databases, and garner any additional information, including gene context, genome assembly is required. Genomes can be assembled de novo or by mapping to a reference. While de novo assembly generally produces more fragmented genomes, it is less biased than reference-based assembly [67]. Several different de novo assemblers have been created targeting either short-read or long-read data (examples in Table 1); however recent benchmarking exercises have identified SPAdes and Flye as the highest performing assemblers for short- and long-read data, respectively [68, 69]. Though relatively costly, if both short- and long-read data has been generated, it is also possible to perform de novo hybrid assemblies, which use the accuracy of Illumina data to correct errors in third-generation sequencing long reads, thereby achieving highly accurate closed genomes [67, 70]. Currently, Unicycler outperforms other tools for hybrid assembly [45, 70]. Once complete, assemblies then undergo gene annotation, typically via automated pipelines such as Prokka [48] or RAST [49].

Assembled genomes in FASTA format can be inputted directly into a growing number of AMR databases (selection provided in Table 1) which use search algorithms (most commonly BLAST) to detect ARGs by aligning the inputted sequence to ARG sequences stored within the database. The notable exception is ARGminer, which additionally utilises machine learning to predict novel ARGs [47]. Once identified, the region containing ARGs can be interrogated using visualisation and analysis software, such as SnapGene and Geneious [63], to ascertain its genetic context, and of particular interest, whether the genes occur on MGEs.

3 Using WGS for One Health AMR Surveillance

In the remainder of this chapter, we present a number of examples focused on key *Enterobacteriaceae*, and in particular *E. coli*, to demonstrate the benefits and challenges of using WGS for One-Health AMR surveillance and management.

4 *Enterobacteriaceae* and AMR

Within the *Gammaproteobacteria* [71], the *Enterobacteriaceae* family is one of the most important taxa. It is taxonomically highly diverse, currently including more than 50 genera and 210 species and continuing to be revised in light of new information from molecular genetics, WGS, and phylogenetic analyses

Table 1 Selection of bioinformatic tools used in bacterial WGS processing and AMR analysis

Assembly				
Name	Type	Core algorithms/ software	Available at	Reference
Flye	De novo for long- reads	Repeat graph	github.com/ fenderglass/Flye	Kolmogorov et al. [40]
GAAP	Reference-based	cGOF	gaap.big.ac.cn	Yuan et al. [41]
Miniasm	De novo for long-reads	Overlap-layout-consensus, minimap2	github.com/lh3/ miniasm	Li [42]
Raven	De novo for long- reads	Overlap-layout-consensus, racon, minimap2	github.com/lbcb-sci/raven	Unpublished
SOAPdenovo2	De novo for short-reads	de Bruijn graph	github.com/ aquaskyline/ SOAPdenovo2	Luo et al. [43]
SPAdes	De novo for short-reads	de Bruijn graph, paired assembly graph	cab.spbu.ru/software/spades	Bankevich et al. [44]
Unicycler	De novo for hybrid assemblies	SPAdes, semiglobal alignment	github.com/rrwick/ Unicycler	Wick et al. [45]
Velvet	De novo for short-reads	de Bruijn graph	github.com/ dzerbino/velvet	Zerbino and Birney [46]

Annotation			
Name	Core algorithms/software	Available at	Reference
DeepARG	Machine learning	bench.cs.vt.edu/ deeparg	Arango-Argoty et al. [47]
Prokka	Prodigal, RNAmmer, Aragorn, SignalP, Infernal	github.com/ tseemann/prokka	Seemann [48]
RASTtk	BLASTn, tRNAscan-SE, Glimmer3, Prodigal, KMA	rast.nmpdr.org	Brettin et al. [49]

AMR databases and arg screening tools				
Name	Type	Core algorithms/ software	Available at	Reference
ABRicate	ARGs, custom	BLAST	github.com/tseemann/ abricate	Unpublished
ARGminer	ARGs, MGEs	BLAST, machine learning	bench.cs.vt.edu/argminer	Arango-Argoty et al. [50]
ARIBA	ARGs, custom	Minimap, Bowtie2	github.com/sanger-pathogens/ariba	Hunt et al. [51]
BacMet	Detergent, metal resistance	BLAST	bacmet.biomedicine.gu.se	Pal et al. [52]

(continued)

Table 1 (continued)

AMR databases and arg screening tools				
Name	Type	Core algorithms/ software	Available at	Reference
CARD	ARGs	BLAST, RGI	card.mcmaster.ca	Alcock et al. [53]
INTEGRALL	Integrons	BLAST	integrall.bio.ua.pt/?	Moura et al. [54]
ISFinder	Insertion sequences	BLAST	isfinder.biotoul.fr	Siguier et al. [55]
KmerResistance	ARGs	KMA	hcge.cbs.dtu.dk/services/KmerResistance	Clausen et al. [56]
MEGARes	ARGs, deter-gent, metal resistance	BWA	megares.meglab.org/	Doster et al. [57]
NCBI AMRFinder	ARGs	BLAST, HMMER	ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder	Feldgarden et al. [58]
PATRIC	ARGs	BALST	patricbrc.org	Wattam et al. [59]
PointFinder	Mutations in chromosomal genes	BLAST, KMA	cge.cbs.dtu.dk/services/ResFinder	Zankari et al. [60]
ResFinder	ARGs	BLAST, KMA	cge.cbs.dtu.dk/services/ResFinder	Zankari et al. [60]

Sequence visualisation and analysis tools			
Name	Note	Available at	Reference
BRIG	Displays circular comparisons between genomes (Fig. 1: 5 iv and v)	brig.sourceforge.net	Alikhan et al. [61]
EasyFig	Displays linear comparisons of genomic loci (Fig. 1: 5 iii)	mjsull.github.io/Easyfig	Sullivan et al. [62]
Geneious	Comprehensive sequence analysis suite, includes WGS visualisation and editing, expression analysis and variant calling	geneious.com	Kearse et al. [63]
Mauve	Aligns multiple genomes and highlights rearrangements and inversions	darlinglab.org/mauve/mauve.html	Darling [64]
SnapGene	WGS visualisation and editing	snapgene.com/	Unpublished

[72, 73]. *Enterobacteriaceae* are disseminated widely in animals (farmed, wild, and companion), humans, soils, water, and vegetation and are notable agents of drug-resistant infections [24, 25, 74–76]. The family includes the most prevalent Gram-negative pathogens isolated in clinical microbiology laboratories, such as *E. coli* and *K. pneumoniae*. These cause a variety of hospital- and community-acquired infections at various anatomical sites including urinary and respiratory tracts, blood, cerebrospinal fluid, the peritoneum, synovial fluid, and wound abscesses, and multidrug resistance rates in these organisms are increasing worldwide [77]. Similarly, *Enterobacteriaceae* in intensively reared food production animals are often resistant to multiple first-generation antibiotics [74, 78] and are less susceptible to clinically important last-line drugs such as the extended-spectrum β-lactams (ESBLs), carbapenems, and colistin, with rates of resistance in many countries rising sharply in recent decades [79, 80]. HGT plays a major role in how these organisms acquire repertoires of virulence and antimicrobial resistance genes (ARGs) and is contributing significantly to the rapid spread of resistance because it effectively allows microbes to share a large gene pool and acquire new genetic material from outside their clonal lineage [81]. This chapter focuses predominantly on *Enterobacteriaceae* member *E. coli*, which is the predominant cause of urinary tract infections globally and a leading cause of bacteraemia and sepsis [77, 82–84]. *E. coli* also accounts for a significant proportion of the current and projected burden of clinically relevant AMR.

5 *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobic bacterial species commonly found in the lower gastrointestinal tract of mammals [85]. A study in which *E. coli* was isolated from the faeces of more than 2,300 nondomesticated vertebrate hosts representing more than 350 species (and all major vertebrate groups occurring in Australia) reported that mammals with hindgut modifications sufficient to allow microbial fermentation are the primary ecological niche of *E. coli* [86]. Subsequent research has shown that although *E. coli* are less prevalent in birds, reptiles, and fish, these can also be important hosts [75, 86–90]. As *E. coli* is primarily found within animal/human hosts, external sources (e.g. surface waters) are often considered to be important in the context of faecal contamination and wastewater management. Yet, although *E. coli* are considered coliforms and their presence is routinely used as indicators of faecal contamination, natural environmental strains of *E. coli* have also been described, challenging existing paradigms about how this organism should be viewed with respect to microbial source tracking [91–93].

Most *E. coli* strains are considered to be commensal organisms, coexisting peacefully within their hosts without harming or helping them. They may also be mutualistic, providing benefits to the host such as vitamin K2 production, aiding in food digestion, and preventing pathogens from attaching and colonising the gastrointestinal tract [94–96]. However, the frontier between commensals and pathogens is

not clear-cut, and *E. coli* is a classic example of a commensal organism that can become pathogenic [95, 97].

6 *E. coli*: Intestinal and Extraintestinal Pathogens

6.1 *Intestinal Pathogenic* E. coli *(IPEC)*

Intestinal *E. coli* are classified as pathotypes based on carriage of specific subsets of virulence genes. As summarised in Table 2 these pathotypes include the enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent *E. coli* (DAEC), and adherent-invasive *E. coli* (AIEC) [98–101]. Carriage of specific combinations of virulence genes that have been acquired by HGT play an important role in differentiating pathotypes from commensal *E. coli* populations. Pathotype designations have been useful for the past 70 years, but WGS studies show that pathotype delineations are increasingly complicated by the emergence of hybrid *E. coli* that carry combinations of virulence-associated genes (VAGs) that are used to delineate different pathotypes [101]. One prominent and well-publicised example of

Table 2 *E. coli* pathotypes

Group	Pathotypes	Refs
IPEC: Intestinal pathogenic *E. coli* or DEC: Diarrheagenic *E. coli* Associated with diarrhoeal disease	EPEC: Enteropathogenic *E. coli* ETEC: Enterotoxigenic *E. coli* EHEC: Enterohemorrhagic *E. coli* EIEC: Enteroinvasive *E. coli* EAEC: Enteroaggregative *E. coli* DAEC: Diffusely adherent *E. coli* STEC: Shiga toxin producing *E. coli* AIEC: Adherent-invasive *E. coli* (not associated with diarrhoea but thought to contribute to the development of Crohn's disease)	Nataro and Kaper [98]; Kaper et al. [99]; Croxen et al. [100]; Robins-Browne et al. [101]
ExPEC: Extraintestinal pathogenic *E. coli* Cause wound infection, urinary tract infection, peritonitis, pneumonia, meningitis, and septicaemia	UPEC: Uropathogenic *E. coli* NMEC: Neonatal meningitis-associated *E. coli* SEPEC: Sepsis-associated *E. coli* APEC: Avain pathogenic *E. coli*	Pitout [102]; Leimbach et al. [97]; Robins-Browne et al. [101]; Rodriguez-Siek et al. [103]

a hybrid *E. coli* lineage is the pathogenic clade of *E. coli* O104:H4 that caused the world's largest food-borne outbreak of haemolytic uremic syndrome, in Germany in 2011 [104]. The outbreak strain also carried an extended-spectrum β-lactamase (ESBL) gene. The causative agent was linked via a European level trace back exercise to sprout seeds sourced from Egypt and was responsible for 54 deaths and 845 cases of haemolytic uremic syndrome out of a total 3,816 infections [104]. The O104:H4 clade responsible for the German outbreak was a highly virulent EAEC that had acquired a Shiga toxin 2 gene (stx_2) [105]. It was followed by a closely related O104:H4 lineage outbreak of food-borne illness in France [106]. These O104:H4 outbreaks were not the first instance of hybrid *E. coli* causing severe disease in humans. A small outbreak of haemolytic uremic syndrome in Picardy, France, in 1998 was caused by a lineage of serotype O111:H2 reported to be a hybrid *E. coli* with a combination of EHEC and EAEC VAGs [107].

6.2 *Extraintestinal Pathogenic* E. coli *(ExPEC)*

ExPEC are *E. coli* that have captured both fitness and VAGs that enable them to successfully colonise sites outside of the gastrointestinal (GI) tract and cause disease. ExPEC are regarded as the most common Gram-negative pathogen affecting human health [83]. Although ExPEC strains comprise many different lineages, only a subset is responsible for the vast majority of infections. The uropathogenic *E. coli* (UPEC) are remarkable in this regard; they are responsible for the vast majority of urinary tract infections (UTIs) that cause bladder (cystitis) and kidney (pyelonephritis) infections and are the leading cause of morbidity in the female population [108, 109]. ExPEC also cause neonatal meningitis and are associated with wound infections, particularly in patients with diabetes mellitus [109, 110].

7 ExPEC Virulence Factors and AMR

ExPEC infections are increasingly resistant to multiple antibiotics and associated with higher case fatality rates thus imparting a significant burden on public health [102]. ExPEC that cause UTIs carry a wide range of VAGs including multiple pili, iron acquisition systems, secreted toxins, and polysaccharide capsules. Some VAGs are located on mobile, pathogenicity-associated islands (PAIs), while others are located on plasmids; thus ExPEC strains are heavily influenced by HGT. There are a large number of plasmids carrying mobile genetic elements that include both ARGs and VAGs [111]. By way of example, we will focus here primarily on ColV/BM plasmids [112].

7.1 ColV/BM Plasmids

ColV plasmids are self-transmissible IncF plasmids whose genetic cargo is contained within a region of DNA comprising about 94-kb. ColV plasmids were recently defined as having at least one gene from four or more of the following: (1) *cvaABC* and *cvi* (ColV operon), (2) *sitABCD* (iron-siderophore and B12 transporter operon), (3) *hlyF* (regulator of outer membrane vesicle biogenesis) and *ompT* (outer membrane serine protease), (4) *iucABCD* and *iutA* (aerobactin operon), (5) *iroBCDEN* (salmochelin operon), and (6) *etsABC* (ABC transport system) [113]. ColBM plasmids are closely related and share significant homology to ColV plasmids; however rather than encoding for the ColV colicin, they encode for colicins B and M [112] (Fig. 2).

ColV/BM plasmids are associated with *E. coli* that cause diverse extraintestinal afflictions both in humans [114, 115] and in poultry [74, 112]. They have been associated with *E. coli* linked to life-threatening clinical presentations including urosepsis [116, 117], haemolytic uremic syndrome (HUS) [118], and neonatal meningitis [117] and are widespread in *E. coli* recovered from poultry with colibacillosis [74, 112, 115]. ColV/BM plasmids are considered a defining feature of avian pathogenic *E. coli* (APEC) [74, 103]. Their presence is linked to enhanced pathogen potential and disease severity. This is illustrated by the success of a recently emerged lineage of *Salmonella enterica* serovar Kentucky that showed an increased ability to colonise the caecum of chickens and express extraintestinal disease in poultry [119]. *S. enterica* serovar Kentucky has only recently emerged as a threat to poultry health, and this has coincided with the acquisition of ColV plasmids [119, 120]. Characterisation of a ColV plasmid with features typical of

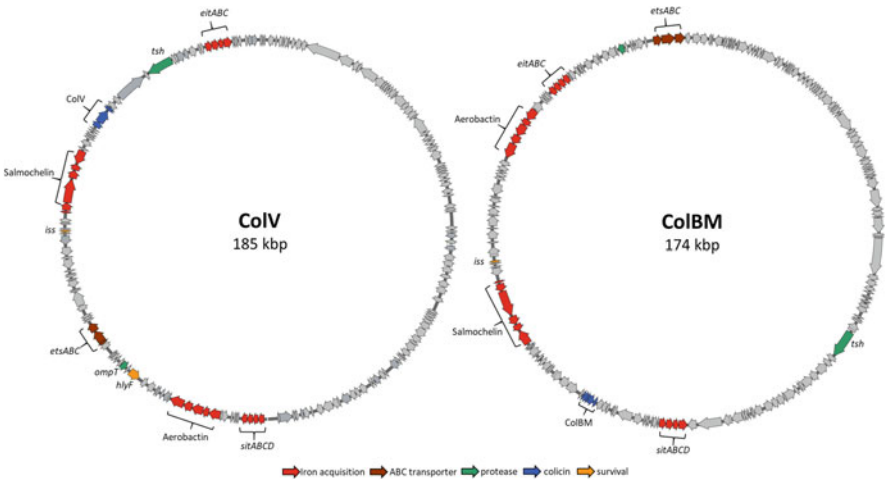

Fig. 2 ColV and ColBM plasmids. Sequences derived from pAPEC-O1-ColV (NC_009837.1) and pAPEC-O1-ColBM (NC_009837.1)

ColV plasmids from poultry in a clade of *S. enterica* serovar Kentucky linked to retail meat has potential zoonotic implications [119, 120]. Related ColV plasmids were also identified in important serovars of *S. enterica* including Typhimurium and Heidelberg [119].

7.2 ST131:H22 Lineages Carrying ColV Plasmids

Whole-genome sequencing (WGS) of 1,923 *E. coli* isolates from retail meat (chicken, pork, and turkey) and 1,188 human-derived isolates from urine and blood cultures undertaken over the same 12 month period in Flagstaff, Arizona, USA, identified 433 STs [113]. ST131 was a dominant ST overall representing 182/1188 (15.3%) human clinical isolates; one lineage represented by *E. coli* ST131-fim type H22 (ST131:*H22*) represented 24/25 (96%) meat isolates and 24/182 (13%) human clinical ST131 isolates. Multiple sublineages of ST131:H22 were identified of which two indicated a human – meat association, particularly with poultry. Notably, most meat isolates (84%) and a quarter of the human clinical isolates carried ColV plasmids, with the human isolates carrying ColV plasmids aligning with the meat isolates using core genome phylogeny. The study by Liu et al. [113] adds to a growing list implicating food, particularly poultry, but also swine [31] as a source of *E. coli*-carrying ColV plasmids that cause disease in humans and identifies that ST131:*H22* lineages are significant in this regard.

7.3 Hybrid Plasmids

Hybrid plasmids carrying multiple incompatibility replicon types often carry substantial antibiotic resistance gene cargo including genes encoding resistance to cephalosporin and carbapenem drugs [121]. It is notable that IS*26* features prominently in most of these chimeric plasmids which have been observed to be an emerging phenomenon in China [121], Australia [122, 123], and elsewhere. The acquisition of a broader number of plasmid replicon types is thought to enhance plasmid maintenance and host range and is a concerning development [121, 123, 124]. IS*26* plays an important role in the formation of hybrid plasmids, generating plasmids with combinations of virulence and antibiotic resistance genes, while at the same time, enhancing plasmid maintenance and host range [30, 124–127]. These fusion events are increasingly complex as demonstrated by the description of plasmids p721005-KPC/p504051-KPC from China in which substantial regions of DNA from different plasmids (including backbone sequences from IncR and IncpA, maintenance and conjugal regions from IncFIIK and IncFIIY plasmids pKPHS2 and

pKOK_NDM-1, and replicon genes representative of IncN1) have assembled on the same entity [121].

8 WGS One Health Insights: ExPEC, APEC, and Zoonoses

Intensive animal production systems underpin efforts to feed a burgeoning global human population and the poultry, pig, and aquaculture industries represent some of the fastest-growing industries globally. ExPEC (APEC) strains are responsible for enormous economic losses in the poultry industry [128] and increasingly linked to significant disease burden in pig and aquaculture industries [129, 130]. Earlier studies alluded to genetic similarities in UTI patient *E. coli* isolates, community-derived human isolates, meat, and production animal isolates suggesting that *E. coli* strains from meat and production animals may pose a zoonotic risk to humans [129, 130] and has led to them being referred to as food-borne UTIs (FUTIs) [131]. Data from WGS analyses have demonstrated that humans and food animals share ExPEC and plasmid cargo [31, 113]. Although similarity in virulence and ARG profiles do not necessarily translate to proof of (unidirectional) transmission [132], several seminal studies point to carriage of ColV and related plasmids in human and animal disease as being important in conferring zoonotic potential [113].

As noted previously, ColV/BM plasmids are common in ExPEC and a defining feature of APEC which, as the causative agents of colibacillosis, are the leading cause of mortality and morbidity in poultry production (chickens and turkeys). Colibacillosis is a multifaceted, economically devastating, and geographically widespread disease that primarily affects broiler chickens 4–6 weeks of age, though birds of all ages are susceptible. APEC are found in the intestinal flora of healthy commercial bird species and cause disease at various anatomical sites including the respiratory tract (aerosacculitis), oviduct (salpingitis), serous membranes affecting the pleura, peritoneum and pericardium (peritonitis, pericarditis, and polyserositis), liver (perihepatitis), blood (colisepticaemia), the yolk sac (omphalitis), bone tissue, and joints [133]. Many systemic infections caused by APEC begin via colonisation of the respiratory tract followed by migration to the pericardium and liver before initiating bacteraemia. Birds raised in suboptimal housing and burdened with chronic viral and bacterial respiratory pathogens have compromised mucociliary defences and are particularly susceptible to infections caused by APEC. As such, APEC were thought to be opportunistic pathogens that target flocks with decreased immunocompetency [134–136]; however, evidence suggests that some lineages of APEC are primary pathogens that cause severe disease and severe mortalities despite high standards of flock management, comparatively low stress levels and the absence of concurrent disease [137–139].

There is clear evidence that combinations of extraintestinal VAGs involved in iron-acquisition, host-cell toxicity and bacteriocidicity, host-cell adhesion and invasion, and host-immune evasion play an important role in APEC disease in poultry and ExPEC disease more broadly. As noted above, many of the putative virulence

genes associated with APEC disease are also associated with human ExPEC, and these observations have led to the hypothesis that poultry-associated *E. coli* may pose a zoonotic threat [140]. While poultry carcasses showing signs of APEC infection are generally condemned post-slaughter, poultry products, such as meat, eggs, and fertilisers, are routinely contaminated with avian faecal flora, a proportion of which carry APEC-associated VAGs and ARGs with zoonotic potential [141]. Genome sequence-based epidemiological approaches to investigate zoonotic potential, although in their infancy, have revealed the genetic diversity inherent in APEC populations. Avian *E. coli* can grow in human urine, resist mammalian complement, and invade human epithelial cells; however, the molecular basis for APEC pathogenesis is complex, remains poorly understood [142–144], and is complicated by the heterogenous nature of VAG profiles for APEC strains [145].

Resistance to antibiotics used for treating ExPEC infections has been steadily increasing [83], and APEC may act as conduits for mobile elements that carry combinations of ARGs and VAGs between human and avian populations. APEC are often resistant to a range of antimicrobial agents, including tetracyclines, chloramphenicol, sulphonamides, aminoglycosides, fluoroquinolones, β-lactam, and ESBL antibiotics [109]. Resistance to first-generation antibiotics is frequently reported globally although different resistance genes predominate in different geographic locations. Recently, WGS of APEC isolates that carry the class 1 integrase gene *intI1* from different geographic locations around Australia revealed carriage of genes encoding resistance to sulphonamides, trimethoprim, ampicillin, streptomycin, and tetracycline but not to antibiotics of major therapeutic significance for human health. The most frequently identified antibiotic resistance genes were the sulphonamide resistance genes *sul1* (59/95, 62%) and *sul2* (31/95, 33%); tetracycline resistance genes *tet(A)* (51/95, 54%), *tet(C)* (18/95, 19%), and *tet(B)* (12/95, 13%); trimethoprim resistance genes *dfrA5* (48/95, 51%) and *dfrA1* (25/95, 26%); ampicillin resistance gene $bla_{\text{TEM-1A/B/C}}$ (48/95, 51%); and streptomycin resistance genes *strAB* (36/95, 38%), *aadA1* (32/95, 34%), and *aadA2* (4/95, 4%) [74]. This supports the suggestion that Australian antibiotic stewardship practices have been effective in minimising carriage of resistance to clinically significant antibiotics in production animals. WGS studies of class 1 integron-carrying commensal *E. coli* from the faeces of swine [78] and from APEC [74] reported no carriage of genes encoding resistance to carbapenems, extended-spectrum β-lactams, or colistin, an observation largely supported by more targeted studies of ExPEC from intensive animal industries where minimal carriage of genes encoding resistance to ESBLs and fluoroquinolones was reported [146]. Unfortunately, the same cannot be said for carriage of clinically important ARGs in synanthrophic wild bird populations. Several studies have shown that Australian gull populations carry genes encoding resistance to a wide spectrum of antibiotics including broad-spectrum β-lactams, fluoroquinolones, and colistin [75, 147].

9 WGS One Health Insights: The Case of *bla*$_{\text{IMP-4}}$ Metallo-Beta-Lactamase (MBL)-Producing *Enterobacteriaceae* in Eastern Australia

Bla-IMP-4 metallo-beta-lactamase (MBL)-producing *Enterobacteriaceae* pose a significant threat to the health of patients visiting hospitals. *bla*$_{\text{IMP-4}}$ is the most common carbapenemase-resistance gene carried by Gram-negative enterobacterial populations that circulate in Australian hospital settings. In one study in a Sydney Hospital, 30 clinical isolates from 23 patients, including those with bacteraemia, wound infections and venous catheter tip infections, and 71 isolates from the hospital shower facilities and various other fomites (objects/materials likely to carry infection) carrying *bla*$_{IMP-4}$ were described [148]. Another notable finding in that study was that the environmental isolates from showers and fomites persisted despite targeted disinfection. This emphasises the challenge of infection reservoir control in complex environments, where resistant microorganisms may also carry accessory genes conferring resistance to important disinfectants and cleaning agents such as chlorhexidine and triclosan [149–152].

Conjugative plasmids play an important role in the capture, assembly, and spread of *bla*$_{\text{IMP-4}}$ and genes encoding resistance to other clinically important drug classes. These same plasmids often retain genes encoding resistance to many first-line antibiotics and heavy metal resistance genes [153, 154]. In hospitals in Sydney, Brisbane, and Melbourne, *bla*$_{\text{IMP-4}}$ forms part of a gene cassette with sequence *bla*$_{\text{IMP-4}}$*-qacG2-aacA4-catB3* within class 1 integron structures that has been found in different genetic contexts and embedded on diverse plasmid backbones including IncA/C, IncL/M, and IncHI2 [155–157]. The *bla*$_{\text{IMP-4}}$ gene has been linked with these and other plasmids including IncF and IncN in gulls [75]. It is notable that IS*26* appears to have been a prominent player in the assembly of complex resistance gene loci carrying *bla*$_{\text{IMP-4}}$*-qacG-aacA4-catB3* [155].

Since *bla*$_{\text{IMP-4}}$ was identified as part of the same cassette array (*bla*$_{\text{IMP-4}}$*-qacG-aacA4-catB3*) in ten bacterial species (including *E. coli*), from a single, large seagull colony off the Eastern Australian coast [75], efforts to understand environmental sources of these clinically important genes have increased. In gulls the *bla*$_{\text{IMP-4}}$*-qacG-aacA4-catB3* cassette array was associated with IncHI2, hybrid IncA/C$_2$, IncL/M, IncF, IncIN, and IncI1 replicons [75, 127]. To date, carriage of *bla*$_{\text{IMP-4}}$ in gulls sampled across Australia seems to be restricted to that specific colony of gulls off the east coast [147]. WGS of 284 *E. coli* resistant to clinically important antibiotics from 562 faecal samples from gulls recovered from coastal regions in all states of Australia except the Northern Territory and the Australian Capital Territory and separated by distances greater than 3,500 km reported carriage of a wide range of ARGs among a diverse collection of clinically important *E. coli* STs including ST69, ST38, ST131, ST10, and ST1193 [147], but although *bla* genes featured prominently (including *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{CTX-M-14}}$), *bla*$_{\text{IMP-4}}$ was not detected [147]. Given these observations, there is a pressing need to better understand how the gulls in that colony acquired *bla*$_{\text{IMP-4}}$ and other

clinically important antibiotic resistance genes. Worryingly, *Enterobacteriaceae* that carry $bla_{\text{IMP-4}}$ are reported in association with human infections with increasing frequency, particularly in China [89, 90, 158]. IncHI2 plasmids, especially those belonging to IncHI2:ST1, seem to be playing a prominent role in the transmission of this carbapenem resistance gene in China with a report of a 314,351-bp IncHI2:ST1 plasmid pIMP-4-EC62 isolated from a MDR *Enterobacter cloacae* isolate from swine that carries a wide range of antibiotic resistance genes including $bla_{\text{IMP-4}}$ [159].

10 WGS One Health Insights: The Role of Environmental AMR

The impacts of human activities on environmental AMR are now widely recognised, and WGS is playing an increasingly important role in understanding these effects. From pharmaceutical manufacturing [160] to farming and food production practices [24, 161, 162], international travel [163], and waste management [164], research has demonstrated the potential to affect the environmental resistome. It is clear that we need to better understand the environmental sources and reservoirs of antibiotic-resistant bacteria and ARGs [165]. As part of this quest, WGS surveillance has a promising role to play in proactively detecting and managing emerging ARG trends. It is not uncommon for surface- and groundwaters used for irrigation to be contaminated with potentially harmful bacteria, nor is it plausible to prevent some level of microbial activity in pipe biofilms and food production systems [166–168]. Where systemic infection in a farming or food production system, industrial process, or water supply is an issue, it can lead to highly complex outbreak patterns as the actual incidence of disease may be quite infrequent, particularly if the contamination is at a constant but low level that is only infrequently leading to disease (e.g. [169]). Such cases will likely have gone unnoticed using previous outbreak detection and strain typing systems such as pulsed-field gel electrophoresis (PFGE) and are one example of how the superior resolving power of WGS is able to cope with the longer time intervals and greater geographical spread of some of the more challenging trace back cases [1]. Nonetheless, WGS is not without challenges when it comes to the complexities of One Health surveillance. One particular challenge when working with potential environmental hotspots is that outbreak strains that have colonised and continued to evolve in the environment will have greater sequence variation than that encountered in a monoclonal point source outbreak [1]. This is a significant challenge to be aware of when using WGS for source tracking and risk assessment and likely requires careful ongoing surveillance and broad testing to compensate. Currently there is much greater availability of sequences from clinical isolates in public databases than from animal or environmental isolates. This is due to the greater prevalence of routine clinical screening programmes, but environmental WGS data are increasingly becoming available as integrated One Health WGS and genomic epidemiology programmes continue to be developed and data sharing platforms are

established. Overall, surveillance is expanding to take a more One Health approach, including animal pathogen and environmental testing, as, for example, now occurs through the GenomeTrakr programme [10]. This international consortium of laboratories organised by the US Food and Drug Administration (FDA) collect and sequence bacterial strains from a variety of food and environmental sources. Other initiatives such as the Global Microbial Identifier (GMI) initiative are also driving things forwards. The GMI was launched in 2011 to help establish global proficiency testing in WGS with the intention to support a global system of DNA genome databases for microbial and infectious disease identification and diagnostics (globalmicrobialidentifier.org/).

WGS case studies based on clinical transmission tracking have demonstrated that although resistance arises frequently during individual infections, most AMR variants are effectively low risk, with limited potential for transmission beyond the index patient in which the new variant evolved [4]. This highlights the value in using WGS to also monitor environmental evolutionary hotspots and transmission pathways to identify, understand, and address emergent high-risk MDR clones with the propensity for rapid spread and impact. In their recent WGS investigation into the clonal composition of MDR *E. coli* in wastewater, Mahfouz et al. [164] found high genomic diversity of MDR *E. coli* with very flexible genomes harbouring a wide variety of virulence genes and resistance determinants. They noted several interesting findings, including that the pan-genome of *E.coli* isolates of clinical origin [170] was smaller than the wastewater *E. coli* pan-genome, and indicated an overall trend of greater resistance to antibiotics that have been available since the 1950s and 1960s compared to the more recently commercialised antibiotics. Interestingly, wastewater treatment did not appear to alter the range and intensity of virulence factors in these genomes (153 and to 155 virulence factors per isolate for inflow and outflow, respectively), and virulence profiles from some effluent isolates were indicative of ExPEC. Mahfouz et al. [164] suggested that the high genomic diversity and large *E. coli* pan-genome they observed in wastewater likely reflected the contribution of multiple sources of animal faeces entering the wastewater *E. coli* population as a result of runoff and storm water and noted that they make an important contribution to the wastewater resistome.

Currently, major efforts to strengthen and standardise global One Health AMR surveillance by the World Health Organisation (WHO), Food and Agriculture Organization (FAO), and World Organisation for Animal Health (OIE) are focusing on *E. coli* – specifically, ESBL-producing *E. coli* – as a key One Health indicator of AMR. Under an initiative known as the ESBL Tricycle AMR surveillance project, ESBL-*E. coli* are measured in identical and controlled conditions in samples representing humans, animals/food-chain, and the environment [171]. This One Health surveillance programme is currently being implemented in a number of pilot Asian and African countries with ESBL-*E. coli* selected as the priority target for standardised surveillance because of their significant global health impacts and associated costs and because they readily cross barriers between humans, animals, and the environment. Importantly, ESBL-*E. coli* host carriage rates vary greatly between different countries and appear to correlate well with stewardship practices

in that efforts to decrease antibiotic use in humans and animals also correspond to decreases in ESBL-*E. coli* carriage [172]. *E. coli* constitute approximately 0.1% of human gut flora and as environmental water sources are prone to bacterial pollution [91, 173] *E. coli* have historically been treated as indicators for presence of faecal contamination. Yet their ecology is complex, and there is now ample evidence that *E. coli* can persist outside of the host gut environment [24, 91–93]. They have been found to form stable populations in soils [174–177], and wastewater [93], and can survive on and within fresh produce [24]. This includes some EHEC strains, including important Shiga toxin-producing strains such as O157: H7 and O111 [93]. Greater knowledge of *E. coli* reservoirs and directional flows and transmission pathways are needed to understand risks and direct mitigation efforts.

11 Outlook

WGS is emerging as a leading approach to support One Health AMR surveillance and research with much to offer in the context of understanding AMR reservoirs, evolutionary hotspots, and transmission. With the discovery that certain plasmids can confer increased fitness and phage can confer hypermobility to genetic regions in some bacterial species, it becomes increasingly important to characterise the entire genetic content of environmental reservoirs in order to predict the risk of ARG and VAG acquisition (pathogenicity) and potential clinical consequences of emerging strains and opportunistic pathogens. Research should focus not only on pathogens carrying AMR but also on commensals and environmental microbes due to the high propensity for HGT. The accumulation and spread of ARGs and VAGs often go hand in hand and delineating their origins, and combining this with evidence-based knowledge of HGT is essential for piecing together the One Health AMR puzzle. Numerous bioinformatics resources are already available to support such endeavours, and efforts are underway to further standardise and streamline ARG screening to support the rapidly increasing user base. Standardisation to ensure reliable prediction of AMR determinants is essential in the move towards supplementing and even potentially replacing traditional phenotypic AMR screening, as is currently under discussion and/or already planned in some jurisdictions.

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Antibiotic Resistance in Pharmaceutical Industry Effluents and Effluent-Impacted Environments

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Contents

Abstract Overuse and misuse of antibiotics are likely the leading causes of antibiotic resistance accumulation in human pathogens, but it has in the last decade been recognized that the discharges from antibiotic manufacturing facilities can also contribute. Such discharges have repeatedly been shown to provide conditions where antibiotics reach concentrations that are selective for resistance enrichment. Manufacturing facilities and environments receiving their effluents are, therefore, key to determining the magnitude of antibiotic resistance, as well as identifying the critical control points to slow its emergence and spread. This chapter endeavours to review the recent research in the extent of pollution from antibiotic-producing

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factories and the effects of this pollution in the external environment. It also provides a case study in Croatia summarizing the discharges from the manufacturing of the antibiotic azithromycin and the subsequent impact these discharges caused to the receiving river.

Keywords Antibiotic resistance, Environmental pollution, Manufacturing, Pharmaceutical effluent

1 Introduction

There are growing concerns worldwide about the risk of antibiotic resistance arising from the anthropogenic releases of antibiotics into the environment. In contrast to the environmental releases from usage and excretion of drugs, direct discharges from pharmaceutical manufacturing have been identified as a source of much higher antibiotic concentrations, sometimes even in the order of mg/L [1–5]. This runs counter to the claims of the pharmaceutical industry that the discharge of antibiotics is unlikely, owing to the high cost of raw materials and, consequently, nonprofitable production in the case of substantial discharge of antibiotics as waste. This was probably one of the reasons why manufacturing sites were generally unexplored until more than 10 years ago. The first paper in the series of papers by a team of Swedish researchers was published in 2007, showing extremely high concentrations of several pharmaceuticals, particularly fluoroquinolone antibiotics in the treated wastewater (effluent) from a wastewater treatment plant (WWTP) treating waste from about 90 drug manufacturers in Patancheru, near the Indian city of Hyderabad. In this effluent, the concentrations for some pharmaceuticals such as ciprofloxacin (a fluoroquinolone antibiotic) were greater than those found in the blood of patients undergoing treatment [6]. The exposure to these effluents was shown to induce various toxic effects in fish and other aquatic organisms [7]. In addition to the release of untreated or inappropriately treated effluents, local media investigations have revealed the practice of illegal toxic waste dumping, where pharmaceutical companies are discarding hazardous waste under the cover of night. Mass die-offs of fish have also made the headlines, and the dead fish were found to contain toxic solvents used during the drug manufacturing process.

High pollution by antibiotics was reported not only to be a problem in India but also in other Asian countries such as China, Korea and Pakistan, as well as, to a lesser extent, in Europe [1]. Therefore, direct discharges from antibiotic factories along with illegal dumping of antibiotic waste resulted in heavy pollution of rivers and lakes and ground- and surface waters with antibiotics and other hazardous chemicals from the production process. This has driven the selective enrichment of the antibiotic-resistant bacteria (ARB) and the antibiotic-resistance genes (ARGs) they carry, thus creating a pool of resistance genes, which can consequently be acquired by pathogens [8–12]. However, in addition to the antibiotics, industrial effluents may contain considerable amounts of ARB/ARGs, particularly after

biological treatment, which consequently significantly influence the amount of resistance found in exposed environments [2, 8, 13, 14]. The increase in resistance genes was invariably accompanied by the increased frequencies of the mobile genetic elements (MGEs) facilitating their mobility [15, 16]. Therefore, the manufacturing facilities and the environments receiving their effluents were identified as hotspots for resistance development, maintenance and/or transmission. The evaluations of WWTPs that treat pharmaceutical wastewaters and environmental compartments impacted by treated or untreated manufacturing waste are key to determining the magnitude of antibiotic resistance, as well as identifying the critical control points to slow its emergence and spread. Still, there are major knowledge gaps about the extent of antibiotic pollution from pharmaceutical production. The next part of this chapter will therefore review the recent research on antibiotics as chemical pollutants and ARB/ARGs as biological pollutants in pharmaceutical wastewater treatment plants. The subsequent section will describe the effects of discharges from pharmaceutical factories on the receiving aquatic environment in terms of the development and spread of the resistance. The fourth section of this chapter will describe a case study on the impact of antibiotic production in Croatia on antibiotic resistance and microbial communities in the receiving Sava River. Finally, the concluding section will describe the discharge management strategies that must be implemented in order to combat the antibiotic resistance crisis.

2 Pharmaceutical Wastewaters and Pharmaceutical Wastewater Treatment

Wastewaters in the antibiotic manufacturing industry usually originate from the synthesis or formulation of drugs. The synthesis of the drugs is usually a very chemical-intensive process which leads to the generation of significant quantities of wastewater containing toxic pollutants. These pollutants usually include organic solvents, catalysts, additives, reactants, intermediates and active pharmaceutical residues, which make wastewaters from drug production complex and difficult to treat. The characteristics of these wastewaters are often variable depending on the raw materials used, the technological processes, and the by-products of these processes [17–19]. In some developing countries, pharmaceutical effluents are often discharged into the environment without any treatment. However, in Western countries, these effluents usually go to municipal WWTPs, with or without pretreatment, or are treated in the WWTP operated by the pharmaceutical industry (PWWTP) [19–24]. Due to the variability of the composition of pharmaceutical wastewaters, it is difficult to specify a particular wastewater treatment system, as it depends on the type of industry and the associated wastewaters [17]. General approaches that have been employed to treat these wastewaters include physico-chemical treatments, aerobic/anaerobic biological treatments and advanced oxidation processes [4, 13, 25–27]. However, as the individual treatment approaches have

shown to be insufficient for the removal of all potentially hazardous constituents of pharmaceutical wastewaters, including antibiotics, it is often necessary to combine different treatment technologies [3, 13, 25]. One of the promising approaches in controlling environmental pollution stemming from pharmaceutical production is zero liquid discharge (ZLD) technology, defined as no effluent discharged into the surface waters from industry, eliminating the risk of environmental pollution. The ZLD usually involves the elimination of the liquid waste effluent stream from the plant using various techniques, including thermal and membrane-based processes, and the recycling of recovered water and solids [28]. However, high energy consumption and capital costs are currently the main barriers for wider implementation of this system. In the future, developing energy-efficient and cost-effective membrane technologies should make the ZLD more affordable and sustainable [28, 29].

Examining the literature for papers related to the treatment of wastewaters from antibiotic production, published in the period 2014–2018, shows that biological treatment technologies are the most commonly employed treatment processes (Table 1), and therefore, we will mostly focus on them.

2.1 The Fate of Antibiotics in Pharmaceutical Wastewater Treatment Plants

The fate of antibiotics in WWTPs treating wastewaters from antibiotic production is influenced by many different factors, including the composition of the raw wastewater (influent) and the design and operation of treatment systems. Different biological treatment methods, including conventional activated sludge, have been the methods of choice for the treatment of pharmaceutical wastewaters [4, 30–32]. The activated sludge (AS) facilities have shown varying efficiency of antibiotic removal, often decreased at high antibiotic concentrations due to the inhibition of the sludge microorganisms [33, 34]. Besides the antibiotic concentration, the type of antibiotics present in the industrial wastewater greatly affects the efficiency of the treatment. For example, the ß-lactam antibiotics appeared to be highly biodegradable due to the hydrolytic cleavage of the ß-lactam ring [3, 4, 31]. On the other hand, macrolides seem to be more recalcitrant towards biodegradation [14]. Furthermore, the high antibiotic selection pressure present in the AS facilities treating wastewater from antibiotic production was shown to significantly decrease the bacterial diversity of the sludge communities, which may affect not only the promotion of antibiotic resistance but also the general treatment efficiency [35].

In an attempt to improve the effectiveness of the AS treatment, many studies were focused on treating the industrial wastewater using membrane bioreactors (MBR), which integrate biodegradation of pollutants by the AS, with direct solid-liquid separation by membrane filtration. The MBR systems provided more effective removal of some antibiotics and other toxic pollutants and therefore offered a better solution to the industries [4, 5, 31, 36]. In addition, there are a number of promising

Table 1 Concentrations of antibiotics in influents and effluents from wastewater treatment plants treating wastewaters from antibiotic production, treatment processes employed by plants and their efficiency in antibiotic removal

Country	PWWTP	Antibiotic	Concentration of antibiotic in influent	Concentration of antibiotic in effluent	% of removal	Treatment process/technology	Reference
China	1	Tetracycline	2.01 mg/L	0.78 mg/L	61.2	CASS	[4]
		Oxytetracycline	41.75 mg/L	0.62 mg/L	98.5		
	2	Tetracycline	11.92 mg/L	0.44 mg/L	96.3	EGSB + MBR	
		Oxytetracycline	34.31 mg/L	3.20 mg/L	90.7		
		Chlorotetracycline	1.78 mg/L	0.31 mg/L	82.6		
	3	Trimethoprim	92.20 mg/L	0.14 mg/L	99.8	EAAS	
	4	Cephalosporin	0.13 mg/L	<LOQ	100.0	CAS	
China	1	Tetracycline	11.9 mg/L	n.a.	73.7	EGSB + MBR	[5]
		Oxytetracycline	334.3 mg/L	32.0 mg/L	62.4		
		Chlorotetracycline	1.8 mg/L	n.a.	62.8		
	2	Tetracycline	61.0 mg/L	2.6 mg/L	77.6	SBR + B-COT	
		Oxytetracycline	2.4 mg/L	n.a.	71.8		
		Chlorotetracycline	0.8 mg/L	n.a.	78.3		
China	1	Vancomycin	54.90 mg/L	500 μg/L	>99	MBBR + sedimentation	[30]
	2	Vancomycin	46.32 mg/L	240 μg/L	>99	Modified A2/O	
China	1	Tetracycline	11.06 mg/L	2.60 mg/L	76.5	SBR + B-COT + DAFT + chemical oxidation	[3]
		Oxytetracycline	0.47 mg/L	0.13 mg/L	72.3		
		Chlorotetracycline	0.15 mg/L	0.03 mg/L	80.0		
	2	Tetracycline	30 μg/L	10 μg/L	66.7	CAS + chemical oxidation	
		Oxytetracycline	210 μg/L	70 μg/L	66.7		
		Chlorotetracycline	50 μg/L	20 μg/L	60.0		
China	1	Ofloxacin	1.61 mg/L	88 μg/L	94.5	MBBR	[32]
	2	Enrofloxacin	5.72 mg/L	42.80 μg/L	99.3	Acidification + A2/O	
		Ciprofloxacin	4.35 mg/L	0.32 μg/L	99.9		

(continued)

Table 1 (continued)

Country	PWWTP	Antibiotic	Concentration of antibiotic in influent	Concentration of antibiotic in effluent	% of removal	Treatment process/technology	Reference
Croatia	1	Azithromycin	n.a.	Up to 3.78 mg/L	n.a.	Neutralization + MBR	[2]
		N-Desmethyl Azithromycin	n.a.	Up to 5.66 mg/L	n.a.		
		Erythromycin-H_2O	n.a.	Up to 2.01 mg/L	n.a.		
	2	Sulfadiazine	n.a.	Up to 20 µg/L	n.a.	Neutralization + mechanical removing of larger, floating solids	
		Sulfamethazine	n.a.	Up to 231 µg/L	n.a.		
		Trimethoprim	n.a.	Up to 5.60 µg/L	n.a.		
		Enrofloxacin	n.a.	Up to 98 µg/L	n.a.		
		Oxytetracycline	n.a.	Up to 29 µg/L	n.a.		
Pakistan	1	Ofloxacin	n.a.	81 µg/L	n.a.	n.a.	[22]
		Moxifloxacin	n.a.	0.7 µg/L			
	2	Moxifloxacin	n.a.	0.9 µg/L	n.a.	n.a.	
	3	Ofloxacin	n.a.	1.2 µg/L	n.a.	n.a.	
		Sparfloxacin	n.a.	19 µg/L			
	4	Ciprofloxacin	n.a.	2.2 µg/L	n.a.	n.a.	
	5	Ofloxacin	n.a.	50 µg/L	n.a.	n.a.	
		Sparfloxacin	n.a.	17 µg/L	n.a.		
Pakistan	1	Ciprofloxacin	n.a.	4.75 mg/L	n.a.	n.a.	[23]
		Ofloxacin	n.a.	3.33 mg/L	n.a.		
		Levofloxacin	n.a.	3.08 mg/L	n.a.		
		Oxytetracycline	n.a.	4.35 mg/L	n.a.		
		Doxycycline	n.a.	4.78 mg/L	n.a.		
	2	Ciprofloxacin	n.a.	4.80 mg/L	n.a.	n.a.	
		Ofloxacin	n.a.	3.20 mg/L	n.a.		

		Levofloxacin	n.a.	2.04 mg/L	n.a.		
		Oxytetracycline	n.a.	2.67 mg/L	n.a.		
		Doxycycline	n.a.	3.79 mg/L	n.a.		
	3	Ciprofloxacin	n.a.	4.65 mg/L	n.a.	n.a.	
		Ofloxacin	n.a.	3.20 mg/L	n.a.		
		Levofloxacin	n.a.	3.68 mg/L	n.a.		
		Oxytetracycline	n.a.	3.09 mg/L	n.a.		
		Doxycycline	n.a.	3.65 mg/L	n.a.		
	4	Ciprofloxacin	n.a.	3.75 mg/L	n.a.	n.a.	
		Ofloxacin	n.a.	2.68 mg/L	n.a.		
		Levofloxacin	n.a.	4.25 mg/L	n.a.		
		Oxytetracycline	n.a.	3.73 mg/L	n.a.		
		Doxycycline	n.a.	3.46 mg/L	n.a.		
	5	Ciprofloxacin	n.a.	3.87 mg/L	n.a.	n.a.	
		Ofloxacin	n.a.	2.66 mg/L	n.a.		
		Levofloxacin	n.a.	3.74 mg/L	n.a.		
		Oxytetracycline	n.a.	4.92 mg/L	n.a.		
		Doxycycline	n.a.	2.34 mg/L	n.a.		
Taiwan		Imipenem	5.18 ng/L	< LOQ	100	MBR	[31]
Tunisia		Norfloxacin	n.a.	226.7 µg/L	n.a.	n.a.	[47]
		Spiramycin	n.a.	84.2 µg/L	n.a.		

PWWTP pharmaceutical wastewater treatment plant, *n.a.* information not available, *LOQ* limit of quantification, *CASS* cyclic activated sludge system, *EGSB* expanded granular sludge bed reactor, *MBR* membrane bioreactor, *EAAS* extended aeration activated sludge, *CAS* conventional activated sludge, *SBR* sequential batch reactor, *B-COT* biocontact oxidation tank, *DAFT* dissolved air flotation tank, *MBBR* moving bed biofilm reactor, *A2/O* anaerobic/anoxic/oxic

new treatments such as advanced oxidation processes that can supplement conventional systems and enhance the treatment of recalcitrant antibiotics and other organic compounds [33, 37, 38].

However, some studies have shown that even when high antibiotic removal efficiencies were achieved (>90%), the residual high μg/L concentrations of antibiotics remained in effluents, thereby posing an ecological risk for the selection of antibiotic resistance [30, 32]. For example, in a study conducted on two PWWTPs that treated wastewaters from fluoroquinolone production industries (approximately 2–6 mg/L of fluoroquinolones in influent), high removal efficiencies of fluoroquinolones (≥95%) were achieved, but nevertheless, up to 88 μg/L of antibiotics remained in effluents [32]. Similarly, despite high antibiotic removal (>90%) of vancomycin, trimethoprim, or tetracycline in Chinese PWWTPs, still hundreds of μg/L of residual antibiotics were measured in the final effluents (Table 1) [4, 30].

In contrast to the above-mentioned μg/L residual antibiotic concentrations in the final, treated pharmaceutical effluents, there are reports that showed very high, mg/L levels of antibiotics, mostly in Indian and Chinese production facilities, as reviewed by Larsson [1]. These two countries manufacture most of the world's antibiotics owing to their cheaper labour costs and weak environmental regulations [9, 39, 40]. A local scandal about environmental antibiotic pollution from the pharmaceutical sector in India that took place more than 10 years ago [6] raised serious concerns about the potential damage to global health and the environment. Consequently, over the last few years, pharmaceutical companies made commitments for change, and reducing antibiotic discharges has been voiced as one of their top priorities, for instance, in the Industry Roadmap presented at the Davos Forum in 2016 [41]. However, recent studies show that today many pharmaceutical companies are still discharging antibiotics into the environment on a mass scale via effluents from their production plants. A detailed overview of antibiotic concentrations measured in final effluents leaving PWWTPs, published in papers in the last 5 years, is presented in Table 1. Major discharges, in the mg/L range, have been reported from Asian countries such as China and Pakistan. For example, the concentrations of tetracycline antibiotics in effluents leaving certain PWWTPs in China ranged from a few mg/L to as much as 32 mg/L [4, 5]. Additionally, a study from Pakistan reported the concentrations of fluoroquinolones and tetracyclines in effluents of PWWTPs ranging from approximately 2 to 5 mg/L [23]. However, the problem of major antibiotic discharges from pharmaceutical production is not confined only to Asia. A recent study by Bielen et al. [2] reported the mg/L levels of macrolide antibiotics in the final, treated effluents from the azithromycin production in Croatia. This study, along with other Asian studies, suggests that it is important to establish a continuous monitoring of antibiotic discharges from manufacturing sites worldwide in order to estimate the proportions of the problem. More importantly, there is an urgent need for the definition of emission limits for individual antibiotics. Such limits can theoretically be implemented by local authorities, but to date it appears that the implementation of such specific emission restrictions has been extremely rare [42]. The laws specifically aimed at curtailing antibiotics in effluents do not exist in Europe, the USA, or India. Notably, India is the

first country that has already voiced an intent to regulate antibiotic discharges nationally [43]. However, a recent Croatian study on macrolide pollution from an antibiotic production facility makes it clear that regulation is urgently needed in Europe as well. A common argument against regulation is a lack of scientific agreement on which emission levels could be considered safe for the functioning of the ecosystem, and against the development of antibiotic resistance. A recent paper has reported a predicted no-effect concentrations (PNECs) for resistance selection for 111 antibiotics [44]. These PNEC values can be used as a good starting point for defining acceptable antibiotic discharge levels and may eventually be refined or supplemented with experimental data as they become available. However, ecotoxicological data for antibiotics is still scarce, and only recently, the standard methods and specific ecological endpoints for such assessment have been proposed [45, 46]. Therefore, further studies that better establish the toxic effects of antibiotics, antibiotic resistance and the relationship between them in environmentally relevant contexts are urgently required as a basis for regulatory efforts.

2.2 *Antibiotic Resistant Bacteria and Antibiotic Resistance Genes in Pharmaceutical Wastewater Treatment Plants*

In addition to antibiotics, effluents leaving PWWTPs are important sources of ARB and their ARGs. Recent studies have shown that the abundance of total heterotrophic bacteria resistant to azithromycin in the final effluents of PWWTP treating the azithromycin production waste was about 10^8 colony forming units (CFU) $(100\ mL)^{-1}$ [2] and around 10^4 CFU $(100\ mL)^{-1}$ in the PWWTP treating waste from the ß-lactam and quinolone production [47]. In addition, the studies on the quantification of ARGs in PWWTPs estimated that about 10^{12}–10^{14} ARG copies could be released per day [13]. Nevertheless, these concentrations are a few orders of magnitude lower than those reported in municipal WWTPs from different world regions. For example, it has been estimated that more than 10^{14}–10^{18} tetracycline or ß-lactam ARGs were released into the receiving environment every day via municipal effluents [48]. However, the above-mentioned data still shows that PWWTPs do not efficiently remove ARGs and, consequently, discharge a large quantity of ARGs into the environment. Besides ARGs, the biological AS processes are not considered especially successful in the ARB removal in PWWTPs; instead, selective increase of ARB was frequently observed throughout the treatment process due to a high selective pressure from the antibiotics [3, 13, 14]. In addition, the high antibiotic selection pressure often present in PWWTPs was shown to favour the selection and amplification of multidrug-resistant (MDR) bacteria. In the Indian PWWTP, which received waste from approximately 90 regional bulk drug manufacturers, a great majority (86%) of the strains isolated from the treatment plant were resistant to 20 or more antibiotics [11]. It has also been suggested that the overall resistance profiles of these strains are, to a large degree, cases of acquired resistance. This conclusion was

made on the basis of comparison of the antibiotic sensitivity profiles of different strains of the same species. For example, when comparing 13 *Ochrobactrum intermedium* strains, there was a difference in the resistance profiles of these strains; one strain (ER-1) was observed to be sensitive to only 1 of the 39 tested antibiotics, resistant to 36 and intermediately resistant to 2 antibiotics, while, on the other hand, the least resistant *O. intermedium* strain demonstrated resistance to 18 antibiotics and intermediate resistance to 7 [11]. In a parallel study, the whole-genome sequencing of the *O. intermedium* strain ER-1 revealed that this strain acquired three regions containing ARGs, a conjugative element carrying a class I type IV secretion system and insertion sequence transposons [49]. MDR bacteria have not been detected only inside PWWTPs but also in their final effluents [21, 47, 50]. Some of these MDR isolates were opportunistic pathogens like *Acinetobacter calcoaceticus*, *Delftia lacustris* and *Citrobacter freundii*, known to cause severe infections under particular conditions [51–53]. All these data suggest that strong selection pressures, as found in PWWTPs, would create the perfect breeding ground for the development of MDR strains, with the potential that a new pathogenic "superbug" would be created in environments like these. While much work has been focused on ARB removal from municipal effluents, and promising results were obtained by applying ozone or UV technologies or membrane filtration [54–57], improved management of discharges from antibiotic production seems to be a more urgent goal in terms of minimizing the leakage of ARB from PWWTPs.

On the other hand, effective inactivation of ARB may not indicate elimination of antibiotic resistance in the effluent, and hence, the fate of ARGs needs to be considered as well. Recent studies have shown that the increase in abundance of ARGs mainly occurs in the biological treatment processes, implying that significant replication of ARGs may be attributable to microbial growth [3, 4]. Studies addressing the link between the presence of antibiotics in PWWTPs and ARGs have shown varying results. A study conducted by Wang et al. [4] on five different PWWTPs showed a correlation between the high concentration of certain antibiotics and the enrichment of corresponding ARGs. Other studies demonstrated the enrichment of corresponding and noncorresponding ARGs in response to the high concentration of specific antibiotic revealing the collateral effects of antibiotics on the resistance development [3, 8, 10]. On the other hand, Li et al. [14] did not find any significant correlations between antibiotics (tetracyclines, quinolones and macrolides) and the corresponding ARGs, except for *sul*1 and *sul*2 genes which had a negative correlation with sulfonamides. This lack of correlation, or negative correlation between the antibiotics and the corresponding ARGs, was assumed to be a consequence of co-localization of different ARGs on the same MGE or in the same cell. After a certain antibiotic is removed, its corresponding ARGs can persist in the PWWTPs in response to exposure to other antibiotics. In addition to the elevated ARGs abundances, the increased abundance of the class 1 integron-integrase gene, *intI1*, was also reported in PWWTPs [4, 14, 58], and its often plasmid localization would facilitate the potential for HGT in these PWWTPs.

Taken together, further research in this area is essential to provide the opportunity to increase the efficiency of the existing strategies or implement innovative new

approaches to minimize the leakage of ARB and ARGs from PWWTPs. Therefore, it has been recommended that the AS treatment of highly antibiotic-polluted pharmaceutical wastewaters be avoided [20, 59] or, if this treatment is used, that the management of resulting waste be improved, e.g. through installing a membrane separation unit for the physical retention of bacterial flocs within the treatment plant [59].

3 Impact of Industrial Discharges on the Receiving Environment

Massive environmental pollution from Indian and Chinese pharmaceutical factories during the past decades has triggered the creation of large reservoirs of bacteria, including pathogens, carrying resistance genes to multiple antibiotic classes [1, 60]. Since the first evidence about heavy environmental pollution from pharmaceutical factories in India [1], and despite intensive campaigning for reducing such pollution, it seems that the situation has not improved and industrial pollution is still rife in South India, raising concerns about the potential damage to global health and the environment. This was confirmed in a recent study conducted by German researchers who examined the extent of pharmaceutical pollution with the proliferation of MDR bacteria in Hyderabad, the central production site for antibiotics worldwide, and its surrounding area [9]. Hyderabad is already known for its contaminated rivers and lakes due to illegal waste dumping and discharges of partially treated/untreated industrial effluents and sewage into the Musi River. In a 2017 study, researchers collected water samples from 28 different sites to cover the direct vicinity of bulk drug-manufacturing facilities, rivers, lakes, groundwater, drinking water, water sources contaminated by sewage treatment plants and the surface water from populated urban as well as rural areas. Importantly, 13 out of 16 samples tested for the presence of 25 anti-infective pharmaceuticals were found highly polluted with antibiotics and antifungals. The concentrations exceeded the suggested environmental regulation limit [44] for antibiotics moxifloxacin (up to 5,500 times), ciprofloxacin (up to 700 times), ampicillin (up to 115 times), clarithromycin (up to 110 times) and levofloxacin/ofloxacin (up to 50 times). In addition, the antifungal fluconazole was detected at a concentration of 236,950 mg/L, which was more than 20 times greater than the therapeutically desired levels in the blood [9]. Besides, almost all samples contained bacteria and fungi resistant to multiple drugs, including last resort carbapenem antibiotics. The PCR screening of samples confirmed the presence of clinically relevant carbapenem-resistance genes, especially $bla_{\text{OXA-48}}$, bla_{NDM} and bla_{KPC}. These findings confirmed those of previous studies demonstrating a strong association between massive environmental pollution with antibiotics and the presence of MDR bacteria, including pathogens. This could have serious implications for global health, as ARB and ARGs can spread around the world through travel and trade with India.

The environmental pollution from the antibiotic manufacturing sector was also recently observed in other Asian countries such as Pakistan and Vietnam. As mentioned in the previous section, the investigations performed in effluents from five Pakistani pharmaceutical manufacturing plants documented high, mg/L concentrations of fluoroquinolone, quinolone and tetracycline antibiotics (Table 1) [23]. A follow-up study reported that soil samples from surrounding areas were also polluted with above-mentioned antibiotics in approximately 10 μg/kg concentrations (total), while their concentrations in groundwater were up to a few hundred pg/L (total) [61]. Besides, antibiotics were also detected in plants (carrot, wheat and spinach) that grew in the soil irrigated by these industrial wastewaters in concentrations up to a few μg/kg [62]. Another Pakistan's study explored the pollution with antibiotics and ARGs at four sites near the drug formulation facilities [63]. The antibiotics oxytetracycline, trimethoprim and sulfamethoxazole were measured in the water samples at 27 μg/L, 28 μg/L and 49 μg/L, respectively, in the site with the highest concentrations of antibiotics (P4). This P4 site also had the highest concentrations of sulfonamide ARGs (*sul*1; 8.0×10^{-1} copies/16S rRNA copies) and trimethoprim ARGs (*dfr*A1; 4.3×10^{-1} copies/16S rRNA copies), as well as class 1 integrons (6.9 *int*I1 copies/16S rRNA copies), indicating selection of resistance in response to high antibiotic concentrations. In the most recent study carried out in Pakistan, Ashfaq et al. [22] quantified different pharmaceuticals, including fluoroquinolone antibiotics, in wastewaters, sludge, solid waste and soil samples near the pharmaceutical formulation facilities in the city of Lahore. The authors reported μg/L concentrations of antibiotics in effluents (up to 81 μg/L; Table 1), while the soil samples collected from agricultural fields which were irrigated with pharmaceutical effluents showed antibiotic levels in the range of tens of μg/kg. On the other hand, investigations performed by Thai et al. [64] in the river/canal receiving wastewaters from four manufacturing sites in Vietnam showed antibiotic levels ranging from low μg/L to approximately 250 μg/L in the surface water close to the discharge point, which were further diluted in downstream river/canal to max. 10–15 μg/L. Importantly, despite moderate pollution levels, the majority of measured concentrations in the recipient waters exceeded the corresponding PNEC values for individual antibiotics, suggesting the risk for selecting antibiotic resistance in the aquatic environment.

In contrast to the situation in selected Asian countries, the emissions from the manufacturing of pharmaceuticals are assumed to be low in Europe; however, there may be exceptions from this general rule. A recent study showed that antibiotic discharges may be very high in Croatia and would not meet the standards set in Europe. In that study, Bielen et al. [2] found up to 10 mg/L (total conc.) of macrolide antibiotics in the treated effluent from PWWTP treating wastewater from the azithromycin-synthesis industry. In partially treated effluents from another Croatian drug formulation industry, the same authors measured up to 231 μg/L of sulfamethazine, up to 98 μg/L of enrofloxacin and up to 29 μg/L of oxytetracycline (Table 1). While environmental pollution from the azithromycin production industry is described in more detail in Sect. 4, the concentrations of antibiotics released from the drug formulation industry did not generally exceed 10 μg/L in the receiving creek

(surface water collected up to 3 km downstream from the discharge), but some of them, like trimethoprim and enrofloxacin, were found in concentrations selective for resistance even 3 km downstream from the discharge. In addition to antibiotics, drug formulation effluents also introduced high proportions of ARB (up to 90% of sulfamethazine-resistant and up to 50% of oxytetracycline-resistant bacteria) into the creek. A follow-up study [65] explored the resistome of this effluent and creek sediment samples taken up- and downstream from the discharge by applying functional metagenomics. The results showed that all of the genes conferring resistance to sulfonamides and tetracyclines were highly similar to previously known genes (amino acid sequence identity $\geq$94%), while among the genes conferring resistance to trimethoprim and ampicillin, some potentially novel genes were identified. Eight of these novel genes derived from the antibiotic-polluted creek sediment at the discharge site and included the dihydrofolate reductase and β-lactamase genes. Known ARGs were often highly similar to ARGs found in pathogens, e.g. β-lactamase genes $bla_{\text{GES-1}}$, $bla_{\text{VEB-9}}$ and $bla_{\text{CMY-10}}$ were similar to those found in pathogens like *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, respectively. The analysis of flanking DNA of all identified ARGs showed that they were often organized in resistance gene clusters and flanked by MGEs such as insertion sequences or integron elements, suggesting their common mobility potential. In addition, a study in Spain [50] investigated the abundance and spatial dynamics of antibiotic-resistant faecal bacteria in water and sediment samples from Bernesga River receiving wastewaters from a cephalexin and amoxicillin production plant. The results showed that effluent discharges increased the cephalexin and amoxicillin resistance in faecal bacteria both in river water and in sediments at the discharge and downstream locations (up to 700 m downstream).

4 Case Study: Effects of Azithromycin Production Effluents on Aquatic Wildlife and Microbial Communities in Receiving Sava River (Croatia)

The most comprehensive studies on environmental pollution from antibiotic manufacturing in Europe and the effects associated with such pollution are those conducted on the azithromycin production industry situated in northern Croatia. This industry is located approximately 25 km northwest of the city of Zagreb and has a long tradition in manufacturing the macrolide antibiotic azithromycin via synthesis from another macrolide antibiotic, erythromycin. The wastewater produced by this industry is discharged into the Sava River after being mixed with the effluent from baker's yeast production and treatment in industry's WWTP (membrane bioreactor). In the treated effluent leaving this PWWTP, very high levels of azithromycin and by-products from its synthesis were occasionally detected, with azithromycin as the most abundant at concentrations up to approximately 4 mg/L [2]. This provided the rationale for an in-depth characterization of the effects on exposed environmental

microbial communities. The samples of PWWTP effluents and river surface water and sediments were collected at the discharge site, one site upstream and four sites downstream from the discharge during 2016 in four sampling campaigns. The collected samples were analysed using chemical, microbiological, molecular and toxicological methods.

4.1 Chemical Pollution from Azithromycin Production Site and Effects on Biota

In the first set of studies, Bielen et al. [2] investigated the pollution levels of antibiotics and heavy metals in industrial effluents and in the Sava River water collected up- and downstream from the industrial discharge point. In the effluents collected during winter and spring, the authors reported 2.1 mg/L and 3.8 mg/L azithromycin, respectively, whereas these concentrations were one to three orders of magnitude lower during summer and autumn (4.9 μg/L and 218 μg/L). Such fluctuations in antibiotic concentrations were assumed to be the consequence of different production cycles and washing of reaction tanks. Nevertheless, all these concentrations are clearly selective for resistance development as they are much higher than the estimated PNEC value for azithromycin (250 ng/L) [44]. In addition to azithromycin, during winter and spring, authors also measured high levels of two macrolide by-products from the azithromycin synthesis, N-desmethyl azithromycin (up to 5.6 mg/L) and dehydrated erythromycin (up to 2 mg/L). Both these compounds have antimicrobial activity and were found at much more modest concentrations during summer and autumn (up to 390 μg/L). As a consequence of strong macrolide pollution of effluents in winter and springtime, all three macrolides were found at the downstream Sava River at enhanced μg/L concentrations (20–30 μg/L total). Importantly, the azithromycin concentrations measured in the receiving river (up to 4.5 km downstream) during winter and springtime were higher than the corresponding PNEC values, suggesting that they might be sufficient to promote macrolide resistance in the Sava River. In addition to macrolides in effluents, the authors also reported the presence of ecotoxic heavy metals in concentrations mostly lower than the maximum legal limits for effluents: arsenic (As) 0.41–0.73 μg/L, cadmium (Cd) 0.07–0.79 μg/L, chromium (Cr) 1.98–15.73 μg/L, copper (Cu) 6.19–26.92 μg/L, lead (Pb) 2.70–4.24 μg/L, nickel (Ni) 12.42–24.77 μg/L and zinc (Zn) 56.35–103.06 μg/L. Nevertheless, these concentrations were up to 85 times higher than those reported to have co-selection potential. All these findings together with the high levels of organic, phosphorus and nitrate compounds detected in effluents were indicative of unsatisfactory treatment of the azithromycin production waste and of unauthorized discharges. In the follow-up study, Milaković et al. [8] recently reported that these discharges led to the pollution of the Sava River sediment with macrolide antibiotics (especially azithromycin), heavy metals and nutrients. For example, the highest levels of azithromycin were detected at the

discharge site (up to 23 mg/kg), with a sharp decrease to about 1 mg/kg at the site located 700 m downstream. The concentrations of dehydrated erythromycin were much lower in the sediments compared to azithromycin, being the highest at the discharge site (up to 1 mg/kg). This difference could be the consequence of the faster biodegradation of erythromycin in comparison to azithromycin [66]. Heavy metals, especially Cu and Zn, were also found accumulated in the exposed sediments, suggesting metal pollution from the incoming industrial waste. Based on the levels found in downstream sites, both Cu and Zn might contribute to the co-selection of metal resistance and antibiotic resistance and also might induce adverse effects on the sediment dwelling organisms [8].

To address the hazard associated with exposure to such highly polluted industrial effluents, eukaryotic algae, crustacean invertebrates and zebrafish embryo were used as models [2]. The results of these experiments demonstrated the toxicity of the treated effluents from the azithromycin production to freshwater algae (*Pseudokirchneriella subcapitata*) and water fleas (*Daphnia magna*). The authors also reported multiple abnormalities in zebrafish embryos such as heart and yolk oedema, scoliosis and lack of pigmentation formation [2].

Taken together, all these data demonstrated that effluents from local azithromycin manufacturing can pose a significant ecological and public health concern.

4.2 *Effects on Antibiotic Resistance and Bacterial Communities in Sava River Sediments*

To identify the ARGs present in the industrial effluent and river sediments collected upstream of and at the effluent discharge site, the functional metagenomic approach was applied [65]. A total of four small-insert metagenomic libraries were prepared and screened for resistance to macrolide antibiotics. Unique macrolide-resistant clones were sequenced (the Sanger method) for the identification of the active resistance genes. Despite a strong selection pressure from macrolides, only one potentially novel macrolide resistance gene was discovered (most similar to 23S rRNA methyltransferase). The majority of identified macrolide resistance genes were similar to known genes such as *msr*E (ribosomal protection proteins), *mph*E and *mph*G (macrolide phosphotransferases) and *mef*C (efflux pumps). Moreover, some clones carried genes for two different macrolide-resistant mechanisms (*msr*E-*mph*E, *mef*C-*mph*G), together with genes for resistance to other class of antibiotics, such as *sul2* (sulfonamide resistance). This suggested that if these genes spread, they are likely spread together. In addition, some of them were flanked with insertion sequence elements (IS6 and IS91), indicating that these genes may also be highly mobile within bacterial populations [65].

To assess the impact of industrial discharges on the abundance of ARGs and MGEs facilitating their spread such as integrons and plasmids, quantitative PCR analysis was performed. In a recent study, Milaković et al. [8] reported a significant

increase of relative abundances of five targeted macrolide ARGs (*mef*C, *mph*G, *mph*E, *msr*E and *erm*B) in the receiving sediments. In addition to the increased abundance of macrolide ARGs, the relative abundance of other ARGs such as sulfonamide (*sul*1, *sul*2), tetracycline (*tet*A) and quaternary ammonium compound ARGs (*qac*E/*qac*EΔ1) was also significantly elevated in the downstream compared to the upstream sediment. Given that only macrolide antibiotics were detected at high levels in the downstream sediments, it was hypothesized that the high levels of *sul*1, *sul*2, *tet*A and *qac*E/*qac*EΔ1 genes could be due to co-selection [67]. In addition to ARGs, downstream sediments contained a significantly higher abundance of MGEs such as class 1 integrons (*int*I1) and broad host range IncP-1 plasmids (*kor*B), indicating increased potential for horizontal gene transfer in these sediments. All these MGEs and ARGs targeted were also detected at high relative abundances (around −1 to −2 log gene copies/16S rRNA gene copies) in effluent, suggesting pollution from the incoming industrial waste. This implied that the industrial discharges enriched the receiving river with the bacteria carrying these resistance genes likely due to the deposition of effluent-associated bacteria or the propagation of indigenous sediment bacteria that acquired ARGs via plasmid-mediated transfer from the effluent bacteria under selection pressure from antibiotics. Indeed, by applying biparental mating experiments, Gonzalez-Plaza et al. [67] demonstrated an increased plasmid-mediated horizontal transfer of ARGs from the effluent-receiving polluted sediments into the *Escherichia coli* CV601 recipient. Most plasmids exogenously captured from the effluent and the polluted sediments belonged to the broad host range IncP-1ε plasmid group, conferred multiple antibiotic resistance and harboured class 1 integrons.

Finally, to assess the impact of industrial discharges on the bacterial communities of the Sava River sediments collected up- and downstream from the discharge point, Illumina-based 16S rRNA amplicon sequencing was applied [8]. The difference in the number of taxa (species richness) between the up- and downstream sediments was surprisingly small during both seasons, although sediments from the discharge site tended to have the lowest diversity among all samples. In addition, community composition in the sediments at the discharge site was clearly distinct from that at upstream and downstream sites. Phyla such as *Firmicutes*, *Bacteroidetes* and *Epsilonbacteraeota* were significantly increased in relative abundance and dominated together with *Proteobacteria* at the discharge site and in the effluent during both seasons. Redundancy analysis and Mantel test indicated that macrolides and copper together with the nutrients significantly correlated with the community shift close to the effluent discharge site. However, the number of taxa that were significantly increased in relative abundance at the discharge site decreased rapidly at the downstream sites, showing the resilience of the indigenous sediment bacterial community. Seasonal changes in the chemical properties of the sediment along with the changes in the effluent community composition appeared to be responsible for the sediment community shifts between winter and summer.

Despite the resilience of the bacterial communities in the river sediments, the abundances of target macrolide-resistance genes were still maintained at elevated levels at downstream sites [8]. This may be an indication of a horizontal transfer of

ARGs to new hosts or a persistence of extracellular ARGs or a combination of both. A previous analysis of the regions flanking the analysed macrolide-resistance genes suggested that these genes likely originated from plasmids [65]. This further suggests that the analysed resistance genes are candidates for dissemination to other bacteria in the river sediment. This is also supported by exogenous isolation of conjugative broad host range plasmids conferring macrolide resistance from the sediments downstream of industrial discharge point [67]. Together, these data indicate that the transferable resistome is likely the primary mechanism for the persistence of macrolide-resistance genes in the downstream Sava River sediments [8].

Taken together, the discharges of insufficiently treated effluents from the azithromycin manufacturing site thus poses a risk for the development and the dissemination of MDR bacterial strains, including pathogens. Macrolides have recently been included in the EU watch list for water monitoring [68] due to properties such as high toxicity, persistence and bioaccumulation potential [2, 44]. In addition, the World Health Organization has recently classified them as the highest priority critically important antibiotics for human medicine [69]. Therefore, it is of critical importance for human health to maintain the efficacy of macrolides, and one of the solutions would be to prevent their leakage from production facilities into the environment.

5 Conclusions and Perspectives

India and China play a key role in global pharmaceutical production and have also played a pivotal role in bringing affordable medicine to millions of people worldwide. However, the failure of pharmaceutical companies to address the environmental impacts of antibiotic pollution could undo much of their good work in improving public health. Indeed, poor management of wastewater along with the illegal dumping of pharmaceutical waste in these countries caused unprecedented antibiotic pollution of surrounding waters, resulting in direct damaging impacts on biota and indirect impacts on human health via the promotion of antibiotic resistance. Local and national authorities are failing to get the situation under control as pharmaceutical companies are continuing to discharge their untreated or inappropriately treated wastewaters into rivers and lakes. In addition, significant discharges have also been recently reported from European antibiotic production facilities (Croatia) despite strong regulations for pharmaceutical production. Although heavy antibiotic pollution in such industrially impacted areas radically increases the abundance of resistant bacteria and their resistance genes, one can argue that there is a low probability that the resistance genes in environmental bacteria are transferred to and maintained by pathogenic bacteria. However, as the types and the abundance of antibiotic resistance genes in the environment increase, so do the risks that it will happen. Moreover, poor water and hygiene standards, such as those in less developed countries like India, further increase the chance of people being infected with antibiotic resistant pathogens. Given that resistant bacteria can often be rapidly

transported across the world through travel and trade, it is clear that bad production practices in one location impact not only the health of local people but also public health all over the world and, thus, need to be addressed globally. It is therefore of utmost importance to improve management strategies for reducing the environmental release of antibiotics from manufacturing sites in order to limit further antibiotic resistance from evolving in pathogens or commensals. These strategies should include the definition of discharge limits for individual antibiotics and the establishment of continuous monitoring of releases from manufacturing facilities worldwide to prove that discharges are kept below the agreed restriction limits. In addition, PWWTPs should be upgraded with proper technology, based on the contents of influent, in order to more thoroughly reduce or eliminate antibiotics, ARB, ARGs and other hazardous pollutants from the wastewater. Furthermore, policy-makers and governments should expand the existing production standards and include environmental criteria in the good manufacturing practice framework to ensure that manufacturers address wastewater treatment. Finally, it is important to introduce more transparency in the pharmaceutical production chain. The details of where and how drugs were manufactured must be clearly displayed on the packaging. In this way the customers would be provided with the evidence of good environmental performance from the factories they are buying from. All these measures are a critical, yet still missing, part of the puzzle in the global strategy to combat antibiotic resistance.

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Antibiotic Resistance in Municipal Wastewater: A Special Focus on Hospital Effluents

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Abstract Hospital effluents contain a hazardous amalgam of drug residues and infectious agents. Qualitative and quantitative evidence shows that hospital effluents are enriched in antibiotics, multidrug-resistant bacteria, antibiotic resistance genes and genetic vectors which could facilitate the horizontal transfer of these genes. This chapter provides an overview of the current status of antimicrobial resistance (AMR) surveillance in hospital effluents and draws comparisons to other AMR monitoring studies in domestic wastewaters and natural aquatic environments. We discuss approaches and standard tools that have been used to measure levels of AMR contamination and provide insights to the latest developments in the detection and profiling of AMR which have yet to gain traction in present surveillance programs.

Keywords Antibiotic residues, Antibiotic resistance genes, Antibiotic resistant bacteria, Hospital effluent, Resistome

Abbreviations

AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic resistance genes
CLSI	Clinical and Laboratory Standards Institute
CRB	Carbapenem
ESBL	Extended spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GC	Gene copy
HPLC-MS/MS	High-performance liquid chromatography coupled with tandem mass spectrometry
HT-qPCR	High-throughput quantitative polymerase chain reaction
MGE	Mobile gene element
MGEs	Mobile genetic elements
MLST	Multilocus sequence typing
qPCR	Quantitative polymerase chain reaction
SPE	Solid phase extraction
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

1 Introduction

Antimicrobial resistance (AMR) is a menace in both community and healthcare facility settings. Hospital hygiene limitation and the overuse and misuse of antibiotics are factors contributing to the spread of antimicrobial drug resistance in hospitals [1]. The mode of transmission is complex and can occur between patients through the healthcare environment (surface, air, clothes), contaminated healthcare workers or others [2, 3]. Other sources of transmission are mediated through the use of invasive medical devices during surgical procedures which may result in hospital-acquired infections [4]. The selection pressure is imposed by the constant presence of antibiotics, which in turn accelerates the transfer of antibiotic resistance genes (ARGs) between bacteria by mobile genetic elements (MGEs) [1, 5, 6]. A transmission model of antibiotic-resistant bacteria (ARB) developed by Almagor et al. [6] showed that frequent antibiotic usage heightens the risk of transmission by increasing the vulnerability of susceptible patients and the contagiousness of colonized patients who are treated with antibiotics. The highest likelihood of AMR emergence and dissemination is through human transmission; however, hospital effluents that are loaded with microbes, infectious agents and pharmaceutical waste, originating from human sources, pose a significant public health risk if not sufficiently treated and discharged into receiving environments [7, 8].

On-site hospital wastewater treatment using advanced technologies (membrane bioreactor treatment, ozonation, granulated activated carbon, UV treatment) is capable of reducing ARGs and eliminating antibiotics in hospital effluents [9]; however, in most countries, there are no specific recommended or standardized treatments of hospital wastewaters. Hospital effluents are often routed for release into community wastewater treatment plants and co-treated with domestic wastewaters [10]. In rural areas of India, Nepal and Bangladesh, where wastewater management is inadequate, domestic effluent is directly discharged into receiving water bodies that are used as drinking water sources [11]. Despite the recognized risk of antibiotic resistance emergence and transmission, there is currently a lack of AMR surveillance in hospital wastewater. There is a clear need to survey current methods and practices, which can be applied to assess the spread of AMR from hospital effluents to the environment. This chapter reviews analytical chemistry methods, microbiological techniques and next-generation sequencing platforms which can be used to measure AMR loads in hospital effluents.

2 Antibiotic Residues

2.1 Antibiotic Residues in Hospital Effluents

Hospital effluents are important sources of antibiotics entering into the aquatic environment [12–19]. To date, analytical methods for determination and

quantification of target antibiotics in hospital effluents have been well developed and validated, in which high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is widely used to identify and quantify antibiotic residues in hospital effluents as well as environmental water samples [20, 21].

2.2 Challenges in Quantification of Antibiotic Residues in Hospital Effluents

To have better understanding of the occurrence, fate and environmental risk of antibiotics in hospital effluents to public health and aquatic ecosystems, the development of robust sensitive analytical methods for simultaneous extraction and analysis of the target antibiotics is critically needed [21]. One of the challenges in the determination of antibiotics in hospital effluents is related to simultaneous extraction of antibiotics from hospital effluent samples, since antibiotics are often present at very low concentrations (ng/L–μg/L) under complex matrices [21]. To date, solid phase extraction (SPE) is widely employed to enrich and purify a wide variety of target antibiotics from environmental samples [21–23]. However, apart from enrichment of target antibiotics, solid phase extraction may enrich some interferences that affect HPLC separation and MS/MS detection. In addition, it is challenging to extract simultaneously multiple classes of antibiotics using a single SPE cartridge as the antibiotics belonging to different classes tend to have different physicochemical properties (i.e. log K_{ow} and pK_a) and molecular structures. For these reasons, it is difficult to select a suitable single cartridge to extract simultaneously all target antibiotics in environmental samples [21].

The selection of a suitable SPE cartridge plays an important role in enhancing recovery of target antibiotics in environmental samples. Normally, the selection is often based on the physicochemical properties of target antibiotics and SPE adsorbent characteristics [21, 24, 25]. For example, Kasprzyk-Hordern et al. [25] chose a strong cation-exchange mixed-mode polymeric sorbent (Oasis MCX) for the simultaneous extraction of selected antibiotics (including ciprofloxacin, doxycycline, sulfamethoxazole, trimethoprim and erythromycin) and other pharmaceuticals and found that recovery for these analytes ranged from 61.6 to 82.5%. In another study, Babić et al. [24] used the Oasis HLB cartridge to extract seven antibiotics (sulfamethazine, sulfadiazine and sulfaguanidine, trimethoprim, oxytetracycline, enrofloxacin and penicillin G). Theoretically, the use of a specific SPE cartridge for each class of antibiotics may provide a good extraction recovery. However, this approach is time-consuming when analysing a large number of antibiotics with different physicochemical properties, and this approach is quite expensive due to SPE cartridge consumption. In a recent effort, Tran et al. [21] optimized the simultaneous extraction of 20 antibiotics and 2 antimicrobial agents belonging to 10 different classes in environmental water samples via using dual cartridges, Chromabonds HR-X (500 mg, 6 mL) [HR-X] coupled with Chromabonds SB (500 mg, 6 mL) [SB].

In addition to extraction, the detection and quantification of antibiotics in hospital effluents are challenging. To date, the use of HPLC-MS/MS is considered to be the best analytical instrument for the detection and quantification of antibiotics in wastewater matrices as it has high sensitivity and selectivity for target analytes compared to other instruments (i.e. HPLC-UV, HPLC-FID, etc.). However, the matrix effect in wastewater samples may lead to reduced detection sensitivity [13]. For example, in a previous study, Gómez et al. [13] found that significant signal suppression (ca. 85%) was observed for erythromycin when using LC-MS/MS for quantification. Hitherto, matrix effects are often corrected using a matrix-matched standard calibration method [13, 26–29], but this approach is challenging to apply for routine monitoring of environmental samples because matrices of environmental samples vary from place to place and from time to time. In such circumstances, the selection of a representative blank with a matrix composition similar to the samples is impossible. Therefore, the accuracy of the analytical methods based on matrix-matched standards calibration approach is limited. To tackle the issues regarding the losses of antibiotics during sample preparation (i.e. storage and extraction) and matrix effects during HPLC-MS/MS, the use of isotopically labelled surrogate/internal standards is deemed to be more accurate for quantification of antibiotics in environmental samples in general and hospital effluents in particular [21].

In short, the use of HPLC-MS/MS coupled with isotope dilution is a recommended option to detect and quantify antibiotics in hospital effluents as well as other environmental water samples (i.e. municipal sewage and surface water), because it allows correcting the losses, matrix effects and instrumental fluctuations during analytical processes.

2.3 *Occurrence of Antibiotics in Hospital Effluents*

The occurrence of multiple classes of antibiotics in hospital effluents has been well documented [13, 14, 17, 30–32]. For example, Gómez et al. [13] reported that the concentrations of trimethoprim and erythromycin in hospital effluents in Spain varied from 10 to 30 ng/L. In another study, Duong et al. [14] found that the concentrations of ciprofloxacin and norfloxacin in hospital wastewater in Vietnam ranged from 1.1 to 44 μg/L and from 0.9 to 17 μg/L, respectively. In a recent study, Thai et al. [17] measured the occurrence of beta-lactams, sulfonamides, macrolides, trimethoprim and fluoroquinolone in hospital effluents in Vietnam and found that the concentrations of detected antibiotics ranged substantially from below detection limit (beta-lactams) to over 40 μg/L (fluoroquinolone antibiotics). Similarly, in an earlier study in Singapore, Le et al. [32] found the presence of macrolides, fluoroquinolones, sulfonamides, beta-lactams, lincosamides, tetracycline and trimethoprim in hospital effluents, in which the maximum concentration of macrolide (clarithromycin) and fluoroquinolone (ciprofloxacin) was greater than >70 μg/L while other antibiotic classes such as lincosamides, tetracyclines and beta-lactams were rarely detected, even though

beta-lactams are known to be one of the most consumed antibiotic classes. The presence and concentrations of antibiotics in hospital effluents tend to depend on the compound and type and size of hospitals.

3 Antibiotic-Resistant Bacteria (ARB)

3.1 ARB in Hospital Effluents

Hospital wastewater contains a mixture of antibiotic residues, disinfectants, metabolized and non-metabolized drugs and bacterial shedding from patients' excreta [33–35]. As a result, hospital wastewater discharged to receiving waters could contribute to AMR dissemination in the natural environment if insufficiently treated [36].

Gram-negative bacteria are of particular concern in hospital settings, with the ability to cause pneumonia, bloodstream, wound, or surgical site infections [4]. In 2017, the World Health Organization (WHO) responded to the burgeoning antimicrobial resistance threat by publishing a priority list of antimicrobial-resistant pathogens which pose problems in human infections, failure to respond to current antibiotic treatment and transmissibility between humans and animals. Within the list, of highest priority are carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and carbapenem-resistant, extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* [37]. These guidelines provide a context to bacterial targets and patterns of resistance which should be incorporated into surveillance strategies.

3.2 Methods to Detect ARB and Commonly Used Susceptibility Testing Method

To isolate ARB in hospital effluents, wastewater samples are serially diluted and filtered through nitrocellulose membranes to trap biomass or spread-plate on media at dilutions required to capture viable bacterial populations within a countable range. Typically, Luria-Bertani medium [32] or selective media such as MacConkey agar [38] or CHROMagar [39] are used to support growth of viable bacteria. Colonies can then be sub-cultured and taxonomically characterized using Sanger sequencing targeting the 16S rRNA gene, multilocus sequence typing (MLST), MALDI-TOF bacterial identification or whole genome sequencing. Isolates identified, subjected to antibiotic susceptibility testing to determine resistance patterns, permit the determination of the ratio between ARB and total number of viable bacteria, which can be designated as prevalence. Alternatively, antibiotics are supplemented into media to directly select for the ARB growth and expressed as total concentrations of viable ARB.

To determine antibiotic minimal inhibitory concentrations (MIC) of bacterial isolates, manual procedures include the broth dilution tests, the antimicrobial gradient method and the disk diffusion test. Amongst the automated instrument systems, the BD Phoenix Automated Microbiology System (BD Diagnostics), the VITEK 2 System (bioMerieux) and the Sensititre ARIS 2X (Trek Diagnostic Systems) are the most commonly used [40]. Manual procedures such as the disk and gradient diffusion methods allow customization and cost savings. All these techniques provide qualitative assessments using the categories: susceptible (S), intermediate (I) or resistant (R). However, reliable interpretation of MIC values requires constant updating of current clinical breakpoints using either the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines of specific bacterial pathogens [40].

3.3 Occurrence of ARB in Hospital Effluents

The AMR selective pressure is particularly high in hospitals. For example, 20–30% of European inpatients receive an antibiotic treatment during their hospitalization [41], and antibiotics as well as antibiotic-resistant bacteria (ARB) excreted from inpatients receiving treatment contribute to the composition of hospital effluent [42, 43].

A study by Le et al. [32] described various taxa of viable ARB cultured from the effluents of two hospitals in Singapore, which showed resistance to different classes of clinically relevant antibiotics. Concentrations of ARB resistant to amikacin (1.06×10^6 CFU/mL), clindamycin (1.37×10^6 CFU/mL), erythromycin (1.24×10^6 CFU/ml), ciprofloxacin (1.14×10^6 CFU/mL) and tetracycline (1.30×10^6 CFU/mL) were at least one order of magnitude higher than those of meropenem (4.79×10^5 CFU/mL), ceftazidime (8.22×10^5 CFU/mL), vancomycin (9.19×10^5 CFU/mL), chloramphenicol (6.08×10^5 CFU/mL) and co-trimoxazole (2.54×10^5 CFU/mL). Using the same hospital effluent samples, Haller et al. [39] used a selective culture-based screening approach on chromogenic agar to specifically target Gram-negative extended-spectrum beta-lactamase (ESBL)-producing bacteria and bacteria with a decreased susceptibility to carbapenems (carbapenem-resistant bacteria, CRB). Concentrations of ESBL producers ranging from 10^3 CFU/mL to 10^6 CFU/mL and mean concentrations of CRB ranging between 10^3 CFU/mL and 10^5 CFU/mL were detected in the hospital wastewaters. Amongst the isolated bacterial strains, 35% were resistant to ceftazidime, and 39% were resistant to ceftriaxone (third-generation cephalosporins), while resistance to ertapenem and meropenem were 19 and 26%, respectively [39]. Another study by Korzeniewska and Harnisz [44] described a ceftazidime resistance rate of 81.6% in *Enterobacteriaceae* strains isolated from hospital wastewater in Poland. Picão et al. [45] measured resistance levels to third-generation cephalosporins ranging between 35 and 79% in hospital sewage in Brazil and levels of meropenem resistance of about 22%, on average.

A wide range of environmental bacteria as well as opportunistic pathogens from the gut microbiota of humans and other animals has been described in hospital wastewater discharge, but this chapter will specifically focus on those published on the WHO priority list.

3.3.1 Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli*

The concentration of *E. coli* in hospital and community wastewaters typically lies within the same range (10^7–10^8 CFU/100 mL), but concentrations of ESBL-producing *E. coli* are generally higher in hospital effluents with percentages ranging between 3.8 and 39% [33, 35, 44, 46, 47]. This is attributed to higher incidence and density of carriage amongst inpatients compared to community carriers [48]. In addition, hospital effluents contain large quantities of antibiotics and antiseptic residues that might favour as well as further the development of ESBL-producing *E. coli.*

3.3.2 Multidrug-Resistant *Pseudomonas aeruginosa*

P. aeruginosa is increasingly recognized as an emerging opportunistic pathogen of clinical importance. It is a widespread hospital-acquired pathogen responsible for respiratory and urinary tract infections especially in intensive care units, where 15% of healthcare-associated infections are attributed to this pathogen [49]. One of its most worrying characteristics is its low antibiotic susceptibility due to its concerted action of multidrug efflux pumps and low permeability of bacterial cellular envelope, as well as the liability to acquire and express antibiotic resistance genes through plasmids, integrons or other mobile genetic elements. *P. aeruginosa* is noted for its intrinsic resistance to certain antibiotics and for its ability to acquire genes encoding resistance determinants [50]. Multidrug resistance in *P. aeruginosa* occurs mainly in clinical settings, which is a result of chromosomal mutations or horizontal gene transfer. As opposed to *E. coli*, *P. aeruginosa* is not a commensal human bacterium, and the frequency of carriage amongst inpatients is low [51]. *P. aeruginosa* is ubiquitous in wastewater; however, the proportion of antibiotic-resistant *P. aeruginosa* is much higher in hospital than in urban wastewater [49, 52–55]. Recent studies reported the presence of multidrug-resistant ESBL-producing *P. aeruginosa* in hospital effluents [39].

3.3.3 Antibiotic Resistant *Acinetobacter baumannii*

A. baumannii can cause various infections like nosocomial pneumonia, bacteraemia, meningitis, and skin and soft tissue and urinary tract infections. The incidence of serious infections (blood stream infections and ventilator-associated pneumonia)

caused by multidrug-resistant *A. baumannii* ranges between 47 and 93%, with mortality rates between 30 and 70% [56]. The overuse of carbapenems has rapidly resulted in the worldwide dissemination of carbapenem-resistant *A. baumannii* strains, as reported in studies from Croatia and China [57]. The observation of multidrug-resistant *A. baumannii* in hospital wastewater has also been previously reported in Brazil, China and Zagreb in Croatia [58–60].

3.3.4 Vancomycin-Resistant Enterococci (VRE)

Enterococci are Gram-positive bacteria, which are part of the natural intestinal microbiota of animals and humans, and are released to the environment through sewage or wastewater [61]. Some members of the genus, such as *Enterococcus faecalis* and *Enterococcus faecium*, are amongst the major causes of nosocomial infections worldwide [62]. One factor contributing to the pathogenesis of enterococci is their resistance to a broad range of antibiotics. This resistance trend has increased in recent years [63]. Vancomycin is a glycopeptide antibiotic used for serious infections by Gram-positive bacteria when treatment with other antibiotics has failed. The excessive use of this antimicrobial agent has led to the appearance of vancomycin-resistant enterococci (VRE). Concentrations of enterococci in urban and hospital wastewater have been found to be similar, although the proportion of VRE was detected in higher concentrations in hospital than in urban effluents [63–66]. Varela et al. [66] reported concentrations ranging between 2.50×10^1 and 2.30×10^3 CFU/mL and between 1.60×10^1 and 2.20×10^3 CFU/mL of enterococci resistant to ciprofloxacin and vancomycin, respectively, in hospital effluents. Hospital effluent constitutes a source of enterococci having multiple resistances to antibiotics, presumably from the faeces of patients, because the rules of biosecurity in medical centres would impede other sources of contamination [67].

4 Antibiotic Resistance Genes (ARGs)

4.1 ARGs in Hospital Effluents

One of the major aspects of understanding antibiotic resistance in hospital effluents is to detect and quantify ARGs. Molecular techniques such as real-time quantitative PCR (qPCR), including singleplex, multiplex and high throughput, and metagenomics have been employed to identify and quantify ARGs in hospital effluent samples.

4.2 Application of High-Throughput qPCR (HT-qPCR) to Measure ARGs and MGE

Characterizing and quantifying resistomes are a rapid method of assessing AMR pollution. Probes and primers designed to target ARGs that confer resistance to different classes of antibiotics provide quantitative information on evaluating the abundance of genes. Unlike the traditional qPCR approach which is limited to a few targets in one assay, high-throughput qPCR (HT-qPCR) arrays are able to simultaneously quantify hundreds of ARGs and other related MGEs in one run [68]. There are a few commercially available platforms, which includes the Fluidigm Array, the Qiagen Antibiotic Resistance Genes Microbial DNA qPCR Array, OpenArray by Applied Biosystems and the WaferGen Biosystems SmartChip Real-Time PCR. Each system allows customization of primers depending on the ARGs or MGE of interest. The utility of HT-qPCR arrays has been demonstrated in environmental surveys aimed at comparing relative concentrations of ARG contamination across different aquatic sources such as lakes and estuaries [69–71], sediments of fish farms [72], drinking water [73] and wastewater treatment plants [74, 75]. Monitoring efforts of ARGs and MGE in hospital wastewaters are predominantly based on data generated from traditional qPCRs targeting the few clinically relevant beta-lactamase genes (e.g. bla_{KPC}, bla_{NDM}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{TEM}, bla_{SHV}) [32, 76–79] with a shifting trend towards using upscaled customized HT-qPCR with increased capacity to detect more ARG targets and markers of MGE [80].

4.3 Prevalent ARGs and MGEs in Hospital Wastewaters Globally and Comparisons with Other Water Sources

High prevalence of sulfonamide (*sul*) and tetracycline (*tet*) genes has been detected in various environments and deeply studied by many groups [32, 81, 82]. In addition, investigations on MGEs such as *intl*1 (class 1 integron-integrase) were included in many studies as integrases have been statistically correlated with anthropogenic sources of ARGs and are potentially involved in ARGs integration in chromosomes or plasmids [32, 81]. However, owing to the rise in importance of beta-lactam resistance and WHO's recent announcement of the global priority list that consists of beta-lactam-resistant pathogens, there is a shift in trend towards studies focused on the detection and quantification of beta-lactamase genes (e.g. bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{OXA}) [9, 82, 83]. Emerging genes such as bla_{KPC}, $bla_{NDM\text{-}1}$ and *mcr-1* are of concern in recent years due to their possible origin from hospitals, their occurrences in plasmids residing in multidrug resistance "superbugs" and the potential for these genes to spread amongst the bacteria community via horizontal gene transfer [84].

A comparison of resistomes in the final effluents of seven European countries (Portugal, Spain, Ireland, Cyprus, Germany, Finland, Norway) using HT-qPCR showed that AMR profiles mirrored patterns of clinical antibiotic resistance prevalence, providing insightful information on country- or region-specific trends of AMR distribution [85]. In a study conducted in China by Li et al. [81], ten *tet* genes (A, B, C, G, L, M, O, Q, W, X), *sul1*, *sul2* and *intl*1 were detected in hospital effluents using singleplex qPCR, with *intl*1 concentrations as high as 10^{11} gene copies (GC) per mL. Compared to residential area effluents studied in parallel with hospital effluents, the total gene abundances from hospital effluents (1.81×10^{11} GC/mL) were slightly lower as compared to residential effluents (2.79×10^{11} GC/mL). In contrast, Lamba et al. [83] compared 12 hospitals and residential effluents in New Delhi, India, where the gene targets were bla_{TEM}, bla_{OXA}, $bla_{\text{CTX-M}}$ and $bla_{\text{NDM-1}}$, and found that all the ARG concentrations had higher relative abundance (normalized to 16S rRNA genes) in hospital effluents than in residential effluents. The differences in ARG abundance could be attributed to antibiotic usage and human demographics where healthy asymptomatic individuals within the community could serve as carriers of ARGs [86].

The effects of discharging untreated hospital effluent into other environments have been evaluated in a few studies. In Tamil Nadu, India, samples were taken from five hospital effluents and five points upstream and downstream of the Cauvery River Basin where genes encoding for beta-lactamase (bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, $bla_{\text{NDM-1}}$) and aminoglycosides (*aadA*) were quantified [79]. Results showed that bla_{SHV} and $bla_{\text{NDM-1}}$ were not present upstream but were detected downstream of the river, indicating that these genes were likely introduced by wastewater. However, it was inconclusive if the genes were derived directly from hospital effluents as the source of wastewater discharge was from a combination of effluents originating from residential areas, industries and hospitals. Rodriguez-Mozaz et al. [82] quantified five ARGs (bla_{TEM}, *ermB*, *qnrS*, *sul1*, *tetW*) from hospital effluent; influent and effluent of a nearby wastewater treatment facility located in Girona, Spain, that receives the hospital effluent; and water upstream and downstream of the river that receives treated wastewater effluent. All the ARG targets in the hospital effluents were found to be of similar concentrations as compared to wastewater influents, ranging from 3 to 7 log GC/mL, but were significantly higher as compared to the other locations (wastewater effluent, river upstream and downstream) sampled. This implies that the ARG concentrations in domestic wastewater were of similar concentrations as compared to the hospital effluents. On the contrary, a massive study done in the Netherlands by Pallares-Vega et al. [87] concluded that healthcare facilities such as hospitals had minimal impact on the concentrations of ARGs entering wastewater treatment facilities. This could be an effect of dilution by domestic wastewater that have lower ARG concentrations resulting in an overall reduction in the abundance of ARGs. It is however worthwhile to note that within the same study, there was an increase in the relative abundance of broad-host-range *IncP-1* type plasmids which are known to carry broad-spectrum ARGs and are

transmissible between Gram-negative bacteria. The demographics and antibiotic usage patterns in humans and animal and differences in regulations governing the sale and use of antibiotics differ from one country to the next which could explain varying global trends in AMR.

To understand AMR trends and occurrence patterns, research groups have designed studies to compare ARG concentrations of effluents derived from different ward types across different hospitals locally to facilitate stewardship efforts. For example, a study by Le et al. [32] concluded that effluents from clinical isolation wards had higher concentrations of ARGs compared to general wards. Another study by Li et al. [81] noted significant differences in total resistance gene abundance across seven hospitals, with concentrations ranging from 10^7 to 10^{11} GC/mL. A detailed study by Lamba et al. [83] correlated the concentration of $bla_{\text{NDM-1}}$ across 12 different hospitals of different capacities and found that larger hospitals were discharging higher concentrations of $bla_{\text{NDM-1}}$. The authors concluded larger hospitals that receive higher volumes of inpatients likely result in higher AMR output in wastewaters.

In contrast to singleplex qPCR, HT-qPCR is able to profile a wider number of ARG and MGE targets in one run which provides higher throughput to comprehensively assess vectors of AMR in hospital effluents. In contrast to metagenomic profiling, HT-qPCR is more sensitive and requires less starting DNA material (PCR reactions at the nanolitre scale) with the ability to detect concentrations of 10^{-4} ARGs/16S rRNA gene [72, 88]. There are four main HT-qPCR platforms currently available in the market, with Biomark Dynamic Array (Fluidigm) requiring the lowest reaction volumes (~10 nL) followed by OpenArray (Biosystems ~35 nL reactions), WaferGen SmartChip (WaferGen ~100 nL reactions) and Bio-Rad CFX384 (Biorad ~3,000 nL reactions) [88].

In a study done in Xinxiang City, Central China, Wang et al. [80] fabricated 258 qPCR primers and utilized a HT-qPCR platform to detect 178 unique ARG targets that confer resistance to seven classes of antibiotics and two MGE targets (*intl1* and *Tn916/Tn1545*) to compare concentrations of wastewater from three tertiary public hospitals in the city. A core of 126 ARGs were detected in all three hospital effluents. Concentrations of 12 frequently detected ARGs (*tetM*, *tetO*, *tetX*, *ereA*, *ermA*, *ermB*, *sul1*, *sul2*, *sul3*, *qnrA*, *qnrB*, *oqxB*) were validated by qPCR yielding results of highest concentrations of *tetO* detected in effluents of two hospitals, with *sul1* detected at high abundance in the effluents of the third sampled hospital. Amongst the five MGEs (*intI1*, *intI2*, *intI3*, *Tn916/Tn1545*, *ISCR1*), *ISCR1* had the highest abundance ranging from 10^7 to 10^8 GC/mL. It would be advantageous to use HT-qPCR for routine monitoring of hospital effluents as it is time-efficient, with a low sample volume requirement per reaction, without the reliance on complicated downstream bioinformatics analyses when compared to using metagenomics.

5 Resistomes and Mobile Genetic Elements (MGEs)

5.1 Uncovering Resistomes by Metagenomics

In the field of water research, integrated multi-omics approaches have been used as bio-monitoring tools for water quality assessment to investigate microbial composition, their functional roles and involvement in water contamination [89]. One of the advantages of metagenomics in AMR surveillance is the collective recovery of genomes from microbes in environmental samples which provides genetic insights to microbial composition (bacterial and viral), ARGs and other MGEs (e.g. plasmids, integrons, transposons). The ability to capture entire genomic profiles to track the distribution of ARGs and MGEs in a variety of environments has made it possible to assess and identify AMR hotspots in different aquatic compartments [96], sources and sinks of ARGs in environmental waters [90], ARG removal in the wastewater treatment process [91, 92] and fate and transport of ARGs in environments receiving treated wastewater effluents [93–95]. There are a handful of studies which have applied metagenomics as an opportunity to create ARG and microbiome catalogues of hospital wastewaters to identify novel carbapenemases [96] and to classify and resolve differences between municipal wastewaters [97, 98] and waters receiving treated hospital wastewaters [99].

Environmental resistomes are profiled by interrogating metagenomic reads or assembled contigs against one of the publically available ARG databases such as the Comprehensive Antibiotic Resistance Database [100], the Antibiotic Resistance Database [101], Resfams [102], ARG-ANNOT [103], ResFinder [104], MEGARes [105] and ARGs-OAP [106]. The relative abundance of ARGs identified from metagenomic datasets is then calculated by normalizing to the ARG reference sequence length (nucleotide) and to the number of 16S rRNA genes [107] or by coverage normalized to the ARG reference gene and size of metagenomic dataset [108].

5.2 Identifying MGEs

Intercellular mechanism of exchange mediated by MGEs such as plasmids, transposons and integrons play a major role in AMR dissemination as they facilitate the capture, transfer and expression of exogenous ARGs [109, 110]. The mobility of plasmid-borne ARGs and the rates of inter- and intraspecies transfer in hospital effluents are largely unknown. In a laboratory-scale experiment, Chen et al. [111] demonstrated plasmid transferability through mating a ceftazidime-resistant strain of *A. baumannii* isolated from hospital wastewaters with a ceftazidime susceptible *E. coli* as a recipient. Whole genome sequencing of plasmids in donor and transconjugants showed highly similar sequences, concluding that plasmid-mediated intraspecies transfer of ARGs had occurred. Interspecies transfer of ESBL-encoding

plasmids between microbial community within a hospital sink environments has been inferred [112, 113]. However, there is limited data available on the frequency and environmental cues that trigger ARG transfer in clinical wastewaters.

Class 1 integron gene cassettes, which are frequently carried by human pathogens, often include ARGs acquired by genetic recombination. Hence, insight to their presence in hospital wastewater may contribute to infer about the risks associated with ARG dissemination [96]. There are a range of bioinformatics tools designed for in silico detection of integrons (I-VIP [114], MARA [115], INTEGRALL [116]) and plasmids which are carried by *Enterobacteriaceae* and Gram-positive bacteria (PlasmidFinder [117]). Inspecting assembled metagenomic datasets (contigs) for co-localization of ARGs and MGE features provides a means of exploring specific mechanisms that mediate the spread of certain ARG types, with this analysis approach proposed as a method to predict ARG mobility incidence in environmental resistomes [118].

5.3 *Examples of the Application of Metagenomics to AMR Monitoring in Hospital Wastewaters*

There is currently more literature on resistome profiles in hospital wastewaters using either qPCR or HT-qPCR, rather than metagenomics. This could be attributed to better sensitivity (detection limits) offered by qPCR platforms that are target specific and easily interpreted [88]. A metagenomics approach in contrast seizes information of known and unknown DNA sequences in a single run, thus providing a greater depth of sequence information without the restraint of a specific targeted sequence [119]. For example, within the context of AMR monitoring in wastewaters, Le at al. [32] used qPCR to detect the relative abundance of four beta-lactamase ARG targets (bla_{NDM}, bla_{KPC}, $bla_{\text{CTX-M}}$, bla_{SHV}). However, a more in-depth metagenomics analysis of the same samples gave a snapshot of the entire ARG diversity within the hospital wastewater samples and allowed the assembly of entire scaffolds providing information on ARG arrangements and co-occurring MGEs within the same gene neighbourhood [97]. Leveraging on the latest DNA sequencing technologies by combining long-read data from third-generation sequencing platforms (Oxford Nanopore) with Illumina short-read data has yielded better sequencing coverage and assembly of ARG bearing plasmids as described in a study of wastewater treatment plants [120].

AMR metagenomics studies conducted in Singapore [97], the Netherlands [99] and France [98] appear to have a consistent pattern of a core microbiome specific to hospital wastewaters. All three studies reported the predominance of anaerobic human gut bacteria belonging to the order *Clostridiales*, *Bifidobacteriales* and *Bacteroidales*. There were however other dominant bacterial taxa (e.g. *Acinetobacter baumannii*, *Enterobacteriaceae*) that contributed to differences in hospital wastewater microbiomes originating from different countries [96–99], which could explain AMR variations globally [119].

As an extension of the utility of rapid AMR and microbiome profiling, Li et al. [121] demonstrated that by integrating metagenomics datasets from different sources with complementary metadata into machine learning classification models, AMR source contribution could be identified to predict putative sources of ARG contamination. This would be particularly useful in tracking the dissemination of AMR originating from hospital effluents.

5.4 *Targeted Metagenomics for Qualitative and Quantitative Resistome Analysis*

One of the challenges with the application of metagenomics within complex microbial communities is the detection sensitivity of low abundant bacterial populations that harbour ARG [122]. To overcome the limitation of heterogeneity, Lanza et al. [123] adopted an in-solution targeted capture platform (TCP), a technique used for diagnosis of human-inherited diseases [124] to develop a targeted metagenomic resistome analysis method coined "ResCap", a TCP based on SeqCapEZ (NimbleGen) technology. The TCP is designed to target ~78,000 nonredundant genes, comprising of ARGs, genes conferring resistance to metals/biocides and relaxase genes as plasmid markers [123]. Briefly, whole-metagenome shotgun libraries are constructed, and DNA captured by the probes are sequenced using Illumina platform and analysed using the ResCap bioinformatics workflow. Comparison of resistomes identified by metagenomic shotgun sequencing versus the ResCap platform showed improved gene abundance detection of 2–83% and increase of gene diversity detection by 300-fold [123]. This underscores the large proportion of ARGs that go undetected by relying on just metagenomics alone. The sensitivity and specificity of the ResCap technology provide qualitative and quantitative means of measuring the levels of ARG contamination which could potentially meet the needs of AMR monitoring and tracking from source to sink.

5.5 *Other OMIC Strategies to Study ARG Expression Levels in Hospital Wastewaters*

To understand the activity and contribution of ARB to AMR dissemination in linked aquatic environmental sources, a combined OMIC approach of metagenomics and metatranscriptomics was used to detect ARG transcripts in wastewaters from hospital and farm effluents into a receiving river in Cambridge, United Kingdom [125]. The authors reported a significant overexpression of bla_{GES} and bla_{OXA} in hospital effluents over a consistent period of 5 months relative to the two other sampled waters which was considered to be due to the levels of antibiotic usage in hospitals [125].

6 Curbing the Spread of AMR

Antimicrobial stewardship in hospitals is a prescribed intervention strategy by the WHO Global Action Plan to contain the spread of AMR beyond the clinical setting [126]. National or regional surveillance networks that monitor antibiotic usage and resistance using standardized methods will enhance knowledge on the extent of AMR severity, region-specific prevalence trends and associated health outcomes [127]. Antimicrobial peptides, probiotics, phage therapy and phage endolysins have been proposed as alternative replacements of antibiotics. However, safety and efficacy in vivo in humans have yet to be determined [128, 129].

7 Possible Treatment Technologies of Hospital Wastewaters

As hospital effluents are recognized as sources of AMR, there is increased awareness in possibly pretreating effluents before discharging them into municipal sewage. Two published studies have attempted to evaluate the removal efficiency of ARB and/or ARGs and MGE.

In Riyadh, Saudi Arabia, Timraz et al. [38] investigated the removal efficiency of wastewater treatment systems placed on site at two different hospitals. Although both treatment plants utilized conventional activated sludge process followed by chlorination, one plant outperformed in terms of log removal values of total viable bacteria and ARGs. The ARGs *sul1* and *intl*1 remained detectable at concentrations of up to 10^5 GC/mL in hospital effluent from the plant with a more interior removal performance. This observation suggests that operational parameters of wastewater treatment plants play a vital role in removal efficiencies of vectors of AMR.

Paulus et al. [9] compared the removal efficiency between an advanced on-site treatment facility (membrane bioreactor/ozonation/activated carbon/UV treatment), in two Dutch cities which received hospital effluent directly, and a municipal wastewater treatment facility, which received both hospital and residential effluents. Data showed significantly higher removal efficiency for the 13 ARG targets by the advanced treatment as compared to the municipal treatment facility. The study recovered the ARGs bla_{KPC} and *vanA* only in hospital effluents, which suggests that healthcare facilities are potential sources of these clinically important ARGs.

Other known methods, such as coagulation [130] and the use of biochar [131], have been demonstrated to effectively remove ARB, ARGs and antibiotic residues, although these methods have only been used to treat other types of waste other than hospital effluents. Nevertheless, these methods have the potential to pretreat hospital effluents before discharge into the main sewers.

8 Conclusion

This review covers the detection and quantification of the three main aspects of AMR (antimicrobial residue, antibiotic resistance bacteria and genes) and their occurrences in multiple hospital discharges. There is a need to step up surveillance systems of wastewater discharged from hospitals which are potential drivers for the spread of AMR. Factors which influence differences in AMR occurrence are dependent on the age and size/capacity of the hospital and the severity and types of infections amongst inpatients. The implementation of the latest molecular and OMIC techniques reviewed in this chapter could provide new standardized methods of qualitatively and quantitatively assessing the dissemination of a wider array of ARGs in different aquatic sources. Physical and chemical treatment processes can be put in place to pretreat hospital discharges in order to reduce the spread of AMR into receiving domestic wastewater treatment facilities or natural water bodies.

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Control Strategies to Combat Dissemination of Antibiotic Resistance in Urban Water Systems

Jianhua Guo, Yue Wang, Yunus Ahmed, Min Jin, and Jie Li

Contents

Abstract The intensive use of antibiotics for medical, veterinary, or agricultural purposes results in the continuous release of antibiotics into the environment, leading to the increasingly widespread occurrence of antibiotic resistance. Although antibiotic resistance has been recognized as a major threat to human health worldwide, the related phenomenon occurring in natural and engineered environments has so far been largely overlooked. The urban (including industrial) water cycle, which

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connects urban life, agriculture, and the environment, is potentially a hot spot for the spread of antibiotic resistance. Therefore, better understanding of the distribution and transportation of antibiotic-resistant bacteria (ARB) and acquisition of antibiotic resistance genes (ARGs) in the urban water cycle is critically important to improve the control of this emerging environmental and human health challenge. In this book chapter, we comprehensively review the occurrence, transfer, and acquisition mechanisms of ARGs in the urban water cycle. Various methods that are used to monitor ARB and ARGs are compared in terms of their strengths and limitations. Opportunities for the development of real-time monitoring methods are discussed, along with possible control strategies for ARB and ARGs in urban water environments. We recommend that three major barriers should be developed to minimize or halt the spread of ARGs in urban water systems, including more efficient water disinfection, advanced wastewater treatment, and optimized sludge treatment processes.

Keywords Advanced oxidation processes (AOPs), Antibiotic resistance genes (ARGs), Antibiotic-resistant bacteria (ARB), Disinfection, Wastewater treatment plants (WWTPs), Water treatment plants (WTPs)

1 The Spread of Antimicrobial Resistance in the Environment

1.1 One Health Perspective

The widespread use of antimicrobial agents in human and veterinary medicine, animal farming, and agri-industrial production and their subsequent release into water, air, and soil have contributed to the emergence and dissemination of antimicrobial resistance (AMR) in the environment. A diverse mixture of antibiotics and other pollutants, their metabolites, and resistant bacteria reach the environment through treated and untreated wastewater, urban litter, hospital waste, aquaculture discharges, and agricultural runoff. On the other hand, the environment is also providing food and crops to human and animal, as well as serving as recreation spots, which in turn facilitate the spread of AMR among communities [1, 2]. Figure 1 illustrates the possible pathways of how AMR disseminates between human, animal, and environments. Bacteria in soil, rivers, and seawater can develop resistance through contact with resistant bacteria, antibiotics, and disinfectant agents released by human activity. People and livestock can then be exposed to more resistant bacteria through food, water, and air from the environment [3]. Therefore, the environment is key to the AMR dissemination and has a significant role in driving resistance transfer and resistant bacteria evolution [4].

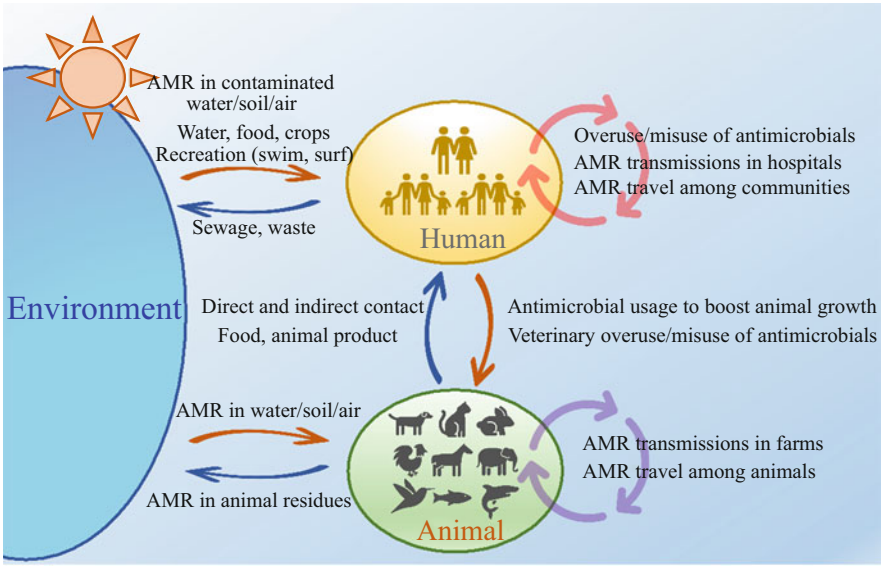

Fig. 1 An illustration of the antibiotic resistance dissemination

1.2 Occurrence and Dissemination of Antibiotic Resistance

Antibiotic resistance can be intrinsic or acquired. Some ARB and ARGs are naturally occurring in environment, for example, the analysis of Beringian permafrost sediments showed that resistance toward β-lactam, tetracycline, and glycopeptide antibiotics existed 30,000 years ago, much earlier than Fleming discovered the first antibiotic in 1928 [5]. Antibiotic resistance can be found in pristine areas unimpacted by anthropogenic activities [6, 7]. While some resistances are intrinsic, many are acquired. This can occur through a mutation in bacterial DNA or by obtaining ARGs through horizontal gene transfer via conjugation, transformation, and transduction.

1.2.1 Mutation and Co-selection

A gene mutation is a permanent alteration in the DNA sequence of bacteria, which can be triggered by factors that display SOS DNA stress response or oxidative stress response. The SOS response is a global response to DNA damage of the cell, while oxidative stress response is induced by overproduction of reactive oxygen species (ROS), including superoxide, peroxides, hydroxyl radicals, and singlet oxygen [8]. The rate of occurrence of spontaneous mutation is very low: in the range of one in one million to one in ten million cells [9]. However, contaminants in urban water systems can facilitate the process of mutation. Major classes of antibiotics, including quinolone, β-lactams, and aminoglycoside, can promote production of

ROS in both gram-negative and gram-positive bacteria, which will induce SOS response and genetic mutagenesis [10, 11]. Moreover, other environmental contaminants, including heavy metals, disinfection byproducts, pharmaceuticals, and personal care products, can increase mutation frequency [12–15]. For example, it was recently shown that both the non-antibiotic antidepressant drug fluoxetine and the non-antibiotic antimicrobial triclosan could induce multiple antibiotic resistance in *Escherichia coli* at environmentally relevant concentrations (0.02 and 0.2 mg/L [16, 17]). After 30 days' exposure, the fold change of mutation frequency toward resistance to antibiotics, including tetracycline, chloramphenicol, and amoxicillin, could be up to 10^7 compared with the control group without any exposure to fluoxetine. The mutants were confirmed to possess heritable multiple-drug resistance. Based on genome-wide DNA sequencing, RNA sequencing, and proteomic profiling, fluoxetine and triclosan were shown to enhance ROS generation, promote multidrug efflux pumps in bacteria, and thus induce mutagenesis toward multiple antibiotics [14, 15].

Bacteria can also develop antibiotic resistance through co-selection. Co-selection is indirect selection that can occur physiologically (cross-resistance) and genetically (co-resistance) [18, 19]. Cross-resistance occurs when one resistance gene can confer resistance to other antimicrobials. For example, metal exposure induces bacterial multidrug efflux pumps to extrude intercellular toxins, and multidrug efflux pumps can decrease susceptibility to both metals and antibiotics [20]. Co-resistance is a genetically linked mechanism, whereby multiple types of resistance genes are located together on the same mobile genetic element (MGE). For example, the sulfonamide resistance gene *sul1* is frequently co-selected via co-resistance as it is often located on integrons (which variable regions often contain ARGs) [21]. The phenomenon of co-selection means that an ARG can be selected in the absence of the antibiotic or other environmental contaminant to which it confers resistance. Numerous studies indicate that aquatic contaminants, including heavy metals, nanomaterials, disinfectants, and personal care products, can accelerate the spread of antibiotic resistance through co-selection [19, 22, 23].

1.2.2 Horizontal Gene Transfer

Horizontal gene transfer refers to the process whereby DNA is transferred (either from a donor cell or external environment) to a recipient cell and becomes part of the recipient genome [24]. This can take place across different bacterial strains and species and thus plays an important role in the spread of antibiotic resistance in aquatic environments. Horizontal gene transfer includes three major pathways: conjugation, transformation, and transduction (Fig. 2).

Conjugation is the transfer of DNA between a donor and a recipient and is an important pathway of horizontal gene transfer [25]. Conjugation is usually accomplished by MGEs (where ARGs may be located) in the donor bacteria, and MGEs will be transferred to the recipient bacteria through a pilus bridge, by direct cell-to-cell contact. Transformation results from direct uptake and incorporation of

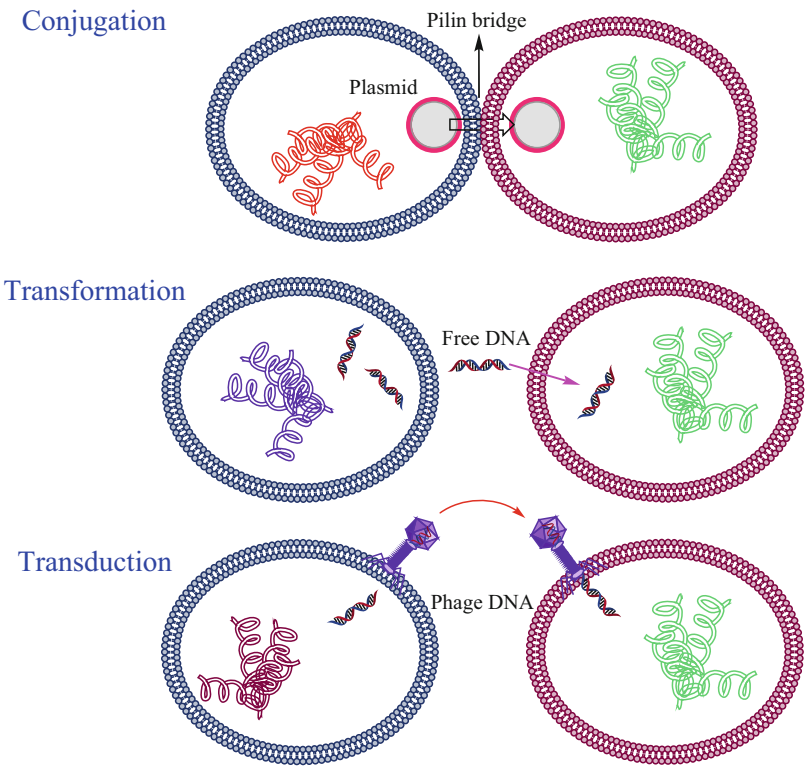

Fig. 2 Mechanisms of horizontal gene transfer

exogenous genetic elements (harboring ARGs) from bacteria's surroundings through the cell membranes. Cells need to be in genetically competent state to accomplish transformation [26, 27]. Transduction is mediated by bacteriophages, in which DNA is transferred to another bacterium by bacterial viruses [25].

In urban water systems (including water treatment plants (WTPs), wastewater treatment plants (WWTPs), and receiving waters), bacteria, MGEs, and bacteriophages coexist; thus, horizontal gene transfer happens with a high frequency and wide range, thus resulting in the spread of antibiotic resistance [28]. Moreover, it is shown that aquatic contaminants can facilitate the horizontal transfer of antibiotic resistance. For example, subinhibitory concentrations of antibiotics, including β-lactams, aminoglycoside, fluoroquinolone antibiotics, and mitomycin C, can enhance the transfer of tetracycline resistance plasmids in *Staphylococcus aureus* or induce transformability in *Streptococcus pneumoniae* [29]. In addition to antibiotics, other frequently detected environmental contaminants are also shown to facilitate horizontal transfer of antibiotic resistance. These contaminants include non-antibiotic pharmaceuticals, nanomaterials, ionic liquids, and even disinfectants and disinfection by-products [30–32]. For example, subinhibitory concentrations of widely applied disinfectants (free chlorine, chloramine, and hydrogen peroxide)

could lead to conjugative transfer increment conjugative transfer up to sevenfold due to overproduction of ROS, enhanced oxidative stress, increased cell membrane permeability, and altered expression of conjugative-related genes [33]. It has recently been demonstrated that disinfection by-products can also increase natural transformation rates of environmental free DNA, e.g., to *Acinetobacter baylyi* ADP1 [34].

2 Comparisons of Various Detection Methods

Until now, various methods have been developed to investigate the emergence, abundance, and diversity of ARB and ARGs in urban water systems. These methods include both culture-dependent and culture-independent techniques. Culture-dependent methods include the direct cultivation of ARB, while culture-independent techniques refer to the analyses of genetic materials (e.g., DNA or RNA), through techniques such as quantitative PCR (qPCR), high-throughput qPCR, or metagenomics.

2.1 Culture-Based Methods

Environmental water samples generally contain 10^8–10^{10} colony forming unit (CFU) per milliliter [35], which is a challenge to culture target microorganisms; thus selective media are commonly used to isolate target bacteria. When assessing ARGs, culture-based methods commonly involve isolating target bacteria on culture media and testing bacterial growth in response to specific concentrations of antibiotics. Isolation of bacteria enables researchers to study phenotypic and genotypic characteristics of isolates, but is time-consuming, and is not practical for large-scale quantitative analysis [36].

2.2 Quantitative PCR (qPCR)

Real-time qPCR measures DNA amplification and by interpolation of a standard curve permits the quantification of copy number within genetic material or the expression level of a specific gene fragment with specific primers in a sample. Because of moderate cost, exquisite sensitivity, and specificity, qPCR has been widely used over the last decade as a feasible approach to investigate the occurrence and abundance of various kinds of ARGs in different environmental matrices [37, 38]. This approach permits the expression of ARGs or MGEs abundance per volume or dry weight of sample, as well as in relative abundance, often per 16S rRNA gene copy number. Nevertheless, amplification bias can occur, e.g., due to

insufficient primer specificity or false negatives caused by amplification inhibitors and may result in incorrect calculations of ARG abundance [39]. In addition, qPCR is time-consuming, especially if a study aims to quantify multiple ARGs and MGEs in environmental settings, and qPCR is not suitable for discovering and identifying new ARGs.

2.3 High-Throughput qPCR (HT-qPCR)

Recently, HT-qPCR has been developed to determine the diversity (i.e., number of positive targets) and abundance of ARGs and MGEs in environmental settings. The Takara SmartChip (previously WaferGen) is a high-throughput qPCR platform with the capability to run 5,184 qPCR reactions within 3–4 h [40], allowing up to 384 primer sets to be analyzed in parallel [40]. Due to its high efficiency, HT-qPCR has been increasingly used to monitor the fate of ARGs in distinct environmental matrices, such as drinking water samples, soil samples, wastewater samples, and sediments [41–43]. On the downside, HT-qPCR shares the major limitations associated with traditional qPCR, including the potential for PCR biases and unsuitability for new ARG discovery and quantification. In addition, the results are normally expressed in terms of relative abundance, given the limited feasibility of obtaining standard curves for all analyzed fragments.

2.4 Metagenomic Sequencing

2.4.1 Short-Read Metagenomic Sequencing

Metagenomics can overcome limitations of selective amplification by obtaining DNA sequences of the total genetic material, thus providing an overview of the entire gene pool in a given sample and eventually inferring about the respective functional potential [44]. The application of metagenomics enables scientists not only to investigate the emergence and relative abundance of known ARGs and their variants but also to infer about possible new ARGs that may exist in a given environment [45].

In recent years, metagenomics has been widely used to investigate the fate of ARGs in WTPs, WWTPs, and surface water and to identify potential new ARGs in various environments [46, 47]. However, as microbial genomes are usually highly complex with repetitive elements such as insertion sequences (IS), integrons, and CG islands, it may be difficult to construct complete genomes or even genes from short-read metagenomics data.

2.4.2 Long-Read Metagenomic Sequencing

Third-generation sequencing technologies such as Pacific Biosciences (PacBio) and Oxford nanopore sequencing are able to deliver reads in excess of several kilobases (up to 2 Mb according to the website of Nanopore). As a result, long-read sequencing can potentially span repetitive regions (e.g., structural variants) and CG islands using a single continuous read, thus eliminating ambiguity as to the positions or size of genomic elements. In addition, long-read sequencing is capable of obtaining more information about the context of ARGs like IS and transposons [48]. Although long-read sequencing makes it easier to accurately decode upstream and downstream elements, it is currently difficult to avoid high error rates. Very recently, increasing studies have used nanopore sequencing technology to investigate the occurrence and abundance of ARGs in wastewater and activated sludge [48, 49].

The advantages and disadvantages of culture-independent approaches are summarized in Table 1. All of the approaches have different strengths and weaknesses, and no single approach is outstanding with respect to all parameters. Overall, metagenomics exhibits an enhanced capacity for surveillance and tracking ARGs in complex environments (e.g., activated sludge). Metagenomic approaches also enable the tracking of plasmids and other gene transfer elements in environment [47, 50, 51]. However, when conducting the data analysis, false negatives and false positives may appear by using current bioinformatics pipelines. False negatives will identify ARGs as non-ARGs, while false positives will identify non-ARGs as ARGs-like sequences. Therefore, the bioinformatics pipeline should be further updated to minimize these false positives and negatives. In addition, the benchmark for bioinformatics pipeline development using next-generation sequencing technologies is needed to systematically monitor and compare the fate of antibiotic resistance in urban water systems.

3 Distribution, Diversity, and Abundance of ARB and ARGs in Urban Water Systems

A complete urban water cycle typically includes water collection and storage facilities at source sites, water transport via aqueducts (canals, tunnels, and/or pipelines) from source sites to water treatment facilities; WTPs, storage, and distribution systems; wastewater collection (sewer) systems and WWTPs; and networks discharging to rivers or lakes (receiving waters) and/or recycled water supply systems. Since some antibiotic resistance genes are naturally occurring, no environment could be completely devoid of ARB or ARGs, and urban water systems are not an exception to this [5]. However, anthropogenic activities, in particular the misuse and overuse of antibiotics, are accelerating the spread of antibiotic resistance, which could be evident from the fact that more ARB and ARGs are occurring in urban water systems. For example, WWTPs are at the interface between human

Table 1 Summary and comparisons of culture-independent detection methods

Index	qPCR	HT-qPCR	Short-read metagenomic sequencing	Long-read metagenomic sequencing
Cost	Low	Low	Medium	High
Time of detection	3–4 h	3–4 h	Depending on methods, but usually days at least	Depending on methods, but usually days at least
Bias	Bias induced by primers, or by amplification inhibitors	Bias induced by primers, or by amplification inhibitors	Bias induced by similar regions among genomes	Abundance bias induced by insufficient sequencing depth for error correction
Able to detect new ARGs	No	No	It is possible to detect new ARGs through identity check with known ARGs	There are more possibilities to detect new ARGs through identity check with known ARGs as it is easier to assemble the ARGs out
Able to decipher the context of ARGs	No	No	It is possible to decipher the context of ARGs if researchers can obtain long enough contigs	It is possible to decipher the context of ARGs if researchers can obtain long contigs, but long-read sequencing is easier for researcher to obtain long enough contigs
Able to detect rare ARGs	It is possible to detect rare ARGs through amplification processes	It is possible to detect rare ARGs through amplification processes	Rare ARGs may be missed due to noise from other genes and elements	Rare ARGs may be omitted during data correction processes due to low sequencing depth
Able to detect variants of ARGs	If specific primers can be designed successfully, variants of ARGs can be detected	If specific primers can be designed successfully, variants of ARGs can be detected	If the data depth is enough to correct reads, variants of ARGs can be detected	If the data depth is enough to correct reads, variants of ARGs can be detected

populations and aquatic/soil environments. A range of diverse sources (e.g., hospitals, households, industries, animal farms), with organic, chemical, and microbiological contaminants, contribute to the WWTP environment. WWTPs are considered to act as a potential source of ARB/ARGs to the environment, since insufficient removal leads to discharge of ARB and ARGs into urban rivers and other receiving environments. ARB and ARGs could also be concentrated in biosolids in WWTPs, which may be used as soil amendments, thus facilitating the spread of ARB and

ARGs from the water environment to the soil environment [52]. In addition, ARB and ARGs are present in urban rivers and source waters and are also in the water intake to WTPs. If the disinfection in WTPs or WWTPs is not efficient enough, there is a risk that ARB and ARGs can spread via the treated drinking water, reclaimed water, or WWTP effluent [53, 54]. In addition, ARB and ARGs are also transported from WWTPs as aerosols and further enter surface water via rainfall [55, 56]. The illustration of transfer and distribution of ARB and ARGs in urban water systems is shown in Fig. 3. Collectively, the presence of ARB and ARGs in wastewater, source water, and drinking water could greatly affect public health and well-being, and these are emerging issues for the ecological environment.

A summary of ARB and ARGs measured in WWTPs is provided in Table 2. Due to different WWTP influent characteristics and operating conditions, ARB and ARG removal efficiencies may also differ. In general, ARB and ARGs abundance in effluent is lower than that in influent, yet this may be partially due to them being concentrated into sludge during the treatment process in WWTPs. For example, based on metagenomic analysis, a total of 42 ARG subtypes belonging to 10 ARG types were identified in aerobic activated sludge, while 51 subtypes belonging to 9 ARG types were detected in anaerobic digested sludge, indicating ARB and ARGs were concentrated during the sludge treatment process [46]. Among the detected ARGs in sludge, they mainly conferred resistance to tetracyclines, fluoroquinolones, β-lactams, sulfonamides, aminoglycosides, glycopeptides, phenicols, and trimethoprim [46, 57, 58].

Another important pathway for antibiotic resistance dissemination in urban water systems is through wastewater reuse (irrigation, agricultural soil fertilization, animal production), which can potentially contaminate surface water, groundwater, and even drinking water distribution networks [59, 60]. For example, multidrug-resistant bacteria, including New Delhi Metallo-β-Lactamase-Encoding Bacteria, can be disseminated through water, including rivers and drinking water systems [61]. Other ARB, such as carbapenem-resistant bacteria, and ARGs, such as *bla*, *sul*, and *tet* genes, are also observed in drinking water worldwide. A summary of ARB and ARGs detected in drinking water systems is shown in Table 3. Moreover, ARB and ARGs are frequently detected in other water bodies around the world, including estuaries, rivers, surface waters, and groundwater. Highly detected genes are *tet*, *bla*, and *sul*, with the abundance of 10–10^5 copies/mL. For example, bacteria isolated from aquaculture sources in Australia were found to be resistant to ampicillin, amoxicillin, cephalexin, and erythromycin, with prevalence of 24 ARGs [62]. These facts indicate that ARB and ARGs are distributed widely in urban water systems.

4 Various Treatment Processes

WWTPs have been suggested as sources of antibiotic resistance because they carry wastewater with antibiotics, ARB, and ARGs, which hypothetically create hot spots for horizontal transfer of ARGs and can disseminate ARB and ARGs into the

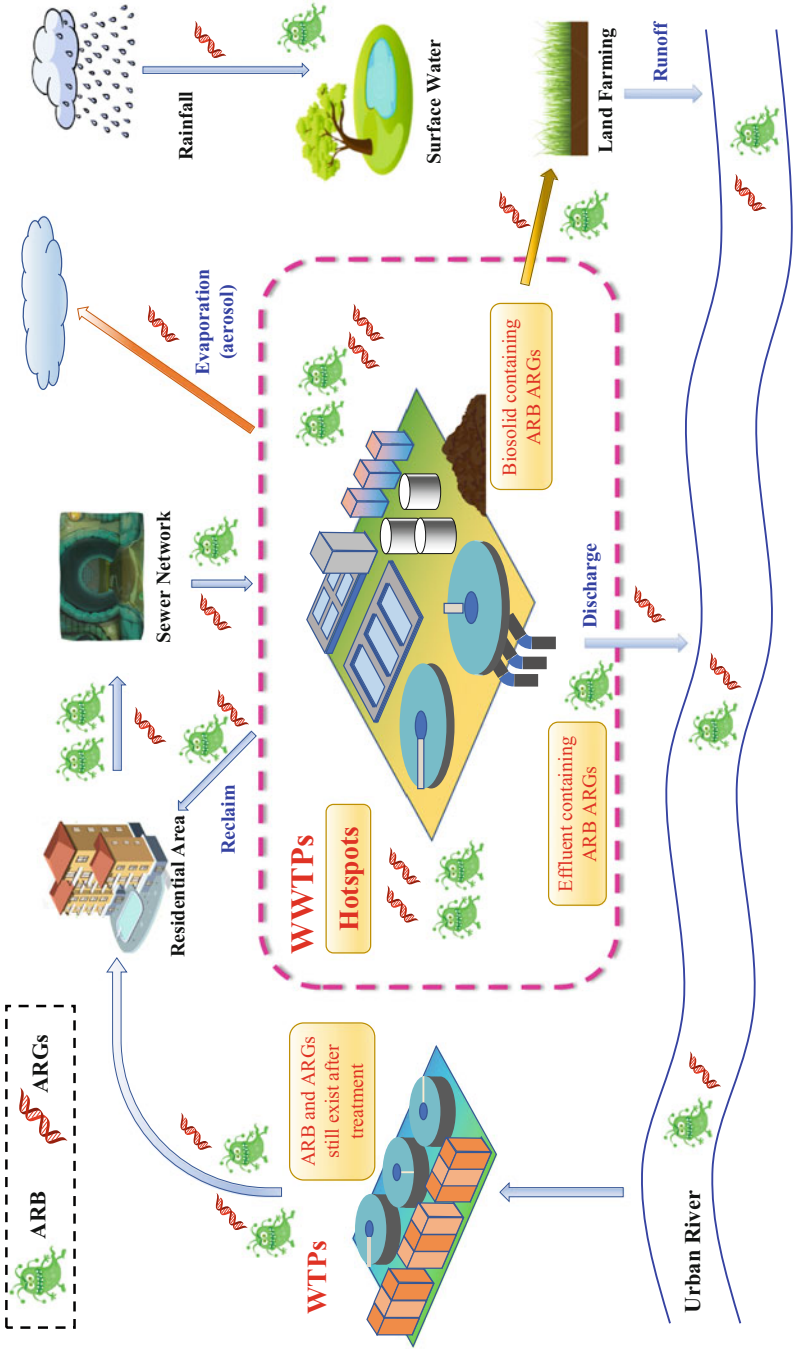

Fig. 3 Transfer and distribution of ARB and ARGs in urban water systems

Table 2 Occurrence, abundance, and diversity of ARB and ARGs in WWTPs within the last 10 years

Continent	Country	Types of ARGs/ARB	ARGs/ARB abundance		Detection method	Reference
			Influent	Effluent		
Europe	Spain	bla_{TEM}, *ermB*, *qnrS*, *sulI*, *tetW*	10^4–10^6 copies/mL; total 10^7 copies/mL	10^2–10^3 copies/mL; total 10^4 copies/mL	qPCR	[63]
	Finland	$bla_{ctx\text{-}m\text{-}32}$, $bla_{oxa\text{-}58}$, $bla_{shv\text{-}34}$, *sul1*, *sul2*, *tetC*, *tetM*	10^6–10^9 copies/mL	10^4–10^7 copies/mL	qPCR	[64]
	Portugal	*intI1*, bla_{TEM}, *vanA*, *marA*	10^3–10^7 copies/mL	10^2–10^6 copies/mL	qPCR	[65]
	Seven countries, include Portugal, Spain, Ireland, Cyprus, Germany, Finland, Norway	Resistance toward aminoglycosides, amphenicols, β-lactams, quinolones, multidrug, macrolide-lincosamide-streptogramin B, sulfonamides, tetracycline, vancomycin	Relative gene abundance (ratio of ARGs copy number to 16S rRNA gene copy) 0.20–1.70	Relative gene abundance (ratio of ARGs copy number to 16S rRNA gene copy) 0.02–1.30	qPCR-array	[66]
	Ireland	*Escherichia coli* strains resistant to ampicillin, streptomycin, sulfamethoxazole, tetracycline, cefotaxime, ciprofloxacin, cefoxitin	10^4 cfu/mL	10^1–10^3 cfu/mL	Culture-based	[67]
	Poland	Fecal coliforms and fecal enterococci resistant to 33 antibiotics	10^6 cfu/mL	10^4 cfu/mL	Culture-based	[68]
	Germany[a]	Heterotrophic bacteria resistant to vancomycin, ceftazidime, cefazolin, penicillin, imipenem	NA[b]	0.6–39% of the total bacteria	Culture-based	[60]
	Denmark[c]	Resistance toward β-lactam, aminoglycoside,	In total 8,540 resistant clones were identified. On	NA[b]	Metagenomics	[69]

		macrolide, tetracycline, phenicol, rifamycin, sulfonamide, and dihydrofolate reductase inhibitor	average, 534 colonies per antibiotic were detected, which ranged from 30 clones for chloramphenicol to more than 2,000 clones for trimethoprim. Core resistome hosts include expression vector, *Sphingopyxis alaskensis*, *Oceanithermus profundus*, *Streptomyces albus*, *Propionibacterium acidipropionici*			
America	USA[d]	Tetracycline resistance determinants (tet^R)	10^6–10^9 copies/mL	10^4–10^6 copies/mL	qPCR	[57]
	USA	$bla_{SHV/TEM}$, *sul1*	10^7–10^8 copies/mL	10^3–10^4 copies/mL	qPCR	[70]
	USA	Seventeen classes of antibiotic resistance include resistance toward trimethoprim, tetracycline, sulfonamide, streptothricin, streptogramin, rifampin, polymyxin, peptide, macrolide, lincosamide, glycopeptide, fosfomycin, fluoroquinolone, chloramphenicol, β-lactam, aminoglycoside, and aminocoumarin	10^7–10^8 copies/mL, the most abundant classes of ARGs were trimethoprim (10.6–49.7%), aminocoumarin (0–18.5%), β-lactam (1.3–38.5%), polymyxin (0.4–11.1%), and aminoglycoside (0.9–9.8%) resistance	10^6 copies/mL	Metagenomics, qPCR	[47]
Asia	China[e]	Tetracycline resistance genes	10^7–10^8 copies/mL	10^4–10^5 copies/mL	qPCR	[58]

(continued)

Table 2 (continued)

Continent	Country	Types of ARGs/ARB	ARGs/ARB abundance		Detection method	Reference
			Influent	Effluent		
	China[f]	Twenty ARG types consisting of 381 subtypes were detected, with 373 subtypes in summer samples and 346 subtypes in winter samples	The three most dominant resistance gene types, conferring aminoglycoside, tetracycline, and beta-lactam resistance, accounted for 54.1% of the total ARGs abundance. For the resistance gene subtypes, genes encoding beta-lactamase, sulfonamide (*sulI*), and tetracycline (*tet40*) were the most common in all sewage. Relative abundance of ARGs in summer, 1.73 copies per bacterial cell; in winter, 1.15 copies per bacterial cell. Absolute abundance of ARGs in summer: 3.27×10^{11} copies/L; in winter: 1.79×10^{12} copies/L. ARB concentration in summer: 1.70×10^{11} cells/L; in winter: 1.21×10^{12} cells/L	NA[b]	Metagenomics	[71]
	Hong Kong, China	Six genera including *Flavobacterium*, *Poriferibacter*, *Bacteroides*,	263 ARG subtypes, resistance genes toward tetracycline and aminoglycoside are with	155 ARG subtypes, sulfonamide and aminoglycoside resistance genes are with the highest	Metagenomics	[51]

		Acinetobacter, *Actinobaculum*, and *Streptococcus* were correlated with persistent ARGs	the highest abundance. In total 595.26 ppm, genes resistant to tetracycline 138 ppm, to aminoglycoside 84.5 ppm[g]	abundance. In total 82.61 ppm, genes resistant to sulfonamide 15.9 ppm, to aminoglycoside 15.4 ppm[e]		
	Singapore	Genes confer resistance to aminoglycoside, β-lactams, chloramphenicol, fosmidomycin, macrolide-lincosamide-streptogramin, polymyxin, quinolone, rifamycin, sulfonamide, and tetracycline	450 kinds of ARG subtypes, average abundance of ARGs normalized to 16S rRNA genes 1.106	325 kinds of ARG subtypes, average abundance of ARGs normalized to 16S rRNA genes 0.605	Metagenomics	[72]
Africa	South Africa	*Pseudomonas* bacteria with resistance to penicillins, rifampicin, sulfamethoxazole	NA[b]	All of the 39 strains isolated were resistant	Culture-based	[73]
Australia	Australia	A total of 40 ARGs, including resistance toward vancomycin, tetracycline, β-lactamase, aminoglycoside	NA[b]	10^3–10^5 copies/mL in reclaimed water from WWTP	qPCR	[74]

[a]Heterotrophic bacteria with resistance to vancomycin, ceftazidime, cefazolin, penicillin, and imipenem were detected in activated sludge, ranging from 2.8 to 44% of the total bacteria.
[b]Data not shown in this study
[c]A total of 15 activated sludge samples were collected over a period of 3 years from five different Danish WWTPs with biological nitrogen and phosphorus removal
[d]Tetracycline resistance determinants (tet^R) were detected in activated sludge, with the concentrations of 10^7–10^9 copies/mL
[e]Tetracycline-resistant *Enterobacteriaceae* were detected in activated sludge, accounting for 32% of the total 109 strains
[f]A total of 116 sewage samples, collected from 32 WWTPs influents in 17 major Chinese cities (summer, $n = 59$, and winter $n = 57$), were studied
[g]ppm, the "abundance" of ARG, i.e., portion of ARG-like sequences in "total metagenome sequences" (one read in one million reads)

Table 3 Occurrence, abundance, and diversity of ARB and ARGs in drinking water systems within the last 10 years

Continent	Country	Site	Types of ARGs/ARB	ARGs/ARB abundance	Detection method	Reference
Europe	Portugal	Untreated drinking water	Carbapenem-resistant bacteria	10^2–10^4 cfu/mL	Culture-based	[75]
America	USA	Drinking water plant	Bacteria resistant to amoxicillin, ciprofloxacin, chloramphenicol, gentamicin, rifampin, sulfisoxazole, tetracycline	1 cfu/mL	Culture-based, qPCR	[76]
	USA	Tap water	Bacteria resistant to amoxicillin, ciprofloxacin, chloramphenicol, gentamicin, rifampin, sulfisoxazole, tetracycline	10–10^2 cfu/mL	Culture-based, qPCR	[76]
	Canada	Drinking water	*ampC*, *tetA*, *mecA*, β-lactamase genes, carbapenemase genes	1–6 copies/ng DNA	Metagenomic, qPCR	[77]
	Brazil	Tap water	*Aeromonas* strains resistant to ampicillin, tetracycline, cephalothin, chloramphenicol, amikacin, ciprofloxacin, sulfamethoxazole, meropenem, cefotaxime	19 of 22 strains were resistant to at least three antibiotics	Culture-based, PCR	[78]
Asia	India	Tap water	bla_{NDM-1} was found positive in *Achromobacter* spp., *Kingella denitrificans*, *Achromobacter* spp., *Pseudomonas aeruginosa*	The gene was detected in 2 of 50 drinking water samples	PCR	[61]
	China	Drinking water	Genes resistant to β-lactam, aminoglycoside, sulfonamide, tetracycline, chloramphenicol, polypeptide, quinolone, macrolide, lincosamide, *Proteobacteria* were the main ARB dominating in the drinking water	Relative abundance 10^7–10^9 [a]	Metagenomic, qPCR	[79]
	China	Drinking water	Genes resistant to aminoglycoside, bacitracin, β-lactam, quinolone, sulfonamide, tetracycline	Average total ARGs abundance: 0.03 copy of ARGs per copy of 16S-rRNA gene	Metagenomic	[80]
	Japan	Tap water in hospitals	*Methylobacterium species* resistant to ampicillin, cefuroxime, gentamicin, erythromycin, vancomycin, chloramphenicol, ofloxacin, fosfomycin	58 strains were found	qPCR	[81]

[a]Relative abundance of target genes was normalized to each total bacterial community

environment. Treatment processes are important potential barriers to control the spread of ARB and ARGs. In the subsequent section, removal efficiencies of ARB and ARGs in a variety of existing treatment processes will be reviewed.

4.1 Biological Treatment Processes in WWTPs

As shown in Fig. 3, WWTPs play multiple roles in the spread and removal of ARGs in the urban water systems. WWTPs are ARG hotspots, as a diverse mixture of ARGs reach the WWTP through the sewer systems and will be (partially) removed in WWTPs. Therefore, WWTPs are also a source of ARGs for downstream environments. Consequently, the operational efficiency of the WWTP is very important for limiting the spread of ARGs to the wider environment. Nevertheless, WWTPs employing conventional processes (including activated sludge, oxidative ditch, rotatory biological contactors), and multiple sludge treatment processes (including dewatering, gravity thickening, anaerobic digestion), are not efficient to remove ARB and ARGs [82, 83]. Compared to conventional processes, membrane bioreactors (MBRs) exhibit more effective removal efficiency of ARGs and ARB due to the integration of membrane separation. For example, higher ARG and ARB removal efficiencies were observed in MBRs (2.57–7.06 logs), compared to those in conventional treatment plants (2.37–4.56 logs) which employ activated sludge, oxidative ditch, or rotatory biological contactors [84].

Sludge digestion is a key factor potentially mitigating the spread of ARGs. Mesophilic and thermophilic types of digestion process are generally used to treat sludge produced in activated sludge processes. Thermophilic sludge digestion has the function of disinfection since it can effectively destroy pathogenic microorganisms in biosolids. Indeed, thermophilic digestion can achieve higher ARG reduction because of reduced microbial diversity compared to mesophilic digestion [85, 86]. In contrast, mesophilic anaerobic digestion process is unable to reduce ARGs efficiently and sometimes even increase ARG abundance [85].

Overall, although biological treatment processes are efficient for the removal of organic substrates and nutrients, these processes for both wastewater and sludge treatment are not optimized for removing neither ARB nor ARGs, and this warrants further research and development.

4.2 Constructed Wetlands

Constructed wetlands (CWs) are artificially designed and constructed to simulate natural processes for the treatment of domestic and livestock wastewaters. CWs are considered as advanced treatment systems due to their capacity to remove contaminants of emerging concern such as antibiotics, ARB, and ARGs [87, 88]. Previous studies showed CW system could potentially achieve higher ARG removal

efficiencies than conventional wastewater treatment processes [89]. For example, one study showed that vertical flow-constructed wetland systems significantly removed several antibiotics (tetracyclines, ciprofloxacin, oxytetracycline, and sulfamethazine) and ARGs (*tetO*, *tet*W, *tet*M, *tet*A, and *tet*X) [90]. Nevertheless, the removal efficiency of CWs depends on the design and operating parameters, including plant species, substrates, hydraulic loading rates, hydraulic retention time, applied pollutant loadings, temperatures, and flow types [88, 90]. Some studies have indicated that CWs might also act as reservoirs for specific ARGs and MGEs and could function as a reservoir for the dissemination of ARGs into the broader environment [88].

4.3 Disinfection Processes

Water disinfection is of paramount importance to the quality of water supply and to human health. In practice, chlorination, UV, and ozonation are widely used in the final process of water or wastewater treatment to remove microorganism, and hence pathogens, and potentially ARB and ARGs. In this section, we discuss their efficiencies for the removal of ARB and ARGs. A comparative summary is provided in Table 4.

4.3.1 Chlorination

Globally, chlorine and chlorine-based compounds are the most commonly used disinfectants in water and wastewater treatment to eliminate waterborne pathogens (reviewed by Deborde et al. [91]). Regarding the effectiveness of chlorination in controlling the spread of antibiotic resistance, opposing conclusions have been reported in literature. On the one hand, chlorination was reported to remove more than 90% of ARGs and prevent the spread of antibiotic resistance by both inactivating the bacteria and damaging ARGs, which were confirmed by analysis of qPCR and natural transformation assay [92–96]. On the other hand, common chlorine-based disinfectants are reportedly less effective on some waterborne ARB [97]. This suggests that higher disinfectant doses may be required, and as a consequence, more toxic disinfection by-products will be generated. More importantly, these disinfection processes are not sterilization methods, and hence they result in incomplete removal of pathogens, ARB, and ARGs in treated water or wastewater. For example, chlorination may inactivate ARB but cause the release of DNA harboring ARGs into the water, creating extracellular ARGs (eARGs). The occurrence of intracellular ARGs (iARGs) may promote ARB dissemination via conjugation and transduction. Additionally, eARGs, which may persist in the aquatic environment, can be taken up by competent nonresistant bacteria in the biofilm and during sedimentation, thereby resulting in the dissemination of antibiotic resistance via transformation. Thus, eARGs in the effluent from WTPs or WWTPs have the

Table 4 Inactivation of ARB and removal of ARGs in water and wastewater through conventional disinfection processes

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
Chlorination					
Cl_2 = 1 mg/L; pH = neutral and treatment time 15 min	4.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli*	Bench-scale setup 250 mL of CAS effluents	[105]
NaOCl = 160 mg/L and treatment time 120 min	NA	2.98- to 3.24-log ARGs	**ARGs**: *sul1*, *tetG*, *intI1*	Bench-scale setup 500 mL of CAS effluents	[94]
NaOCl = 30 mg/L and treatment time 1,200 min	NA	1.53- to 1.93-log ARGs	**ARGs**: *sul1*, *tetG*, *tetW*, *intI1*	Bench-scale setup 1800 mL of CAS effluents	[93]
Cl_2 = 0.05–2 mg/L; and treatment time 10 min	<5.0-log ARB	0.14- to 4.0-log *tetW*	**ARB**: *Aeromonas* sp. 14S232, *Acinetobacter* sp. 14W115, *Chryseobacterium* sp. 14S111, *E. coli* sp. 28 W121, *Pseudomonas* sp.28S434, and *Serratia* sp. 14S224 **ARG**: *tetW*	Bench-scale setup 20 mL of CAS effluents	[95]
Cl_2 = 3 mg/L; pH =7.4 and treatment time 3 min	<7.85-log ARB	Unable to inactivate *vanA*	**ARB**: vancomycin-resistant *Enterococcus faecium* (ATCC 51559) **ARG**: *vanA*	Bench-scale setup 100 mL of PBS buffer	[106]
Cl_2 = 0.11–376 mg/(min·L); pH 7–8	4.0-log ARB	4.0-log *e-amp*^{R}, *e-kan*^{R}, *i-amp*^{R} and *i-kan*^{R}	**ARB**: *E. coli DH5α* **ARGs**: *amp*^{R} (850 bps) and *kan*^{R} (806 bps)	Bench-scale setup 100 mL of CAS effluents	[96]
NaOCl = 30 mg/L and treatment time 15 min	1.0-log ARB	1.0-log ARG	**ARB**: *E. coli DH5α* **ARG**: multiresistance gene (*pB10*)	Bench-scale setup Synthetic wastewater	[92]

(continued)

Table 4 (continued)

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
UV irradiation					
UV fluence (120–400 mJ/cm^2)	5.0-log ARB	1.0- to 4.0-log ARGs 1.6- to 2.9-log iARGs 1.4- to 2.9-log eARGs	**ARB**: methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE), *E. coli* SMS-3-5 and *P. aeruginosa* 01 **ARGs**: *mecA*, *vanA*, *tetA*, and *ampC*	Bench-scale setup Solar simulated light 10 mL sterile PBS buffer or filtered wastewater effluent	[110]
UV_{254} fluence (5 mJ/cm^2)	1.1- to 1.4-log ARB	0.83- to 5.54-log ARGs	**ARGs**: *ereA*, *ereB*, *ermA*,*ermB*, *tetA*, *tetB*, *tetM*, and *tetO*	Bench-scale setup Solar simulated light 160 mL biological aerated filter effluent	[139]
UV_{254} fluence (12,477 mJ/cm^2)	NA	2.48- to 2.74-log ARGs	**ARB**: - **ARGs**: *sul1*, *tetG*, *intI1*	Bench-scale setup Solar simulated light 1.8L of CAS effluents	[94]
UV_{254} fluence (249.5 mJ/cm^2)	NA	0.36- to 0.60-log ARGs	**ARB**: - **ARGs**: *sul1*, *tetG*, *tetW*, *intI1*	Bench-scale setup 1800 mL of CAS effluents	[93]
UV_{254} fluence (108 mJ/cm^2) at pH 7	NA	0.25- to 1.5-log e*tetA* 0.25- to 2.3-log ebla_{TEM-1}	**ARB**: *E. coli* TOP10 **ARGs**: *tetA* (LA = 1,191 bps; SA = 216 bps) bla_{TEM-1} (LA = 861 bps; SA = 209 bps)	Bench-scale setup Solar simulated light	[116]
UV_{254} fluence; pH 7 and treatment time 30 min	~3- to 4-log ARB	~2-log 16S rRNA and *intI1*	**ARB**: enterobacteria, total heterotrophs, and *Enterococcus* **ARGs**: *sul1*, *qnrS*, bla_{TEM}, *vanA*, *intI1*	Bench-scale setup 1 L of synthetic or CAS wastewater effluents	[111]

(continued)

Table 4 (continued)

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
UV_{254} fluence (69.8–279 mJ/cm^2)	4.15- to 5-log ARB	0.02- to 1.72-log *tetW* from ARB	**ARB**: *Aeromonas* sp. 14S232, *Acinetobacter* sp. 14W115, *Chryseobacterium* sp. 14S111, *E. coli* sp. 28W121, *Pseudomonas* sp. 28S434, and *Serratia* sp. 14S224 **ARG**: *tetW*	Bench-scale setup Solar simulated light 20 mL of CAS effluents	[95]
UV_{254} fluence (61–132 mJ/cm^2) at pH 7 UV_{254} fluence (71–142 mJ/cm^2) at pH 8	NA	4.0-log eamp^R and ekan^R 4.0-log iamp^R and ikan^R	**ARB**: *E. coli DH5α* **ARGs**: amp^R (850 bps) and kan^R (806 bps)	Bench-scale setup Solar simulated light 100 mL of CAS effluents	[96]
UV_{254} fluence (90–120 mJ/cm^2) at pH 7	NA	4.0-log eamp^R and iamp^R	**ARB**: *E. coli* DH5α **ARG**: amp^R (851 bp)	Bench-scale setup Solar simulated light 100 mL of PBS buffer	[140]
UV/H_2O_2					
$H_2O_2 = 0.588$–2.205 mmol/L; UVA energy, $Q_{UV} = 5.93$–7.92 kJ/L, pH = 4 and treatment time 120–150 min	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli*	Pilot-scale setup Natural solar irradiation 8.5 L of autoclaved CAS effluent	[123]
$H_2O_2 = 20$ mg/L; UVA energy $Q_{UV} = 6.29$–14.86 kJ/L, pH = neutral and treatment time 120–240 min	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, ofloxacin, and tetracycline-resistant *E. coli* and *Enterococcus faecalis*	Pilot-scale setup Natural solar irradiation 8.5 L of autoclaved CAS effluent	[141]
$H_2O_2 = 50$ mg/L; UVA energy	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and	Bench-scale setup	[105]

(continued)

Table 4 (continued)

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
Q_{UV} = 8 kJ/L, pH = neutral and treatment time 90 min			tetracycline-resistant *E. coli*	Natural solar irradiation 250 mL of CAS effluents	
H_2O_2 = 0.01 mol/L; UV_{254} irradiation (16 W mercury lamp), pH = 3 and treatment time 30 min	NA	2.63- to 3.48-log ARGs	**ARGs**: s*ul1*, *tetX*, and *tetG*, *intI1*	Bench-scale setup UV irradiation 1800 mL of CAS effluents	[136]
H_2O_2 = 10 mg/L, UV_{254} fluence (44–146 mJ/cm^2) at pH 7–8	4.0-log ARB	4.0-log eamp^R, ekan^R, iamp^R and ikan^R	**ARB**: *E. coli DH5α* **ARGs:** amp^R (850 bps) and kan^R (806 bps)	Bench-scale setup Solar simulated light 100 mL of CAS effluents	[96]
H_2O_2 = 20 mg/L; UV dose = 0–2.5 × 10^4 μW s/cm^2, pH = neutral and treatment time 120 min	4.0-log ARB	*qnrS* and *tetW* genes were undetectable in qPCR	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli* **ARGs**: $bla_{TEM.}$ *qnrS, tetW*	Bench-scale setup Solar simulated light 500 mL of sterile DNA-free water	[142]
H_2O_2 = 20 mg/L; UV power of 1,200 W/m^2 pH = 7 and treatment time 90 min	6.0-log ARB	NA	**ARB**: streptomycin-resistant *E. coli*	Bench-scale setup Solar simulator light 100 mL of synthetic wastewater	[138]
H_2O_2 (0–100 mmol/L) under 12–120 mJ/cm^2 UV_{254} fluence	2.5- to 3.7-log ARB	1.4- to 2.7-log *mecA* 2.3- to 2.9-log *ampC*	**ARB**: methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* **ARGs**: *mecA* and *ampC*	Bench-scale setup Solar simulated light 50 mL of PBS buffer	[131]

(continued)

Table 4 (continued)

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
$H_2O_2 = 10$ mg/L, UV_{254} fluence (60–120 mJ/cm^2) at pH 7	NA	4.0-log *e-amp^R* and *i-amp^R*	**ARB**: *E. coli DH5α* **ARG**: *amp^R* (851 bp)	Bench-scale setup Solar simulated light 100 mL of PBS buffer	[140]
Ozonation					
$O_3 = 31$–33 mg/L and treatment time 15 min	2.0-log ARB	2.0-log ARGs	**ARB**: *E. coli DH5α* **ARG**: multiresistance gene (*pB10*)	Bench-scale setup Synthetic wastewater	[92]
$O_3 = 50$ g/Nm^3 pH 7 and treatment time 30 min	~3- to 4-log ARB	~2.0-log 16S rRNA and *intI1*	**ARB**: enterobacteria, total heterotrophs, and *Enterococcus* **ARGs**: *sul1*, *qnrS*, bla_{TEM}, *vanA*, *intI1*	Bench-scale setup 1 L of synthetic and CAS effluents	[111]
$O_3 = 27$–178 mg/L; pH 7 and treatment time 30 min	NA	1.68- to 2.55-log ARGs	**ARB**: - **ARGs**: *sul1*, *tetG*, *intI1*	Bench-scale setup 6 L of CAS effluents	[94]

Note: NA. Data not shown in this study

potential to promote ARGs and ARB transmission in environmental settings. A recent study demonstrated that 8–9 mg/L chlorine dioxide (ClO_2) increased the abundance of both eARGs and iARGs up to 3.8-fold and 7.8-fold, respectively [97]. In addition, there is potential that even at low concentrations, disinfectants act as a selective pressure for antibiotic resistance and cause increased occurrence of antibiotic resistance [79]. For example, *Pseudomonas aeruginosa* that survived chlorination of drinking water ($\leq$500 μg/L) was resistant to a range of antibiotics, including nalidixic acid, gentamicin, cefotaxime, and amikacin, indicating that suboptimal chlorine treatment leads to the selection of multidrug resistance [23]. Furthermore, plasmid DNA and MGEs associated with ARGs such as *ampC*, $bla_{TEM\text{-}1}$, *tetA*, *tetG*, *ermA*, and *ermB* were found to be enriched in chlorinated waters [79].

Regarding removal efficiencies of ARB and ARGs by chlorination disinfection, some contradictory results are reported in literature. For example, Murray and co-workers [98] observed that chlorination is able to decrease the initial amount of ARB in treated wastewater but may substantially increase the proportions of ARB [99–101]. In addition, some ARB, such as chloramphenicol, trimethoprim, and cephalothin-resistant bacteria, are reported to be tolerant to chlorine, weakening

the disinfection effect of chlorination. In some cases, chlorination significantly removed one or two ARGs from wastewater but was unable to control the spread of other ARGs [102, 103]. Some reports also showed that lower chlorine doses (0.5 mg/L) could significantly reduce the viability of bacteria but was less effective in removing ARGs or controlling their regrowth [95, 104–106]. After the treatment with 4 mg/L of chlorine (5 min) and chloramine (15 min), several antibiotic resistance relevant genes (*soxR*, *gadA*, and *katG*) were detected in bacteria that were in a viable but nonculturable (VBNC) state, yet cells were physiologically active [104]. However, an increase in chlorine dose (30 mg/L of chlorine) resulted in higher removal efficiency of ARGs (*sul1*, *tetX*, *tetG*, *intI1*) from 1.30- to 1.49-log unit [93]. According to the World Health Organization (WHO), 0.92 mg·min/L of hypochlorite ion is required to inactivate 99% bacteria (*E. coli*) in oxidant demand-free systems [107]. This can be as high as 112 mg·min/L when dealing with pathogens according to Centers for Disease Control and Prevention (CDC) [108]. Here 30 mg/L of chlorine was used to degrade 1.30- to 1.49-log of ARGs (*sul1*, *tetX*, *tetG*, *intI1*) with a contact time of 1,200 min. However, there are no specific guidelines for the usage of chlorine to inactivate ARB and ARGs. Although the concentration of chlorine is within the range of guidelines, such a long contact time is not technically feasible for full-scale WWTPs. The detailed information about the removal efficiency of ARB and ARGs during chlorination is shown in Table 4.

4.3.2 UV Irradiation

Ultraviolet (UV) disinfection is an alternative to chlorine since it does not produce toxic disinfection by-products, prompting many WWTPs to switch from chlorine to UV, or applying chlorine and UV in combination. UV radiation damages bacterial cells by targeting the DNA molecule, which may result in inhibition of replication and a subsequent decrease in proliferation [109]. In drinking water disinfection processes, energy-rich UV-C light with wavelengths of 200–260 nm has been successfully used to inactivate microorganisms for decades [96, 110, 111]. However, the application of UV disinfection is limited in turbid wastewater, because high levels of suspended particles significantly decrease the efficiency of the UV radiation. In addition, microorganisms can possess several mechanisms such as photoreactivation, base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) to ensure cell survival after UV-light treatment [112]. DNA damage also can be tolerated by bacteria to a certain extent by use of DNA repair mechanisms [113]. Additionally, double-strand break repair (by homologous recombination and nonhomologous end joining), SOS response, cell-cycle checkpoints, and programmed cell death (apoptosis) are also operative in various organisms with the expense of specific gene products [112].

As a disinfectant, UV irradiation inactivates most microorganisms and achieves reduction from 1 to 6 logs (Table 4). Relatively high inactivation efficiencies (3.4–4.2 logs) were observed for total bacteria as well as ARB in swine wastewater

at fluence of 220 mJ/cm^2 [114]. The results confirmed that UV light can penetrate UV-transparent structures into the cell and is primarily absorbed by the nucleobases comprising DNA and RNA [115]. However, very low ARG removal efficiencies (0.36–0.60-log of *sul1*, *tetX*, *tetG*, *intI1*) were observed at UV fluence of 249.5 mJ/cm^2 [93]. Higher eARG (3–4 logs) removal efficiencies could be achieved, compared to that of iARGs, at the UV dosage of 400 mJ/cm^2, indicating that UV was limited in its potential to damage iARGs [110]. Although bacteria can be inactivated, DNA may be still present in the treated water, which can be helpful for the regrowth of the surviving cells and may contribute to the spread of antibiotic resistance [95, 96, 116]. In order to improve the disinfection performance for real wastewater, H_2O_2 has been incorporated with UV radiation to generate highly reactive free OH• to damage the bacterial cell structure by increasing the oxidation potential and degrading the intracellular DNA after 90 min treatment [117].

4.3.3 Ozonation

Ozone is a powerful oxidizing agent, which has been used as disinfectant in WTPs and WWTPs to destroy microorganisms and DNA and which can potentially reduce the ability of bacteria to acquire antibiotic resistance via transformation [118]. Until now, there have been very few studies focusing on the inactivation of ARB and ARGs using ozone. Oxidative damage to the plasmid DNA of multidrug-resistant *E. coli* was induced at higher doses (4 mg/L) of ozone, and complete disappearance was observed using gel images [119]. A much more differentiated picture of ARG removal was reported in lab-scale experiments, in which elevated oxidant doses ($[O_3]$ =27–178 mg/L) led to a significant removal of 16S rRNA, while some ARGs including *tetG*, *sul1*, and *intI1* persisted after exposure to ozone, and their abundance further increased after storage [94]. According to WHO, 0.02 mg·min/L of ozone is required to inactivate 99% bacteria (*E. coli*) at 5°C with pH 6–7 [107]. Here 27–178 mg/L of ozone was used to remove the 16S rRNA gene significantly. Considering there is no specific guidelines for the use of ozone to inactivate ARB and ARGs, the concentration applied here is high and may not be feasible for full-scale WWTPs.

4.4 Advanced Oxidation Processes (AOPs)

Advanced oxidation processes (AOPs) are oxidation technologies that generate reactive free radicals, mainly hydroxyl radicals (•OH), at ambient temperature and atmospheric pressure. Mostly, these methods employ •OH radicals that can be produced in situ by either one or a combination of oxidants (e.g., oxygen, ozone, and hydrogen peroxide) with catalysts, such as transition metals, iron, and semiconductor oxides. AOPs are mostly used in both lab-scale and pilot-scale, to inactivate a

range of microorganisms such as bacteria, viruses, and parasites, as well as ARB and ARGs (reviewed by Oturan et al. [120], Wang et al. [121]).

4.4.1 Photocatalytic Processes

Photocatalysis has attracted increasing attention as an effective form of water disinfection by using natural sunlight and artificial solar simulated light. Titanium oxide (TiO_2) is a well-known efficient semiconductor with strong oxidizing power and long-term photochemical corrosive resistance that has been used in photocatalytic processes for the inactivation of microorganisms. Previous studies have reported that TiO_2-based photocatalysis driven by simulated solar light could achieve high inactivation efficiency of multidrug-resistant *E. coli* and *Enterococcus* spp. strains in urban wastewater (5–7 logs), yet there is no evidence regarding the regrowth potential after photocatalysis treatment [122–124]. However, compared to solar light, coupling TiO_2 with UV could lead to a higher removal efficiency of ARB and possible remove ARGs [122, 125].

As the photocatalytic inactivation efficiency of TiO_2 is restricted for visible light or natural solar light due to its low activity, increasing efforts have been devoted to develop visible-light-active catalysts by using dopants, chemical modifiers, and chemical additives [126]. These strategies aimed to narrow the band gap of semiconductors, to retard the recombination of the photo-generated electron-hole pairs, to enhance the visible light adsorption capacity, as well as to increase the reaction ratio between the photocatalysts and contaminants [127]. These dopants materials were directly added to the bacterial solution as immobilized materials. 200 mg/L of nitrogen (N)-doped TiO_2 and 250 mg/L of Mn/Co-doped TiO_2 were added to inactivate 5–6 logs of ARB, and the treatment time were 60 and 30 min, respectively. However, higher amount of leached dopants materials may enter the environment from the treatment solution and thus reduce its sustainable usage.

These catalysts can be nonmetal dopants or transition metals. Nitrogen (N)-doped TiO_2 and Mn- and Co-doped TiO_2 were synthesized to improve the solar radiation absorption and increased the inactivation efficiency of ARB. These new catalysts were shown to be capable of 4–6 logs bacterial removal efficiency after 90 min of exposure under simulated solar irradiation [128, 129]. In addition, faster inactivation efficiency (60 min) was observed by the addition of H_2O_2 at lower cumulative energy (TiO_2/H_2O_2 = 10:100, Q_{UV} = 3 kJ/L) due to faster charge separation on the surface of the catalyst and hence greater •OH generation [130]. Increasing the dosage of H_2O_2 improved the photocatalytic inactivation efficiencies against ARB and ARGs [131]. Furthermore, an H_2O_2/TiO_2/UVA treatment system was able to damage bacterial cell walls by chemical and catalytic oxidation and to degrade both eARGs and iARGs at low UV fluence (120 mJ/cm^2). Complementary information with respect to the operating conditions, the target ARB and ARGs, and the removal efficiency are shown in Table 5.

4.4.2 Fenton and Photo-Fenton Processes

The Fenton process is a promising AOP. This process has been used not only for degrading antibiotics but also to inactivate microorganisms such as bacteria, fungi, virus, and yeast [132–134]. The Fenton process has also been demonstrated as an effective treatment solution for inactivating ARB in both laboratory-scale and pilot-scale studies [134]. This process is enhanced in the presence of H_2O_2 and solar light by generating •OH that attack the external membrane of bacteria, leading to increased cell membrane permeability, inducing internal cellular reactions, and eventually inactivating the cells [135]. The efficiency of the photo-Fenton process depends on the reagent concentrations, solution pH, and generation of •OH. Inactivation efficiency was significantly increased by increasing the Fe^{2+}/H_2O_2 molar ratio from 0.033 to 0.1 [136]. In the photo-Fenton process, the regeneration of ferric iron (Fe^{2+}) from ferrous iron (Fe^{3+}) is a rate-limiting step; thus the process has high dependence on Fe^{3+} concentration. Under alkaline conditions, since more ferrous irons are precipitating, low concentrations of Fe^{2+} will achieve in solution. When pH was lower (pH < 2.5), the majority of the microorganisms examined were not viable, and slower inactivation rates were observed due to the formation of $[Fe(II)(H_2O)]^{2+}$, which reacts more slowly with H_2O_2 and then produces less amount of reactive •OH radicals [136]. However, at higher pH (pH >4), the rate of oxidation decreases because of the decrease of the free iron species in the solution, which is probably due to the formation of Fe^{2+} complexes with the buffer inhibiting the formation of free •OH radicals or the precipitation of ferric oxyhydroxides inhibiting the regeneration of Fe^{2+} [136, 137]. To enhance the regeneration of Fe^{2+}, researchers recommend the solar-based photo-Fenton process for the inactivation of ARB in the presence of UV-visible sunlight [134]. However, UVA has a minimal effect on DNA, due to the low absorption of DNA nitrogen bases. In the solar photo-Fenton process, reliance on UVA does not achieve complete inactivation and hence results in regrowth risks, whereby the surviving bacteria could proliferate and spread antibiotic resistance to the surrounding environment [138]. A number of Fenton-related studies are summarized in Table 6.

5 Perspectives

5.1 Real-Time Detection Methods and Risk Assessment

Currently, both culture-dependent and culture-independent methods are used for ARB and ARGs detection. As discussed in Sect. 2 in this chapter, each method has its pros and cons, and no single approach is outstanding with respect to all parameters. Based on different purposes (e.g., fundamental and applied research or practical surveillance) or different research questions, an appropriate single method or a combination of various methods can be selected for investigating ARB and ARGs

Table 5 Inactivation of ARB and removal of ARGs in wastewater effluents through the photocatalytic process

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
TiO_2 = 50 mg/L; H_2O_2 = 100 mg/L; UVA energy Q_{UV} = 4 kJ/L, pH = neutral and treatment time 60 min	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli*	Bench-scale setup Natural solar irradiation 250 mL of CAS effluents	[105]
TiO_2 powder (Degussa P25) = 50 mg/L; simulate solar radiation (250 W lamp) and treatment time 60 min	7.0-log ARB	NA	**ARB**: ciprofloxacin, cefuroxime, tetracycline, and vancomycin-resistant *E. coli*	Bench-scale setup Solar simulated light 500 mL of CAS effluent	[122]
TiO_2 = 50–100 mg/L; H_2O_2 = 0.147–0.588 mM; UVA energy Q_{UV} = 3.79–7.63 kJ/L, pH = 4 and treatment time 80–180 min	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli*	Pilot-scale setup Natural solar irradiation 8.5 L of autoclaved CAS effluents	[123]
TiO_2 powder (Degussa P25) = 50 mg/L; simulate solar radiation (250 W lamp) and treatment time 60 min	7.0-log ARB	NA	**ARB**: tetracycline-resistant/sensitive *Enterococcus* (TRE/TSE)	Bench-scale setup Solar simulated light 500 mL of CAS effluent	[124]
TiO_2 = 62.5–125 mg/L; UVA energy Q_{UV} = 400–800 μW/cm^2 and treatment time 180 min	1.0- to 3.0-log ARB	NA	**ARB**: methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Acinetobacter baumannii* (MDRAB) and vancomycin-resistant *Enterococcus faecalis* (VRE)	Bench-scale setup UVA irradiation	[125]
N-doped TiO_2 powder = 200 mg/L; simulate solar radiation (250 W lamp) and treatment time 60 min	5.0-log ARB	NA	**ARB**: ciprofloxacin, cefuroxime, tetracycline, and vancomycin-resistant *E. coli*	Bench-scale setup Natural or solar simulated irradiation 500 mL of CAS effluent	[128]

0.04 wt% Mn/Co- TiO_2, UV irradiance $Q_{UV} = 1.31 \times 10^2$ W/m^2, and treatment time 30 min	6.0-log *K. pneumoniae*	Only *sul1* and *a*mpC remained in the reaction solution	**ARB**: ampicillin and cefaclor, sulfonamides, and tetracycline-resistant *K. pneumoniae* **ARGs**: *tetA*, *tetM*, *sul1*, *blaTEM*, and *ampC*	Bench-scale setup Natural or simulated solar irradiation (150 W) 300 mL of CAS effluents	[129]
$TiO_{2\ thin\ film}$ + H_2O_2 (100 mmol/L) under 12–120 mJ/cm^2 UV_{254} fluence	4.5- to 5.0-log ARB 5.5- to 5.8-log ARB	5.7- to 5.9-log *mecA* 4.6- to 4.8-log *ampC* 4.4- to 5.2-log iARGs 2.6- to 3.3-log eARGs	**ARB**: methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* **ARGs**: *mecA* and *ampC*	Bench-scale setup Solar simulated light 50 mL of PBS solution	[131]
Crystallizing dish coated with TiO_2; UVA energy Q_{UV} = 6–8 mW/cm^2 and treatment time 86–145 min	3.0-log ARB	NA	**ARB**: ampicillin and streptomycin-resistant *E. coli* (ATCC 700891)	Bench-scale setup UVA/LED lamps (3 W, 365 nm) 30 mL sterile distilled water	[143]
Photocatalytic (TiO_2) ozonation + Two 10 W UV high intensity LED light	5.0-log enterococci, enterobacteria, and fungi 4.0-log heterotrophs	3.0- to 4.0-log 16S rRNA 5.0-log *intI1* 3.5- to 6.0-log *blaTEM* 7.0-log *qnrS* 8.0-log *sul1*	**ARB**: enterococci, enterobacteria, fungi, and heterotrophs **ARGs**: *intI1*, *blaTEM*, *qnrS*, *sul1*	Bench-scale setup UV LED light irradiation 100 mL CAS effluents or 250 mL surface water from drinking water treatment plant	[144]
TiO_2-rGO-PH or TiO_2-rGO- hydrothermal (HD) (100 mg/L) under simulated solar radiation (63 W/m^2). pH = 5.2–6.2 and treatment time 120 min	6.0-log ARB	2.4-log *ampC* 0.4- to 0.5-log *ecfX*	**ARB**: clarithromycin, erythromycin, and sulfamethoxazole-resistant *E. coli* **ARGs**: *sul1*, *ampC*, *ermB*, *mecA*, *ecfX*, 23S rRNA	Bench-scale setup Solar simulated light 300 mL of MBR effluent	[145]

Note: NA. Data not shown in this study

Table 6 Inactivation of ARB and removal of ARGs in wastewater effluents through the photo-Fenton process

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
Solar photo-Fenton (Fe^{2+}: H_2O_2 ratios (10:20 and 20:40 mg/L); pH = neutral and treatment time 240 min)	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli*	Bench-scale setup Natural solar irradiation 250 mL of CAS effluents	[105]
Solar photo-Fenton (Fe^{2+} = 0.090 mmol/L; H_2O_2 = 0.294 mmol/L; UVA energy, Q_{UV} = 0.98–1,534 kJ/L, pH = 4–8.72 and treatment time 20–240 min)	5.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, and tetracycline-resistant *E. coli*	Pilot-scale setup Natural solar irradiation 8.5 L of autoclaved CAS effluents	[123]
Solar photo-Fenton (Fe^{2+} = 1 mg/L; H_2O_2 = 10 mg/L); UV power of 1,200 W/ m^2 pH = 6.5 and treatment time 75–120 min)	6.0-log ARB	NA	**ARB**: methicillin-resistant *S. aureus* (MRSA ATCC 43300), *β*-lactam-resistant *E. coli* (ESBL 8543), and *K. pneumoniae* (ESBL 8534)	Bench-scale setup Solar simulator light 40 mL of ultrapure water	[135]
Solar photo-Fenton (Fe^{2+}: H_2O_2 ratios 0.1–0.5); UV_{254} irradiation (16 W mercury lamp), pH = 3.0 and treatment time 120 min)	NA	*2.57- to 5.30-log* ARGs	**ARGs**: *sul1*, *tetX*, and *tetG*, *intI1*	Bench-scale setup No irradiation (dark Fenton) 500 mL of CAS effluent	[136]
Solar photo-Fenton (Fe^{2+} = 1 mg/L; H_2O_2 = 20 mg/L); UV power of 1,200 W/ m^2 pH = 7 and treatment time 60 min)	6.0-log ARB	NA	**ARB**: streptomycin-resistant *E. coli* W1485	Bench-scale setup Solar simulator light 100 mL of synthetic wastewater	[138]
Solar photo-Fenton ([Fe^{3+}] = 5 mg/L, [H_2O_2] = 50 mg/L,	5.0-log ARB	NA	**ARB**: clarithromycin and sulfamethoxazole-	Pilot-scale setup Natural	[146]

(continued)

Table 6 (continued)

Treatment process, catalyst doses, and treatment time	ARB log removal efficiency	ARG (i and e) log removal efficiency	Targeted ARB and ARGs	Note	Reference
pH = 4 and treatment time 180 min)			resistant *Enterococcus* spp.	solar irradiation 60 L of CAS effluents	
Solar photo-Fenton ($[Fe^{3+}]$ = 875 mg/L, $[H_2O_2]$ = 500 mg/L, pH = 3 and treatment time 60 min)	2.0-log ARB	NA	**ARB**: ampicillin, ciprofloxacin, gentamicin, tetracycline, and chloramphenicol-resistant *E. coli*	Bench-scale setup Natural solar irradiation 300 mL of CAS effluent	[147]
Solar photo-Fenton (Fe^{2+} = 5 mg/L; H_2O_2 = 50 mg/L); UV power of 30 W/ m^2 pH = 2.8 and treatment time 240 min)	5.0- to 6.0-log ARB	1.56-log *sul1* 1.53-log *ermB*	**ARB**: erythromycin, clarithromycin and sulfamethoxazole-resistant *E. coli*, *P. aeruginosa*, and *Klebsiella* spp. **ARGs**: *ermB*, *sul1*, *mecA*, *ampC*, *Enc*, *ecfX*	Pilot-scale setup Natural solar irradiation 60 L of MBR effluent	[148]

Note: NA. Data not shown in this study

[149]. Metagenomic analysis could simultaneously investigate the broad profile of ARB and ARGs, if the research aims to obtain a comprehensive overview of ARB and ARGs in the complete urban water cycle. In addition, metagenomic analysis can overcome the drawbacks of amplification-based methods, including the limited availability of primers, the possible bias in the amplification process, and the false-negative results caused by the enzyme inhibitor in the environmental samples. More interestingly, the application of a hybrid short- and long-read metagenomic sequencing approach will enable identification of the genomic context of ARGs as well as the potential to assemble plasmids, near-complete, or even complete bacterial chromosomes. It should be noted that metagenomic analyses only infer the presence of an enzyme that may encode antibiotic resistance, but it is unable to confirm whether a gene is functionally expressed in the bacterial host. Therefore, metatranscriptomics and metaproteomics should be further considered for the validation of functional ARGs in environmental samples [45]. In some cases, we expect to obtain timely information about ARB and ARGs for effective pathogen control in drinking water or wastewater treatment. Thus, it is very significant to develop and employ real-time detection method in the future. Nanopore-based sequencing might

offer an opportunity for identification of ARB and ARGs in real time, in which the sequencing data is available for base calling as soon as a library strand passes through the nanopore.

In addition, the surveillance and assessment of antibiotic resistance in the urban water cycle is incomplete, since most studies have focused on a given component but neglecting the urban water cycle as an integrated water system. It is of significance to investigate the fate and transfer of ARGs in the complete urban water cycle, including water collection and storage facilities at source sites, water transport via aqueducts (canals, tunnels, and/or pipelines) from source sites to water treatment facilities; WTPs, storage, and distribution systems; sewer systems and WWTPs; and networks discharging to rivers or lakes (receiving waters) and recreational water sites (e.g., swimming pool and surfing beach). Such a comprehensive surveillance will help us identify those prevalent and persistent ARB and ARGs. Through large-scale survey-based studies, it is also expected to identify the indictors of ARB and ARGs, which is very important to develop an efficient and executable monitoring strategy. More importantly, a quantitative risk assessment regarding the transmission of antibiotic resistance from various water sources to human or animal is needed, as it has been suggested (e.g., reviewed by Larsson et al. [150], Vikesland et al. [151]). Although it is challenging to develop risk models, it can help policymakers or health protection organizations to gather information about the emergence and spread of antibiotic resistance and develop policy, regulation, strategy, and technology that effectively protect public human health.

5.2 Advanced Disinfection Methods

Theoretically, disinfection processes in WTPs or WWTPs could be used to remove ARB, and the ARB accumulating in sludge could be reduced by improved sludge treatment processes. However, as has been documented in this chapter, conventional treatment systems generally demonstrate low ARG removal efficiencies. Therefore, it is critical to develop control strategies or advanced processes not only for ARB inactivation but also for ARGs elimination. On the one hand, we can further optimize the current operational conditions to improve both ARB and ARG removal efficiencies in WTPs and WWTPs. For example, we can focus on the optimization and upgrading of biological filters, micro-/ultrafiltration, ozonation, chlorination, and UV disinfection, which are already available in most of WTPs and WWTPs. By optimization and comparison, it is expected to identify what operational conditions or which operational configurations will have enhanced removal efficiency of ARB and ARGs. Thus, we can employ these optimized conditions or configurations to reduce antibiotic resistance burden. On the other hand, we need to develop more advanced disinfection methods to remove ARB and pathogens and also effectively damage/inactivate eARGs and iARGs, aiming at minimizing microbial risks. Such advanced water disinfection processes should be safe, environmentally friendly, low-cost, and highly effective at removing both ARB and ARGs from water. One

of major reasons to develop advanced water disinfection processes is attributed to the fact that common disinfection processes could promote the spread of antibiotic resistance via mutation or horizontal gene transfer. Photocatalysis will likely be further developed and employed as an effective advanced technology for inactivation of ARB and ARGs from various water streams, e.g., drinking water and wastewater. Compared with conventional disinfection techniques, photocatalysis-based disinfection is beneficial because it avoids generation of disinfectant by-products while generating strong radicals, e.g., superoxide and hydroperoxide, which can inactivate ARB and ARGs and potentially achieve mineralization of ARGs, thus minimizing microbial risks. It should be also noted that photocatalysis sometimes promotes the formation of toxic transformation products that are more toxic than parent compounds, which should be avoided for practical application by process optimization [152]. Further studies should be performed, including thorough assessment of the relative merits of photocatalysis and other advanced treatment options using multi-criteria analysis to consider capital cost, operational cost, operational complexity, process robustness, and environmental impacts/benefits.

6 Summary

The urban water cycle, which connects urban life, agriculture, and the environment, is also implicated in the spread of problematic ARGs. Therefore, a better understanding of the distribution, transportation, and acquisition of antibiotics, ARB, and ARGs in the urban water cycle is critically important to improve the control of this emerging environmental and human health challenge. This book chapter has reviewed the occurrence, fate, and transmission of ARB and ARGs in the urban water cycle and also summarized ARB/ARG removal performance in various processes, including biological wastewater treatment units, sludge digestion, chlorination, ultraviolet, ozonation, and AOPs. We recommend that three major barriers should be developed to minimize the spread of ARGs in urban water systems, including advanced water disinfection, advanced wastewater treatment, and optimized sludge treatment processes. The mitigation solutions for efficient reduction of ARB/ARGs during water, wastewater, and sludge management not only will be beneficial for the development of policy, strategy, and technology for protecting public health but also would bring additional environmental and economic benefits world widely.

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Antibiotic Resistance, Sanitation, and Public Health

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Contents

Abstract Antimicrobial resistance (AMR) poses a serious threat to global health. In countries with poor sanitation conditions, the situation is worrisome. In this chapter, worldwide data, particularly from Brazil, supports a discussion about the risks of sewage and livestock manure on spreading antibiotic resistance, calling attention to the relationship between poor sanitation conditions, water pollution, and public health. The role of wastewater treatment plants (WWTP) and different treatment technologies in reducing AMR from municipal and livestock wastewaters are

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discussed based on the information available. It has been observed that municipal WWTPs with tertiary treatment systems can be decisive in the prevention and control of AMR spread and thus contribute to the maintenance of environmental and public health. Considering the information provided, there is a potential for antibiotic-resistant bacteria and antibiotic resistance gene dissemination through conventional WWTP effluents and sludges, especially when the latter are used as biosolids. By reaching surrounding aquatic environments, antibiotic-resistant bacteria may arise as a threat for public health since WWTP and water treatment plants (WTP) are not normally designed to specifically remove AMR. In summary, globally and in particular, Brazil has a lot of challenges to monitor and control AMR not only in municipal WWTPs but also in clinical and natural environments. Accurate information provided by research and routine monitoring, political engagement, new policies, and multidisciplinary actions will be vital to tackle this problem. In the short term, the control of the antibiotic prescription and their use by the population and farmers (already in place) and the increase of sewage collection and treatment are strategic actions to reduce AMR and guarantee public health in the country.

Keywords Antibiotic resistance in Brazil, Livestock wastes, Tertiary treatment, Wastewater treatment, Water treatment

Abbreviations

AMR	Antimicrobial resistance
AR	Antibiotic resistance
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
AOPs	Advanced oxidative processes
BOD	Biological oxygen demand
COD	Chemical oxygen demand
ESBL	Extended-spectrum β-lactamases
HGT	Horizontal gene transfer
UV	Ultraviolet
WWTP	Wastewater treatment plant
WTP	Water treatment plant

1 Introduction

Antibiotic resistance (AR) is a global health problem that affects any person in any country independent of age or economic situation. It demands multidisciplinary and complementary actions and combined efforts from different countries in order to tackle and reduce this problem. This is a complex problem, with the urban water cycle playing an important role as potential disseminator of AR. The urban

population collects water from natural rivers or reservoirs that are treated for consumption and transformed into wastewater (sewage). The sewage produced is collected by wastewater treatment plants (WWTPs), treated, and discharged back into rivers. In addition, natural waters might receive wastes and wastewaters produced by farmers and livestock activities that might contribute to disseminate AR (such as antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes) among other pollutants (such as nitrogen- and carbon-based compounds in high concentrations). Therefore, if the wastewaters and wastes are not properly treated, they can disseminate AR in the environment. In this sense, the sanitation conditions are very important to understand the challenges that each country will face in order to tackle this problem related to AR and ensure good public health.

The goal of this chapter is to provide and discuss information about the antibiotic resistance situation worldwide and particularly in Brazil, calling attention to the relationship between poor sanitation condition and public health. Brazil is the largest country in Latin America and has high economic and geographic diversity. In Sect. 2, data about AR collected around the world and also in Brazil is presented. Information about the sanitation condition in Brazil is presented in this section as well. Then, the occurrence and removal of AR elements (antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs)) in municipal WWTPs are presented and discussed in Sects. 3 and 4, respectively. Whenever available, data gathered in Brazil were included in these sections. In Sect. 5, the importance of livestock as source of AR dissemination is discussed, because Brazil has an intensive animal production, and thus actions in this sector must be taken to control AR spread to the environment. In Sect. 6, information about AR element occurrence in Brazilian natural and drinking waters is shown. Finally, we discuss the challenges Brazil faces to monitor and control AMR not only in municipal WWTPs but also in clinical and natural environments.

2 Outlook of Antibiotic Resistance Situation Worldwide and in Brazil

Antimicrobial resistance (AMR) is the ability of microorganisms (bacteria, viruses, and some parasites) to survive and multiply, even in the presence of antimicrobial agents such as antibiotics, antivirals, and antimalarials [1]. Currently, AMR is one of the biggest threats to global health, food security, and human development, with 70% of the drugs (antibiotics and growth promoters) being consumed worldwide in the food and livestock sectors [1]. This situation can affect anyone, at any age, in any country, mainly due to the ease and frequency with which people travel nowadays [1, 2].

In 2015, the World Health Organization (WHO) has launched the Antimicrobial Action Plan, which aims to reduce the overuse of antibiotics in both human and animal health systems and agriculture. By 2016, 67 countries had already completed

this plan, and another 62 countries were in the draft process. Still in 2016, WHO recommended that farmers and food industry stop using antibiotics routinely to promote growth and prevent diseases in healthy animals [1].

In 2018, data were presented at the 13th World Economic Forum Global Risks [3], showing that AMR is intensifying and that antimicrobial resistance can lead to 100,000 deaths annually in the USA, 80,000 in China, and 25,000 in Europe, within 10 years. Still according to this forum, the world currently faces two scenarios, which may lead to even worse consequences: new classes of antibiotics are not being developed successfully, and the AMR continues to spread inexorably, so that if nothing is done, it could be the end of modern medicine [3].

In Brazil, the consumption of antimicrobials and the resistance scenario have been inducing a great concern to human health. According to the World Health Organization [4], the number of antibiotic doses (as "defined daily doses" – DDD/1,000 inhabitants) consumed in this country is among the highest in the world, surpassing the average in Europe, Canada, and Japan. Brazil has a median consumption of 22 DDD/1,000 inhabitants per day, which places the country as the 19th largest consumer of antimicrobial medicines among the 65 surveyed nations. Europe has an average consumption of 18 DDD/1,000 inhabitants per day, while in Canada and Japan, the measured consumption was 17 and 14 DDD/1,000 inhabitants per day, respectively.

According to the latest report of the Brazilian Health Regulatory Agency (*Agência Nacional de Vigilância Sanitária – ANVISA*) [5], in Brazil, 23,000 people die each year by hospital infections, and this number may be even higher, as it is known that many cases are not reported. Regarding bacterial resistance, the same report mentioned that *Acinetobacter* spp., *Klebsiella pneumoniae*, coagulase-negative *Staphylococcus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were the bacteria identified as the major cause of infections in Brazilian hospitals between 2012 and 2015 [5].

Actions have been taken in Brazil through a partnership between ANVISA, the Pan American Health Organization (PAHO), and the Ministry of Health through the General Coordination of Public Health Laboratories to monitor and control AMR in health services. Among these, it was launched in 2017 the National Network for Monitoring Microbial Resistance in Health Services, whose main objective is to make health care more effective through the adequate use of antimicrobials and the detection, prevention, and control of the emergency of AMR in health services. In addition, actions are planned to qualify antimicrobial medical prescriptions and reduce the indiscriminate use of this type of drug, as well as to improve regulatory actions regarding the presence of residues of these products in food [6].

Overuse and misuse of antibiotics are some of the main factors of accumulation and spread of resistance [7]. However, resistance originates from random processes – mutation and acquisition of resistance genes mobilized from the chromosomes of other bacteria [8]. Water is essential to maintain population health, and, at the same time, poor water quality can contribute to the spread of different forms of pollution and pollutants (including AMR). The water cycle in urban environments can be divided in two major parts: (1) freshwater (that can be used as water source and

therefore will be treated and distributed for drinking water purposes) and (2) wastewater (sewage) produced by the population that must be collected and treated in municipal wastewater treatment plants (WWTP) before being discharged into the environment. These two major components are interconnected, and the level of basic sanitation within a region (or country) will affect considerably the population health.

The municipal WWTPs receive domestic sewage (and sometimes industrial and hospital effluents) and therefore are common places to harbor organisms found in human and animal feces, pathogens, ARB, and ARGs. In fact, they are considered reservoirs for the proliferation of AR. High biomass and nutrient concentrations that are constantly mixed with antibiotic residues create ideal conditions for the proliferation and transfer of AR in municipal WWTP and also lead to the development of multidrug-resistant microbes [9, 10]. Many authors have reported that municipal WWTPs with secondary treatment systems (based upon biological processes) are not effectively removing ARB, ARGs, and antibiotic residues [10–13] and, therefore, are important sources of AR dissemination into the environment. In fact, in countries with poor sanitation conditions, the environmental spread of AMR might be worrisome [14].

In this aspect, it is important to understand the dimension of Brazil and mention its basic sanitation situation. Brazil is the largest country in Latin America; it has continental proportions and high economic and geographic diversity. In terms of drinking water, 93% of the Brazilian urban population is supplied by treated drinking water. This value varies depending on the region of the country: in the center-west, south, and southeast part of Brazil, around 98.1%, 98.4%, and 95.9%, respectively, of the population have treated water, whereas in the north and northeast part, the values are lower, 70.0% and 88.8%, respectively [15]. In relation to sanitation, 56% of the urban population is connected to a sewer system, and approximately 70% of the sewage collected in networks is treated [16]. In terms of overall sanitation coverage, around 40% of the sewage generated in Brazil is treated in about 2,800 WWTPs in operation around the country [16]. The configurations most widely adopted in Brazil for sewage treatment are anaerobic pond followed by facultative pond; UASB (upflow anaerobic sludge blanket) reactor; activated sludge; anaerobic or facultative ponds followed by maturation ponds; and septic tank followed by anaerobic filter. An assessment of the actual performance of 166 Brazilian sewage treatment plants showed a great variability in the effluent quality parameters (such as COD, BOD, suspended solids, among others) and in the removal efficiencies, with performances that were usually inferior to those reported in the technical literature [16].

Monitoring data about ARG and ARB concentrations in raw and treated sewage from full-scale municipal WWTPs in Brazil are scarce. Most of the studies had reported ARB and multidrug-resistant isolates in hospital effluents [17, 18]. Nevertheless, unpublished data from our research group revealed that cultivable ARB abundance in the raw sewage (from two municipal WWTPs located in Belo Horizonte, Minas Gerais) is in the order of 10^6 to 10^8 CFU/L. Considering that 45% of the Brazilian population (i.e., 94.5 million inhabitants) do not have sewage collection and treatment and that one person generates 160 L of sewage per day [15],

it is estimated that the load of cultivable ARB discharged into Brazilian rivers and water bodies falls in the range of 10^{16} to 10^{18} CFU per day. These numbers call the attention for the urgent need to increase sewage treatment in Brazil in order to improve population health and preserve natural water quality.

With regard to the taxonomic composition of cultivable ARB present in raw sewage and treated effluent from municipal WWTPs in Brazil, unpublished data from our group revealed that most of the ARBs were members of *Enterobacteriaceae* (*Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Salmonella enterica*, *Shigella* spp., *Escherichia coli*, *E. fergusonii*, *Citrobacter freundii*). Multidrug-resistant bacteria belonging to *Enterobacteriaceae* were identified in hospital effluent and also in the WWTP that receives this effluent for treatment [18]. Interestingly, some of these species (*K. pneumoniae*, *E. coli*, and *Enterococcus faecalis*) are resistant pathogens reported previously in Brazilian hospitals [19]. For instance, Rossi [19] pointed out that local resistance to vancomycin was first related to *Enterococcus faecalis*. Carbapenem resistance among the *Enterobacteriaceae* family is also a major problem, and carbapenem-resistant *K. pneumoniae* isolates have been reported in different states [19]. Although these results might suggest that the routes of antimicrobial resistance from hospital and municipal effluents to the environment and from the environment to the community are likely interconnected, there is still a need to assess the relatedness of strains collected (from both environment and clinical samples). Therefore, contact with contaminated water containing antibiotic-resistant pathogens, whether for recreational activities or direct consumption, can lead to serious public health problems. It is also evident that more research should be performed and aquatic natural environments should be investigated and monitored for antibiotic resistance for a better understanding of the prevalence, distribution, and transmission of ARB and ARGs.

Actions in Brazil must be taken as soon as possible and must involve efforts not only from the Ministry of Health and the National Health Surveillance Agency but also from the National Secretariat of Environmental Sanitation (related to the Ministry of Cities) and from the research institutes and universities. These last can provide accurate information about AMR dissemination into the environment in different parts of the country and research technologies to mitigate this public health problem.

The role of WWTPs in the dissemination and removal of ARB and ARG will be discussed in more details in the next sections.

3 Occurrence of Antibiotic Resistance in Municipal WWTPs

Municipal WWTPs play a fundamental role in the urban hydrological cycle, aiming for the preservation and quality of water bodies, in addition to maintaining public health. WWTPs essentially aim for the removal of solids, organic matter, and

nutrients from the municipal wastewater (including domestic sewage), promoting the protection of the environment from the adverse effects caused by these elements. In addition, they are important agents in limiting health risks to the population, as they considerably reduce the amount of pathogenic microorganisms present in wastewaters. Conventional biological technologies employed in these treatment plants are unable to successfully remove micro-contaminants from the sewage, such as antibiotic-resistant microorganisms, resistance genes, and antibiotic residues [11, 12, 20]. Therefore, they act as potential reservoirs, amplifiers, and disseminators of resistance elements into the environment by combining diverse ecological conditions (chemical, physical, and biological), favorable to the intimate interaction of the microorganisms that form the microbiome of biological reactors [10, 21, 22]. It has been also argued that they can stimulate the horizontal gene transfer (HGT) [23], because they have units with high microbial density, high nutrient and heavy metal concentrations [22], and subinhibitory concentrations of antibiotics [20] and other biocides [21, 24, 25]. Thus, it is proposed that municipal WWTPs, biological sludge, and final effluent can cause an imbalance in the three pillars of the One Health concept (that recognizes that people's health is connected to the health of animals and the environment) by triggering uncontrolled spread of antimicrobial resistance through its final disposal sites [11].

By-products from sewage treatment plants are considered valuable resources, as the sludge presents high concentrations of bioavailable organic matter, while the treated effluent represents large amounts of water containing nutrients with potential for reuse [26]. With the increasing need for natural fertilizers (in detriment of chemical ones) and water recycling (thus reducing the exploitation of water resources), in order to maintain ecosystem sustainability and reduce environmental impacts, the interest in the use of sewage sludge and treated effluent for agricultural purposes (fertilization and irrigation, respectively) has intensified. It is of great concern that there is a possibility of transferring resistance elements present in these by-products to foods intended for public consumption. In addition, there is also concern about the farmers that handle the sludge used for fertilization and about the workers exposed to treated sewage during crop irrigation [27]. This concern is due to the fact that even after biological treatment of the sludge, biosolids contain ARB and ARGs [28].

In addition to the dispersion of resistance elements in the terrestrial environment, attention is needed for the possibility of contamination of water bodies that receive treated domestic effluents, among several rivers, lakes, and oceans [29]. These elements can be assimilated by marine and freshwater animals and may eventually reach humans by the ingestion of contaminated fish products or downstream recreational contact. This hypothesis is supported by a study carried out in the southern coast of Brazil, in which the presence of antibiotic-resistant enterococci isolated from seabirds, turtles, and marine mammals was detected in environments considered as wastewater receptors, with a high level of pollutants and pathogens [30].

Several studies around the world have shown the occurrence of AR and discharge to the environment of a variety of resistance elements from domestic sewage systems. For instance, Nnadozie et al. [31] mentioned that antibiotics such as

cephalosporins, diaminopyrimidines, fluoroquinolones, lincosamide, macrolides, beta-lactams, sulfonamides, and tetracyclines have been reported to be present in WWTPs. Li et al. [32] reported that most of the target antibiotics were detected in the secondary and tertiary effluents, with concentrations that ranged from 4.8 to 1,106 and 0.3 to 505 ng/L, respectively. They also reported that fluoroquinolone antibiotics showed relatively high concentrations in all samples (782–1,814 ng/L).

With regard to ARB in municipal WWTPs, an enormous variety and abundance of species, some of them potentially pathogenic (such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Aeromonas hydrophila*, *Escherichia coli*, *Enterococcus faecium*, *Shigella flexneri*, *Acinetobacter baumannii*, *Salmonella* spp., *Vibrio cholerae*, *Aeromonas* spp., and *Mycobacterium tuberculosis*), was extensively documented in sewage and even in treated effluents [31]. With exception of the first three species (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*), all the other species were detected in both sewage and treated effluents [31]. In addition, multidrug-resistant bacteria such as the carbapenemase-producing *Klebsiella pneumoniae*, (KPC)-2-producing *Aeromonas* spp., and several *Enterobacteriaceae* species, including *Kluyvera* spp., were identified in hospital sewage and in different sites of the WWTP that receives this effluent for treatment [18]. KPC producers were not recovered from secondarily treated sewage. However, neither primary nor secondary treatment was efficient in eliminating multidrug-resistant bacteria from sewage [18].

With regard to the occurrence of ARGs in WWTPs, genes that confer resistance to a wide range of antibiotics (tetracycline, penicillin, ampicillin, quinolone, vancomycin, erythromycin, sulfonamide, beta-lactams, and macrolides) have been evidenced in raw and treated sewage [33–36]. Moreover, a study carried out in 32 municipal WWTPs in China revealed the presence of 381 resistance genes conferring resistance to different antibiotics, demonstrating the complexity and variety of these elements in the raw sewage that reached the treatment plants [37]. In another study [22], it was reported that the prevalence of resistance genes was correlated with biotic and abiotic factors in WWTPs. For instance, total organic carbon, total prokaryotic cells, and the number of bacterial aggregates were positively correlated to the concentrations of ARGs, throughout the treatment [22].

With regard to the removal of AMR by the WWTPs, it would depend on the type of biological system applied and the tertiary treatment and/or disinfection technique used. For instance, Lamba and Ahammad [38] found, in lab-scale experiments, that aerobic systems performed better than an anaerobic reactor in removing fecal coliforms, ARB, and ARGs and that the removal efficiency was positively related to hydraulic retention time (HRT). The fact that higher removal rates were observed in the aerobic systems may be related to the configuration of the anaerobic system (that was a flow through reactor) which was not efficient in retaining suspended solids [38]. They also mentioned that the use of aerobic biological systems followed by disinfection techniques, such as UV, ozonation, or chlorination, was effective in the removal of coliforms and ARB, but none of them allowed the complete removal of the ARGs [38]. However, ozone and UV treatment resulted in higher removal (~10-fold) of ARGs compared to chlorine treatment [38]. On the other hand, Harb

and Hong [39] observed that anaerobic membrane bioreactor (at lab scale) was much more effective to remove pathogens from municipal wastewater, in comparison to an aerobic membrane bioreactor (at full scale). They concluded that the risks associated with the anaerobically treated effluent reuse would be lower compared to the use of the aerobic effluent (which in this case needs to be submitted to disinfection treatment prior to be used for irrigation activities). Another study [40] reported that the physical-chemical conditions in the treatment systems and the type of disinfectant used (e.g., chlorine and UV radiation) can affect the occurrence of HGT between microorganisms in sewage.

The aforementioned studies suggest that, in fact, physical-chemical, biological, and operational conditions are capable of influencing the fate of resistance elements present in sewage. Thus, the presence of resistance genes in sewage under such conditions may contribute to its spread through HGT [23, 40]. Moreover, the inefficiency in the removal of these elements by secondary treatment systems [41] calls attention for the need to implement posttreatment technologies with a disinfection step, in order to reduce the concentration of resistance elements in sewage [38, 42]. However, it has been argued and suggested at lab or pilot scale that depending on the disinfection agent used, such as chlorine treatment [43] or ultraviolet radiation [44], they can increase the relative abundance of some ARGs and promote selection and dissemination of resistant bacterial strains [44], thereby disseminating AMR to the environment [10]. The efficiency of different tertiary techniques for the removal of ARB and ARGs will be discussed in more detail in the next section.

Although some studies strongly corroborate the occurrence of the dissemination of resistance elements by WWTPs, specifically downstream from the WWTP discharge point [9, 29, 42, 45], other studies show opposite results. Munck et al. [46] compared in silico the metagenomes and the functional resistome of WWTPs and reported that only six (8%) of the resistance genes present in WWTPs were found in the metagenomes of other environments, suggesting that the resistance character present in the treatment plants is distinct from those of other ecosystems, due to the limited mobility of the genetic elements. LaPara et al. [47] reported that high concentrations of resistance genes discharged into the water body had little or no effect on the abundance of these elements along the course of the Mississippi River. Taučer-Kapteijn et al. [48] reported the discharge of *Enterococcus faecium* resistant to ampicillin and vancomycin in the receiving water by a WWTP. However, these strains were not detected downstream (in surface water used as water supply sampling sites). Therefore, these studies suggest that the large river flow rate compared to that of the discharged effluents, along with the natural decay and settling of resistance elements, is responsible for minimizing their widespread in the environment. Thus, although the correlation between WWTPs and dissemination of resistance elements into the environment is strongly documented in the literature, the existence of studies with opposite results suggests that the characterization of these treatment plants as hotspots for this dissemination may be questionable.

Nevertheless, the real consequences of the AR increase and dissemination by WWTPs are still uncertain, and clear evidence demonstrating the evolution of

resistance in WWTPs is not yet widely documented [49]. Furthermore, the distinct climatic, environmental conditions and sanitation conditions of the different regions of the planet may contribute to the different results obtained in the studies previously discussed. In fact, temperature can influence the virulence and antibiotic resistance of some pathogens, such as *Acinetobacter baumannii* [50] and HGT efficiency [51]. Therefore, future studies should be conducted to investigate how environmental conditions, such as temperature, affect the spread of antibiotic resistance [51].

In summary, several studies have demonstrated that municipal WWTPs that use up to the secondary treatment discharge to the environment effluents containing high doses of ARB and ARGs. On the other hand, several authors have shown that WWTPs (with tertiary treatment and disinfection step) can reduce the concentration of antimicrobial agents, ARB, and ARGs, depending on the type of treatment used [42–44, 52]. This will be discussed in more detail in the next section.

4 Removal of Antimicrobial Resistance (ARB and ARGs) in WWTPs

Conventional wastewater treatment technologies, which include the primary and secondary stages, are commonly based on physical principles for retention and sedimentation of solids; and biological, for the conversion of dissolved or suspended organic compounds, resulting in effluents with concentrations of solids and organic matter lower than those of the raw sewage. Although municipal WWTPs are not specifically designed for the removal of contaminants present in small concentrations, such as contaminants of emerging concern which include antibiotics, resistance genes, and resistant bacteria, it may occur incidentally by sorption and co-metabolism, among other mechanisms.

Tertiary treatment technologies that may involve oxidation processes (e.g., ultraviolet photolysis, chlorination, ozonation, advanced oxidative processes), modified biological treatment (e.g., constructed wetlands and biological aerated filters), and conventional (e.g., coagulation) or advanced physical processes (e.g., filtration in membranes) have been increasingly employed for the complementary removal of microorganisms, including ARB, ARGs, and antibiotics. Such technologies are capable of removing the burden of pathogenic microorganisms present in domestic sewage. On the other hand, some of them (as in the case of chlorination) may act in the selection of multidrug-resistant strains and might not be efficient in controlling AR in full-scale WWTP (since some antibiotics are not degraded or efficiently removed and cell lysis might increase the release of intracellular resistance genes to the bulk solution [43]). Thus, it might be expected that WWTPs discharge, into receiving water bodies, effluents containing significant loads of resistance elements. Yet, there is no doubt about the overall benefits that WWTPs bring to society (in terms of prevention of aquatic pollution and environmental protection by

removing most of the pollutants), and the results presented in this topic indicate WWTPs are cost-effective.

Different studies [53, 54] have shown that conventional WWTPs can reduce the amount of ARB and ARGs by one to three orders of magnitude, and it seems that the degree of removal is dependent on the technology employed and also on the type of wastewater and size of the treatment system. Tertiary treatment systems are overall efficient in the complementary removal of resistant elements from sewage. This was demonstrated by McConnell et al. [52] who found the ARG marker abundances decreased by 1.77 log units ($p < 0.05$) when using an aerated lagoon and ultraviolet posttreatment and by 2.69 log units ($p < 0.05$) in a treatment plant which employed a biological nutrient removal system coupled to an ultraviolet disinfection step. Some advanced treatment systems can perform worse or better than others, as demonstrated by Chen and Zhang [55] when comparing UV disinfection to constructed wetlands and biological aerated filter, or may result in adverse effects due to the formation of hazardous disinfection by-products in the case of chlorination and ozonation (i.e., nitrosodimethylamine and bromate) [56].

For instance, UV radiation, a disinfection technology employed in some parts of the world as a final step of sewage treatment (before municipal WWTP effluent disposal or reuse), is not always able to achieve satisfactory removal of ARB and ARGs [44]. An increase in the efficiency of this treatment is normally observed when preceded by a filtration step or when combined with advanced oxidative processes (AOPs, H_2O_2, Fenton reactions, peracetic acid (PAA), and TiO_2). Chlorine and ozone treatments, when applied in a unitary way, besides not promoting satisfactory efficiencies in the removal of resistance elements from sewage, may result in the formation of by-products, which might be toxic to humans and animals [57, 58]. Indeed, Liu et al. [43] found that the relative abundance of intracellular ARG increased up to 7.8 orders of magnitude in the final effluent after the application of chlorine, whereas for some extracellular ARG (such as *tetA*, *tetB*, *tetQ*, *sul2*, among others), the median concentrations increased 2.1 to 3.8 folds, while only the concentrations of *tetM*, *gyrA*, and *dfrA1* decreased after chlorination.

Table 1 presents data about the technologies that have been applied in WWTPs aiming the removal of ARB and ARGs from domestic sewage. It is important to note that several WWTPs operate by combining two or more tertiary treatment technologies, in order to improve the removal of antimicrobial resistance elements from sewage. From Table 1, it can be seen that the ARB and ARG removal efficiency ranged from 1 to 3 log units. The best efficiencies were obtained by treatments based on AOPs that tested combined treatments (peracetic acid and UV-C; heterogeneous photocatalysis with TiO_2 and H_2O_2; combined process with UV, chlorination, and ozonation; photo-Fenton; among others) [49, 81].

Tertiary treatments are more sophisticated and costly systems which overall contribute for a significant increase in the removal efficiency of antibiotic resistance elements such as ARB from the sewage. Such effective disinfection systems might, on the other hand, contribute to a strong selective effect that can promote the selection of opportunistic pathogenic bacteria, as demonstrated by Alexander et al. [62]. These authors reported the selection of a robust population of bacteria

Table 1 Mechanisms of action and efficiency of tertiary treatment technologies for the removal of ARB (CFU/mL) and ARG (gene copies/mL) from sewage

Treatment	Mechanisms of action/limitations	Log removal[a]		References
		ARB	ARGs	
Ultraviolet	Cellular inactivation via photochemical reactions; photolytic degradation of purines and pyrimidines contained in genetic material, intra- or extracellular [38, 59]	–	–0.2 to 0.5	[60]
		–	2.48–2.79	[59]
		3.5–4.8	1.0–1.5	[38]
			–0.25	[44]
			2.0–6.0	[61]
		–	1.77–2.69	[62]
		0.73–0.86	<1.0	[63]
		1.0–2.4	–	[64]
Chlorination	Chlorine compounds are oxidizing agents with reactive action on cellular constituents (tryptophan, tyrosine, cysteine, glutathione, and guanine), thus acting on the degradation of nucleic acids and cell membranes of microorganisms [38, 59]	–	0.95–2.67	[65]
		–	0.42–0.02	[66]
		–	2.98–3.24	[59]
		–	1.20–1.49	[67]
		3.5–4.8	0.5–1.0	[38]
		–	0.6–4.4	[68]
		–	<1.0	[44]
		–	–1.5 to 7.8	[43]
		0.24–0.26	≤1.0	[63]
Ozonation	Oxidation of cell membrane constituents, causing increased cell permeability and lysis. Ozone has low penetration into the cytoplasm and, therefore, little action on ARG degradation [42, 59]	–	1.68–2.55	[59]
		–	≤1.0	[62]
		1.4–1.6	0.5	[42]
		3.5–4.8	1.0–2.0	[38]
			1.5–7.0	[61]
		2.1–2.46	<1.0	[63]

(continued)

Table 1 (continued)

Treatment	Mechanisms of action/limitations	Log removal[a]		References
		ARB	ARGs	
Constructed wetlands	Removal of ARB, ARGs, and antibiotics by biodegradation, adsorption on packing media, and uptake by plants [55, 69]	–	<1.0	[69]
		–	1.0–3.0	[55]
Coagulation	Neutralization of negatively charged particles – such as antibiotics, ARB, and ARGs – by coagulation agents, leading to the formation of flocs subsequently removed by settling and/or filtration [70]	–	1.0–3.1	[70]
Membranes	Retention (by physical or chemical interaction) of contaminants such as antibiotics, ARB, and ARGs present in the liquid mass. It depends on the type (micro-, ultra-, nanofiltration, or reverse osmosis) and characteristics (contact surface, porosity, material) of the membrane and the properties of the material rejected (hydrophobicity, sphericity, size) [71]	–	≤2.7	[72]
		2.7–5.4	0.1–4.8	[71]
		–	0.6–5.6	[73]
		–	3.3–3.6	[74]
Advanced oxidative processes (AOPs)	Oxidation of the contaminants (such as bacterial cells and genetic material) by the action of free radicals (e.g., hydroxyl radical,·OH). Non-irradiated (e.g., Fenton) or irradiated with UV light (e.g., TiO_2, photo-Fenton, O_3) processes have been widely employed [56, 75–77]	4.0–5.0	–	[56]
		3.0	–	[78]
		2.0–3.0	1.0–3.0	[75]
		–	<1.0	[76]
		–	1.55–3.79	[77]
		–	<1.0	[44]
		1.0	<1.0	[79]
		2.5–5.8	2.7–3.4	[39]
		6.0	–	[80]
Biological aerated filter	Biological retention and degradation of substrates in biofilms [55]	–	0.6–1.2	[55]

Negative values indicate that ARG concentration increased during treatment

[a]Removal = log ($C_{\text{raw sewage}}/C_{\text{final treated effluent}}$)

(including strains of enterococci and *P. aeruginosa*) that were poorly reactive to ozone, besides an increase in the relative abundance of the ARGs *van*A and bla_{VIM} in the treated effluent, since ozonation preferentially removed the ARGs *ampC* and *ermB*. Similarly, a research conducted in China has identified an increase in the proportion of bacteria resistant to sulfadiazine, vancomycin, rifampicin, and tetracycline after UV disinfection, step that was placed after the secondary treatment at a municipal WWTP [64]. In another study [44], it was observed that when high doses of disinfectants (UV, chlorine, and PAA) were applied, the process becomes

aggressive, imposing stress on the bacterial community and promoting the selection of resistant phenotypes.

Although total removal of AR elements in sewage still represents a great challenge in WWTPs, the implementation of tertiary treatment should be targeted and optimized. This is because the adverse effects caused by the different technologies mentioned in this section have a strong correlation with the dose of physical and/or chemical agent as well as the sewage characteristics, e.g., types of ARB and ARGs, organic matter, nutrients, pH, and temperature. In addition, the cost effectiveness of each technology should also be evaluated and changes in the population of resistant bacteria and concentrations of resistance genetic elements periodically assessed. Combined processes, which integrate both biological and physical-chemical systems (e.g., membrane separation, advanced oxidation, adsorption, disinfection), have potential to satisfactorily remove ARB and ARGs from sewage; however, more detailed studies are still needed to find out the most effective process setup for this purpose. All these issues may help fill knowledge gaps and mitigate the spread and accumulation of AR elements in domestic sewage.

5 Livestock as an Important Source of Antibiotic Resistance Dissemination into the Environment

In the last years, Brazil has been standing out in the production and exportation of meat. In 2018, the country reached the first position in the ranking of exports of chicken meat and fourth position for export of pork, besides being the second largest producer of beef in the same year [82]. Intensive animal production has increased the use of veterinary drugs and growth-promoting substances in ways that maximize feed efficiency in order to improve performance and reduce raising time, thereby lowering production costs [83]. The European Centre for Disease Prevention and Control has published in 2009 that livestock is the largest sector of antibiotic consumption used for growth-promoting [84, 85]. The Brazilian Association of Swine Producers has estimated that Brazil spent approximately U$53 million in 2015 with antimicrobials used for therapeutic or sub-therapeutic purposes [86].

Whatever the form, as an integral molecule or its metabolites/products of degradation, antimicrobials are dispersed in the terrestrial and aquatic environment by the application of manure and effluents in agricultural areas or directly through the excretion of animals in pastures. Even when the antibiotic molecule is metabolized, some of the excreted degradation products may remain bioactive [87]. Zhao et al. [88] have measured the concentration of oxytetracycline in fresh manure and found up to 17.6 mg/kg in chicken manure and up to 59 mg/kg in swine and cattle manure, whereas Frey et al. [89] have found 243 μg/g in swine manure in Canada. Indeed, antibiotics used in the "animal protein industry" have been reported in different environmental compartments. For instance, Pinheiro et al. [90] have found a range of 0.04 to 0.36 μg/L of chlortetracycline and 0.018 to 0.082 μg/L of sulfadimidine in a

farm soil. Either in soil or in fresh manure, antibiotics can be leached to water bodies, including groundwater [91].

As mentioned before, the risks for public health of an increase on ARB and ARG exposition have led to a number of governmental actions motivated by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organisation for Animal Health (OIE). The aim is to control the use of antibiotics, understand their impact on the environment, and develop new drugs. As a result, the Brazilian National Program for the Prevention and Control of Antimicrobial Resistance in Agro-Livestock was established in 2016 [92]. The most recent regulation of authorized and non-authorized antibiotics for growth-promoting purposes was published in 2018 [6]. According to the Brazilian Agriculture and Livestock Ministry, the only antibiotics allowed to be used as growth promoters are avilamycin, enramycin, flavomycin, monensin, narasin, ractopamine, and salinomycin.

The continuous and prolonged use of antibiotics results in the sharing of resistant extrachromosomal plasmids with nonresistant organisms. This was observed by Linton and Hinton [93], in 1988, where the prevalence of olaquindox resistance in pig *Escherichia coli* increased from 0.004% to 6% in 3 years after introduction of olaquindox as a growth promoter. In addition, the manure and effluents used for irrigation may be enriched with ARB, causing dissemination of resistant pathogens and compromising animal and human health [87, 94]. Arias and Murray [95] have shown that the use of avoparcin, a glycopeptide used in the USA for animal feed in the 1980s, was correlated with the emergence of *Enterococcus* spp. strains resistant to vancomycin in hospitals. Nowadays either olaquindox or avoparcin is not allowed to be used as growth promoter in several countries, including Brazil.

The dissemination of ARGs may occur between ARB and nonresistant environmental microorganisms [96]. Migliore et al. [97, 98] verified the accumulation of a large amount of sulfadimethoxine in several cultivated plant species and the toxic effect of enrofloxacin on seed germination. Therefore, considering the risk associated with livestock on spreading AR, researches around the world have raised awareness of the risk associated with such spread by monitoring ARB and ARGs in manure and effluents. As an example, resistance to the antibiotic colistin, one of the last line of therapy for infections caused by multidrug-resistant Gram-negative, was investigated by Mobasseri et al. [99]. They found that 1/3 of the *Klebsiella pneumoniae* strains isolated from swine manure in Malaysia were colistin-resistant and also multidrug resistant. In another study, Amador et al. [100] assessed the contamination of livestock manure from poultry, pig, dairy farms, and slaughterhouses in Portugal with resistance determinants. Overall, a high prevalence of multidrug-resistant *Enterobacteriaceae* was found in the livestock manure. The high ARG diversity identified in this study highlights the risk of multidrug resistance spread within the environment through manure use [100].

Measurements of ARG abundance are usually made by real-time PCR through which is possible to estimate the number of copies of the target gene per mass (g) or volume (mL) of a sample. A literature survey including studies carried out around the world has demonstrated that tetracycline, erythromycin, and sulfonamide

resistance genes (*tet*, *erm*, and *sul* genes, respectively) are the most frequently detected ARGs in swine, bovine, and chicken fresh manure [101–111].

It has been estimated for tetracycline resistance gene from 5 to 9 log units of gene copies per g of swine manure [112–114] and 3 to 4 log units of gene copies per mL of swine effluent [89, 114, 115]. A similar range was obtained for erythromycin gene, being 5 to 10 log units of gene copies per g of swine manure [114, 116] and 1 to 4 log units of gene copies per mL of swine effluent [115, 117]. The resistance to sulfonamide was detected in the order of 6 log units of gene copies per g of swine manure [114]. Considering the cattle manure, the estimated gene copies per g of sample reached values of up to 10, 3, and 6 log units of gene copies of *tet*, *sul*, *erm* genes, respectively, per g of sample [116, 118, 119]. Although no Brazilian data on erythromycin-resistant bacteria or gene have been reported so far, it is known that the use of erythromycin is not allowed since 2012 [6]. In several countries, erythromycin has been banned from being used as growth promoter; nevertheless, resistance genes and resistant bacteria have been found in livestock industries around the world.

Considering that crude livestock wastes are sources of ARB and ARGs which can be spread out in natural ecosystems, a proper treatment should occur in order to minimize the dissemination effect. In Brazil, about 180 million tons of waste and effluent from stables (pigs, cattle, and poultry) are produced per year. The technology encouraged by the Brazilian government involves the anaerobic digestion (anaerobic reactors or anaerobic ponds) of these organic residues resulting in products that represent a source of income, i.e., biogas and biosolids. Another option is the composting process, in which the solid livestock waste is biodegraded into dry mounds, generating a fertilizer with high nutrient content, especially phosphorus [120].

Some studies around the world have been carried out to evaluate the biological treatment efficiency on ARB and ARG removal. Most of them have shown that the biological treatment such as stabilization ponds [119, 121–123] and wetlands [124, 125] was not effective on ARGs' removal for swine manure treatment. Similarly, no removal was observed for cattle waste treatment by anaerobic digestion [126, 127] and aerobic composting [128]. Actually studies performed by Sun et al. [127] and Wallace et al. [129] have shown that the biological treatment for cattle waste promoted an increase in the relative abundance of resistance genes.

In general, the most frequently detected genes in treated livestock wastes are the same as those previously reported for raw wastes, *tet*, *erm*, and *sul* [121–125, 130–134]. Considering swine manure treatment, aerobic systems seem to promote decay on ARGs, as demonstrated by Wan and Chou [133] which have found a 1 log copy/μg DNA decay on activated sludge system applied for swine effluent. Investigating combined treatment systems, Tao et al. [132] found that an activated sludge system after an anaerobic reactor promoted a 0.5 to 2.0 log decay for *tet* genes, whereas Wang et al. [130] found a 3 log decay by adding a composting unit for the sludge generated by a pond treatment. From ARG monitoring it was also noticed a persistence of *sul* genes in wastewater treatment plants for livestock production even when other ARGs are removed [129, 132]. Therefore, the removal efficiency of ARGs by conventionally used techniques varies according to the type

of gene and the system operating conditions. It was possible to observe the presence of many genes in the raw manure, evidencing the need to use effective treatment techniques to reduce their spread to the environment. In order to enhance the biological treatment of poultry waste, Zhang et al. [110] showed that by applying surfactants during the residue composting, there was a significant reduction in the relative abundance of class 1 integron (from 0.58 log to 0.12 log of *int1*/16S rRNA gene copy). Similarly, Li et al. [111] reported that the addition of coal during 26-day composting decreases the relative abundance of most ARGs (tetC, *tetG*, *tetW*, *tetX*, *sul2*, *drfA1*, *drfA7*, *ermB*, *ermF*, *ermQ*, and *ermX*) and intI by 21.6–99.5%, but not for sul1 gene, which increased by 7.5–17.7 times.

Regarding ARB, Schmidt and Cardoso [135] evaluated the survival of *Salmonella* spp. in pig manure submitted to biological treatment, by successive stabilization ponds, on a pig breeding farm in Brazil. Most *Salmonella enterica* serovar Typhimurium strains (94.5%) were resistant to four or more antibiotics, and the multidrug resistance level and the variability pattern of these strains were similar at the beginning and at the end of the stabilization ponds system.

6 Antibiotic Resistance in Natural and Drinking Waters

Surface water is an important component of the urban water cycle. It can harbor natural bacteria that can eventually carry resistance to antibiotics, and it can also receive raw and treated sewage and other wastewaters (such as hospital and pharmaceutical effluents) that may contain antibiotic residues, ARB and ARGs.

The conventional process that takes place at the majority of water treatment plants (WTP) normally employs water coagulation with an iron or aluminum salt so that the flocs formed (flocculation unit) are removed from the liquid phase by settling (sedimentation unit) and filtration (usually sand filters). Then the clarified water is submitted to disinfection, usually with a form of chlorine (chlorination) before being distributed. Therefore, a conventional WTP has the potential of removing ARB and ARGs from water both in the clarification and disinfection steps.

Recent studies [136, 137] have shown that conventional water treatment can remove ARGs with an efficiency that varies from 1 to 3 log units, i.e., from 90% to 99.9%. In addition, other studies [136–138] indicated that the conventional water treatment exhibited similar removal efficiencies in removing ARGs when compared to an advanced process which consisted of oxidation with ozone coupled to adsorption with powdered activated carbon.

Regarding ARB, some studies have reported that different genera (*Salmonella*, *Shigella*, *Staphylococcus*, *Escherichia*, *Enterococcus*) have been detected in untreated [139, 140] or drinking water samples [139, 141] around the world. It is noteworthy the presence of antibiotic-resistant *E. coli* in treated or distributed water in different countries whether developed and developing ones.

In Brazil there are few studies about the occurrence of ARB in surface waters [45, 142–144], in coastal recreational waters [145, 146], and in drinking water

samples [147, 148]. Freitas et al. [144] reported the presence of multidrug-resistant pathogenic strains in Lake Água Preta (an Amazonian lake near to the city of Belem). They also observed a high dissemination of ESBL (extended-spectrum beta-lactamase)-producing bacteria in this lake. Scoaris et al. [148] isolated *Aeromonas* spp. from drinking water samples and reported that most of the isolates were multidrug resistant. They also observed that the majority of the isolates were not killed by chlorine at 1.2 mg/L (at 1 min of contact time). In another study, Silva et al. [147] isolated *Pseudomonas aeruginosa* from drinking water samples and reported that although all the drinking water isolates were susceptible to aztreonam, cefepime, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin-tazobactam, and polymyxin, the *P. aeruginosa* isolates were resistant to one or more antibiotics. They also reported that 3 out of 10 and 2 out of 10 isolates from tap water and mineral water, respectively, were not killed by chlorine at 0.6 mg/L (at 1 min of contact time). This is noteworthy because such chlorine concentration is three times higher than the minimum level (0.2 mg/L of free chlorine) recommended to be maintained (all the time) at any point of the water distribution network (and in reservoirs), according to the Brazilian drinking water legislation [149]. Results of these studies suggest the need for a re-evaluation of the criteria used to determine the microbial quality of drinking water.

The microbiological standard adopted in most countries, and also recommended in the WHO Drinking Water Guidelines (World Health Organization), involves the monitoring of *E. coli* in the water distributed by WTPs since its presence must be interpreted as an unambiguous sign of contamination. In addition, in order to evaluate the integrity of the distribution system (reservoir and network), the Brazilian drinking water guidelines require the analysis of heterotrophic bacteria which presence should be kept below 500 CFU/mL. Sudden changes in heterotrophic bacteria counts in the distribution system should be interpreted as suspecting dangerous events such as infiltration or biofilm growth in the water distribution network, which can contribute to the development of opportunistic pathogenic bacteria. The possibility of bacterial regrowth and selection of pathogenic microorganisms in breached distribution systems are reasons pointed by some researchers [150] who advocate the inclusion of ARB monitoring in drinking water. The latest version of the drinking water guidelines published by the World Health Organization [151] does not mention this aspect, and, as far as we know, currently no country requires the monitoring of ARB in distributed water. Although this action contributes to guarantee safe water to the population and to ensure public health, the costs involved might preclude the adoption of such policy in the developing world.

7 Conclusions

Municipal WWTPs with tertiary treatment play a key role in the prevention and control of environmental pollution (including AR spread) and are strategic to contribute to maintain public health. In countries that have high economic and

geographic diversity, such as Brazil, there is an urgent need to increase the sanitation coverage and improve the quality of treatment of the municipal WWTPs that are already in operation, in order to prevent AR spread to the environment. In addition, data gathered in Brazil about ARGs and ARB occurrence and removal in municipal WWTPs as well as in WTP are scarce. Therefore, research concerning this topic should be encouraged, in order to provide accurate information and data to support the revision of the criteria used to set the microbiological standard quality of water and wastewater.

Livestock is an important source of AR spread into the environment and has also been discussed. Considering that Brazil is one of the largest producers and exporters of all varieties of meat (beef, chicken, and pork) and that most of the biological treatments applied for livestock wastes and effluents are not effective to remove ARB and ARGs, there is a need to monitor and control AR spread by this type of effluents and wastes in the country.

Based on the data discussed, further research on the mobility of genetic resistance elements, and the environmental conditions that promote their transport by pathogenic bacteria, should be performed. It should also be ascertained which ARB and ARG concentrations represent, in fact, a risk to public and environmental health and to establish the potential for acquiring these elements through the management of treated sewage, sludge, and livestock effluents. Long-term studies should be carried out to compare the AR profile in WWTPs with different technologies and operational conditions in different climatic zones, as well as to evaluate the relevance of the posttreatment application due to effluent characteristics. Moreover, it is important to assess, in situ, the persistence of resistance elements in water bodies (especially those that are explored for recreational or water supply purposes) and sewage-receiving soils, in order to investigate their survival conditions in the environment, their accumulation in the food chain, and probable interaction with environmental bacteria.

Therefore, it can be concluded that the current scenario in Brazil, regarding the environmental spread and control of AMR, deserves attention and implementation of measures that should not only increase sanitation coverage but also improve the final effluent quality. Thus, a better surface water quality may reduce environmental contamination and ensure the maintenance of population health.

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Antibiotic Resistance and Sanitation in India: Current Situation and Future Perspectives

R. Sasikaladevi, V. Kiruthika Eswari, and Indumathi M. Nambi

Contents

Abstract Antimicrobial resistance (AMR) is a global threat as the existing health care may become ineffective. Antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) considered as emerging contaminants are the three major components of AMR. India is one of the largest consumers of antibiotics with defined daily dose (DDD) of 4,950 per 1,000 population in 2015. By 2030, therapeutic and nontherapeutic use of antibiotics in veterinary animals is projected to increase by 18%. Antibiotics, ARB, and ARGs in the solid and liquid waste generated enter the environment via different pathways. The major sources of antibiotics, ARB, and ARG include domestic, hospital, and pharmaceutical industry wastewater apart from the solid/liquid waste generated from veterinary and food animals. Existing conventional wastewater treatment technologies like activated

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sludge process (ASP) do not ensure complete removal of antibiotics, ARB, and ARGs from wastewater. Similarly, the sludge generated find its way to agriculture land and eventually spread resistance in the environment. Once introduced in the environment, elimination of these contaminants is difficult. India's action plan on AMR in 2017 regulates antibiotic use for human and animal and addresses environment AMR spread from all possible sources and containment. In 2020, the Government of India introduced discharge standard for 121 antibiotics in the effluents of bulk drug manufacturing industries, formulation industries, and common effluent treatment plant (CETP) receiving pharmaceutical wastewater.

Keywords AMR, Antibiotics, ARB, ARG, Discharge standards, Environment, India, Nontherapeutic, Solid and liquid waste, Therapeutic, Wastewater treatment

1 Introduction

Antibiotic resistance is a major public health concern at the global level as the existing health-care services may become ineffective. To exemplify, chloramphenicol is no longer a preferred choice for treating patients with antibiotic-resistant bacterial infections [1]. By 2050, two million deaths are projected to occur in India because of antibiotic resistance [2]. In developing countries like India urbanization and population growth are in increasing trend and this could lead to high consumption of antibiotics. In 2010, India was one of the top 5 countries with the largest shares of global antibiotic consumption. According to the Center for Disease Dynamics, Economics, & Policy (CDDEP), the antibiotic consumption was 2,645 DDD per 1,000 population in the year 2000 and has increased to 4,950 DDD per 1,000 population in the year 2015 [3]. Increase in antibiotic consumption could increase the antibiotic resistance burden and failure of treatment [4]. Hence, containment of antibiotic resistance spread is important. However, it is important to understand that AMR containment is not specific to only health-care services but also to the environment.

Antibiotics, ARB, and ARGs are the three major components of antibiotic resistance. The major sources of antibiotics, ARB, and ARGs in environment are wastewater from hospital, domestic use, and pharmaceutical industries [5–7]. Apart from wastewater and solid/liquid waste generated from animals, nontherapeutic use also introduce antibiotics, ARB, and ARGs into the environment [5, 6]. The western part of India has 47% of nation's pharmaceutical manufacturing units, but the effluent characteristic data including antibiotics from the manufacturing unit is scarcely available for this zone. While southern India hosts 18% of the pharmaceutical manufacturing units, concentration of the pharmaceutical compounds in the effluent is well reported compared to other zones [8]. The data is very important to understand the contribution of manufacturing units in polluting the environment

with antibiotics. However, the data on contribution from animal and nontherapeutic use is limited due to lack of strict rules and stringent monitoring. Similarly, quantitative data on ARB and ARGs entering environment due to anthropogenic activities are also limited in India.

To frame policies and treatment standards for an emerging concern like antibiotics, ARB, and ARG contamination, a holistic study approach is required. The study may include data on (1) antibiotics use in hospital, veterinary antibiotics in farms, and in other nontherapeutic applications; (2) source, pathway, or fate and transport of antibiotics, ARB, and ARGs; (3) occurrences of antibiotics, ARB, and ARGs in different environment matrices; (4) efficiency of current wastewater treatment practices in removing antibiotics, ARB, and ARGs; (5) national policies and plans implemented by the Government to contain AMR spread; and (6) current status and future recommendations. This book chapter attempts at presenting the Indian scenario of the abovementioned holistic approach.

2 Antibiotic Consumption for Therapeutics Purpose

Antibiotic use is one of the major determining factors of resistance gain and transfer. In India, antibiotics are prescribed in excess [9], and consumption is increasing steadily [10]. The most commonly consumed antibiotics are β-lactams while commonly prescribed includes fluoroquinolones, macrolides, cephalosporins, tetracyclines, and co-trimoxazole apart from penicillins [11]. It was reported that global antibiotic consumption has increased by 36% of which Brazil, Russia, India, China, and South Africa (BRICS) countries contribute 76%. About 23% of overall retail sales in BRICS countries was attributed to India. Between 2010 and 2015, there was a substantial increase in consumption of cephalosporins, broad-spectrum penicillins, and macrolides [3]. Cephalosporin and broad-spectrum penicillin consumption have significantly increased from 330 and 691 to 1822 and 1,055 DDD per 1,000 population between 2000 and 2015, respectively. Similarly, the consumption of macrolides has increased from 247 in 2000 to 469 DDD per 1,000 population in 2015 (Fig. 1). The consumption of trimethoprim has dropped from 332 to 186 DDD per 1,000 population between 2000 and 2015. However, consumption of the fluoroquinolones in 2000 was 492 and increased to 1,033 DDD per 1,000 population in 2006 and has dropped to 762 DDD per 1,000 population in 2015 (Fig. 1). Pattern change in antibiotic use could be attributed to rapid economic growth, rising income, and easy availability of antibiotics.

The use of antibiotics varies due to different reasons ranging from availability [12] to self-medication. Antibiotics consumption also varies with season, and in India, the average consumption was reported to peak during September, the end of the monsoon [13]. Inappropriate consumption of antibiotics for acute diarrhea [13], viral dengue fever [14], and acute respiratory infections [12] is widely reported in India. Poor sanitation practice [13], unregulated over-the-counter private pharmacy sales [14, 15], inappropriate prescription by medical practitioners for incentives [16],

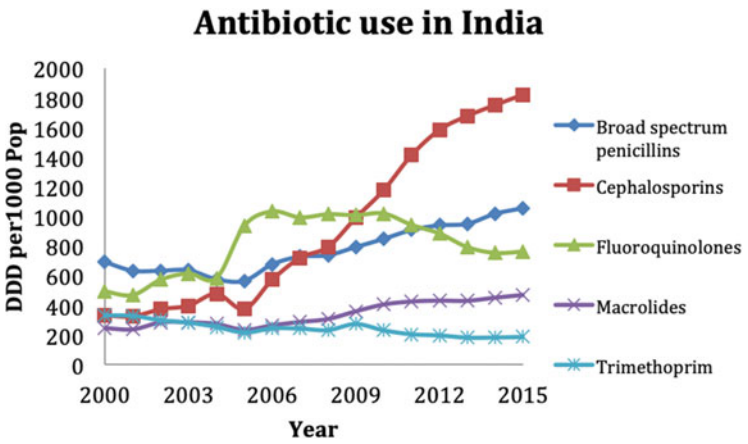

Fig. 1 Pattern change in antibiotic consumption in India, 2000–2015 [3]

and pharmacy sales in hospitals [17] are also reported in India. Over 50% of antibiotics consumed for presumed tuberculosis in Nagpur were dispensed without prescription over the counter [14]. A study conducted by Peripi et al. in Vijayawada reported that broad-spectrum antibiotics are highly prescribed by private practitioners compared to the public doctors [9], while Kumari Indira et al. observed high antibiotic prescription by rural practitioners compared to urban practitioners [12]. The prescription pattern also depends on nonclinical factors such as drug availability, type of hospital and department, and patients' request. Drugs available in the reserve (for specific treatments) are commonly prescribed in private hospitals, while in public/government hospitals, availability at the pharmacy is the deciding factor of prescription [12]. In some situations, antibiotics are prescribed based on patients' request. About 11–35% of doctors prescribed antibiotics due to patient pressure in a study conducted in Tamil Nadu [18]. However, irrational prescription of antibiotics is quite common in private and public sector hospitals across India [19]. Nevertheless, education on rational use of antibiotics is mandatory for both medical practitioners and patients.

3 Antibiotics Use in the Animal Food Industry

Over the years, the purpose of antibiotics use in livestock industry has changed. The antibiotics are used in animal farms as therapeutic agents, growth promoters, and prophylactic agents [20]. Antibiotics and antimicrobials constitute more than 70% of veterinary medicine [21]. Global consumption of antibiotics in 2013 in animal food industries was estimated to be 131,109 tons and is expected to increase more than 50% by the year 2030 [22]. In 2010, India was the fourth largest consumer of

antibiotics in food animal, which accounts for about 3% of the global consumption. By 2030, antimicrobial consumption for animals is expected to grow by 99% in BRICS countries [23].

Antibiotics are mixed in the feed to make animals resistant against disease and to gain more weight in a short time, which increases productivity. India is one of the largest producers of milk, producing 165.4 million tons in the year 2016–2017 primarily from smallholders [24]. An easy way for a smallholder to increase the yield is to make use of uncontrolled availability of antibiotics for therapeutics and to promote growth as it is economical [25]. This could result in high concentration of antibiotics in milk products and meat and animal wastes exceeding the maximum residue limits. Lack of regulatory framework to curb the use of antibiotics in livestock and food animals is one of the reasons for resistance spread in India. It is also estimated that in 2030, the antibiotics consumption in livestock in India may increase by 18% [23]. Chicken consumption in India is rapidly increasing. To meet the growing demand addition of unregulated quantity of antibiotics to animal feed as a growth promoter in poultry is practiced. The chicken meat was reported to have antibiotic residues such as tetracyclines (sum of oxytetracycline, chlortetracycline, doxycycline) and fluoroquinolones (sum of enrofloxacin and ciprofloxacin). Concentration of tetracyclines and fluoroquinolones was in the range of 16.01 to 46.02 μg/kg and 3.37 to 196.34 μg/kg, respectively [26]. In European countries, food quality controls on chemicals including antibiotics are in place. In India, the tolerance limit for antibiotics is set for seafood under Food Safety and Standards Regulation 2011, but no such regulation is put in place for chicken meat. The chicken meat that is exported has to comply with EU standards, while the domestic consumption has no regulation [26].

Nontherapeutic use of antibiotics in food preservation, apiary, aquaculture, agriculture, poultry, and pig farming is one of the sources of antibiotic resistance gain, and it spreads to the larger environment [22, 23]. 90% of the antibiotics are administered to farm animals at subtherapeutic concentration, of which 30% constitute growth promoters. Over-the-counter sale of veterinary antibiotics as growth promoters is quite common in India [27]. Antibiotics at sublethal concentration could increase the chance of resistance acquisition and transfer in animal gut bacteria [28]. Up to 90% of the antibiotics are excreted via urine and animal feces either unchanged or as active metabolized products [29]. Apart from the antibiotic residues and ARB in animal products, animal waste in all forms (carcasses, urine, feces) serves as a medium for AMR spread in the environment.

4 Source and Pathways of Antibiotics, ARB, and ARGs in the Environment

Figure 2 represents the source and pathway of antibiotics, ARB, and ARGs into the environment. Direct contamination of surface and groundwater with components of AMR is due to direct and indirect discharge of treated/untreated wastewater from

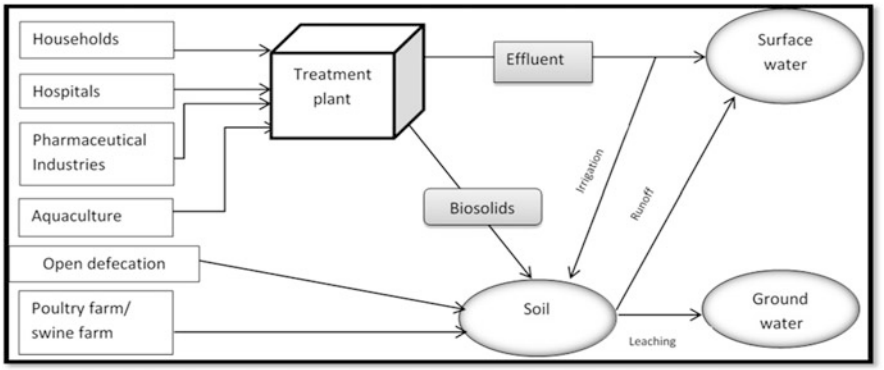

Fig. 2 Source and pathway of antibiotics into the environment

residence, industries, and solid/liquid waste generated from livestock farms, aquaculture, etc. In recent years, rapid urbanization has led to the implementation of decentralized treatment plants, and the treated effluent from such plants is commonly used for landscaping, watering lawns, gardening, and irrigation. The application of treated effluent for irrigation and recreational activities could contaminate the soil and indirectly contaminate the surface and groundwater [29, 30]. The sludge generated from wastewater treatment plant (WWTP) or sewage treatment plant (STP) and solid/liquid waste from livestock farms containing antibiotics, ARB, and ARGs are widely used as soil amendment to improve the nutrient content of the agriculture soil [31, 32]. Leaching of antibiotics, ARB, and ARGs over time from amended soil may reach groundwater and contaminate it indirectly [33]. Poor sanitation practice is also reported as one of the reasons for AMR and spread [34]. According to the UNICEF monitoring data 2012, 59% of 1.1 billion people who practice open defecation in the world reside in India [35]. Open defecation contaminates the soil, surface water, and groundwater directly and indirectly.

In India, surface water serves as the source of drinking and irrigation of agriculture fields. Contamination of the water sources with antibiotics, ARB, and ARGs can disseminate resistance genes to the environment [36]. Direct and indirect human exposure to contaminated environment such as water and soil is risky. Similarly, the prevalence of antibiotic resistance in the environment can be life-threatening when there is a disease outbreak making treatment of the resistant organism's infection difficult.

5 Antibiotics in Different Environmental Matrices

Lack of sufficient infrastructure and proper waste management practices are the main reasons for introduction of antibiotics into natural streams. The concentration of each antibiotic in different environmental matrices vary due to properties of antibiotics

such as octanol-water partition coefficient, biodegradability, bioavailability, etc. (Table 1). The direct source of antibiotics in surface water results from the discharge of untreated or partially treated wastewater from hospitals, industries, and WWTPs [37]. The concentration of antibiotics in sewage is directly proportional to prescription quantity [38]. In India, antibiotics are sold as nonprescription drug with a frequency of 18% [18]. Hence, there is no control over the residual concentration ending up in sewage. High concentration of fluoroquinolones was detected in hospital effluents and in river water samples especially ciprofloxacin [38, 39]. The concentration of ciprofloxacin found in effluent from a CETP that treats wastewater from bulk drug manufacturing units was 31,000 μg/L [6].

The Yamuna River is polluted with antibiotics including ampicillin, ciprofloxacin, gatifloxacin, sparfloxacin, and cefuroxime and is continuing to receive effluents from 17 STPs and 17 storm water drains [40]. Cochin estuaries receive 250 m^3 of domestic sewage because of improper waste management. This estuary is reported to have antibiotic-resistant *E. coli* counts higher than that reported in estuaries in France and Portugal [41, 42]. Sediments collected from Mutha River immediately after discharge point of treated wastewater from major hospitals and residential zone are enriched with genes conferring resistance to last resort antibiotics like carbapenems as well as metals and biocides [37, 43]. Contamination of Mutha River is due to the malfunctioning of WWTPs in Pune city and 50% of city's untreated sewage being discharged into the river [38].

The discharge of conventionally treated wastewater into streams has a significant effect on all forms of surface water. Efficient tertiary treatment of wastewater, proper management of sludge, and changes in disposal ways could control antibiotic resistance transmission to different environmental matrices.

6 Seasonal and Spatial Variation of Antibiotics in the Environment Matrices

Antibiotic concentration in the environment is reported to vary with seasons. Antibiotics concentration is reported to be highest during winter season, less during summer, and least during monsoon in River Yamuna [40]. Kshipra River water was found to have high concentration of sulfamethoxazole during autumn (2.75 μg/L) and winter (2.15 μg/L) compared to summer (1.39 μg/L) and rainy season (0.04 μg/L) [48]. The change in antibiotic residue concentration could be due to change in pH. pH of the water bodies can influence the solubility of the antibiotics [50]. Sulfamethoxazole concentration in Kshipra river sediments is reported to be influenced by solubility, which in turn is influenced by pH [48]. High concentration of antibiotics in river water during winter could be due to low biodegradation rate, which is influenced by temperature and other operational parameters of the treatment plant [45]. The lower concentrations of antibiotics observed during monsoon could be due to the dilution effect of rainwater. During summer, photo degradation, temperature,

Table 1 Occurrence of antibiotics in various environmental matrices

Type of sample	Location	Antibiotics	Concentration (μg/L)	Analytical instrument	Reference
Wastewater	Hospital wastewater, Madhya Pradesh	Ofloxacin	4.5	LC-MS/MS	[44]
		Ciprofloxacin	218.3		
		Norfloxacin	6.4		
		Levofloxacin	5		
	CETP for pharmaceutical wastewater, Andhra Pradesh	Ofloxacin	150–160	HPLC	[6]
		Ciprofloxacin	28,000–31,000		
		Norfloxacin	390–420		
		Enrofloxacin	780–900		
		Lomefloxacin	150–300		
		Enoxacin	150–300		
	Okhla STP, Delhi	Ciprofloxacin	8	HPLC-PDA	[40]
		Gatifloxacin	1.22		
		Sparfloxacin	0.14		
		Cefuroxime	0.22		
		Ampicillin	12.68		
	STPs, South India	Chloramphenicol	<0.01	HPLC-MS	[10]
		Trimethoprim	0.04–0.29		
		Sulfamethoxazole	0.40–0.64		
		Ofloxacin	0.21–2.47		
	WWTP influent from Metropolitan city, Western India	Levofloxacin	150	HR-LC-MS/MS	[45]
		Norfloxacin	20		
		Azithromycin	300		
River	Yamuna River, Delhi	Ciprofloxacin	1.44	HPLC-PDA	[40]
		Gatifloxacin	0.48		
		Sparfloxacin	2.09		
		Cefuroxime	1.7		
		Ampicillin	13.75		
		Ofloxacin	1.51	LC-ESI-MS/MS	[46]
		Erythromycin	0.10		
		Azithromycin	0.16		
		Norfloxacin	0.20		
		Ciprofloxacin	4.88		
		Moxifloxacin	0.16		
		Amoxicillin	0.18		
	Musi River, Telangana	Ofloxacin	1.55–318.1	LC-MS/MS	[47]
		Ciprofloxacin	6.59–5,528		
		Norfloxacin	16.14–217.5		
		Pefloxacin	0.74–44.34		
		Enrofloxacin	2.57–123.4		
		Difloxacin	0.47–37.74		

(continued)

Table 1 (continued)

Type of sample	Location	Antibiotics	Concentration (μg/L)	Analytical instrument	Reference
		Lomefloxacin	3.59–10.32		
	Kshipra River, Madhya Pradesh	Norfloxacin	0.66	LC-MS/MS	[48]
		Ofloxacin	0.99		
		Sulfamethoxazole	0.04–2.75		
	Cauvery River, Tamil Nadu	Carbamazepine	0.13	GC-MS	[49]
	Tamraparni River, Tamil Nadu		0.01		
	Vellar River, Tamil Nadu		<0.01		
River sediments	Kshipra River, Madhya Pradesh	Ofloxacin	0–9.74	LC-MS/MS	[48]
		Sulfamethoxazole	0–8.23		
Aquifer	Delhi	Ofloxacin	4.34	LC-ESI-MS/MS	[46]
		Erythromycin	0.11		
		Azithromycin	0.17		
		Norfloxacin	0.05		
		Ciprofloxacin	5.90		
		Moxifloxacin	0.21		
		Amoxicillin	0.18		

and microbial activity play important role in reducing the antibiotic concentration [48].

In India, the concentration of antibiotics is comparatively more in river tributaries than in the main river because the tributaries are the immediate discharge points of wastewater. The antibiotics concentration reduces due to dilution effect when it reaches the main river [47]. Also, the contamination level of antibiotics in surface water such as river is positively correlated with land use pattern [39, 43]. This could be due to discharge of treated/untreated wastewater containing high concentration of antibiotics resulting from high domestic consumption. High concentrations of antibiotics are reported near the discharge points in surface water [51] and in rivers traversing through urbanized area as the infusion of sewage and industrial effluent are inevitable in densely populated areas [52]. There are several other physic-chemical properties such as photosensitivity, co-metabolism, nature of active metabolites/by-products, etc. that determines the spatial distribution and concentration of antibiotics in different environments. However, reports available on seasonal and spatial variation of antibiotics in different Indian environment are limited. Antibiotics in any environment could increase the chance of resistance spread, disease burden, and treatment failure from direct and indirect human exposure to resistant pathogens [36].

7 Antibiotic-Resistant Bacteria in the Environment

ARB are inherently present in the environment. ARB and antibiotics enter the environment from different sources [44] such as human and animal excreta, discharge form wastewater treatment plant, and direct sewage discharge. It is supposed that antibiotic residues that enter aquatic environment can promote resistance in aquatic microbial communities [53]. High concentration of antibiotics may select ARB in the environment [54]. Reports suggest that antibiotics at low and subminimum inhibitory concentration can also select ARB [55]. ARB mutants selected at low and subinhibitory concentrations exhibit higher stability than the ones selected at high antibiotic concentration [56].

WWTP acts as a connecting bridge between wastewater generated and aquatic environments. WWTP provides the right environment for mutation and exchange of genes resulting in resistance spread because a large number of bacteria constantly encounter antibiotics at subinhibitory concentration [56, 57]. Proportion of ARB may increase during course of wastewater treatment mainly when treatment processes involve one or more biological methods [54]. Akiba et al. reported that increased prevalence of resistant bacteria in the STPs was due to inflow of hospital wastewater. Also, the strains isolated were resistant to antibiotics quantified in the STP samples [10]. Partially treated or untreated wastewater is usually discharged into surface water leading to contamination of aquatic environment with ARB. ARB could share resistant genes with environmental bacteria. When antibiotic resistance genes are shared, the environmental ARB (eARB) could become pathogenic ARB (pARB) if the eARB carries virulence traits [58, 59]. This signifies that inadequate wastewater treatment and poor maintenance of STPs in hospitals could contribute to the spread of AMR in the environment [60, 61]. ARB are reported in different environments including hospital wastewater, untreated sewage, STPs, drinking water, river water and sediments, coastal waters, marine water, and sediments (Table 2). Bacteria exhibiting different levels of resistance are widely reported in various environments across India (Table 2). However, distribution of ARB in environment varies with season [48], and elimination of ARB from any environment can be difficult [5]. Hence, ARB introduced into the environment through anthropogenic activities are of concern.

From Table 2, it can be seen that in India, domestic/hospital wastewater and effluents from WWTPs/STPs are the widely reported sources of ARB. This is because wastewater and effluents are direct source of ARB introduction. Indirect/neglected sources of ARB include application of STP/WWTP sludge, livestock waste on land as soil amendment, open defecation, and direct dumping of animal waste in the vicinity water sources, which are widely practiced in India [29, 82–84]. The indirect sources ARB are least studied. Runoff water from agriculture lands and places of open defecation also serve as a medium for ARB introduction in the environment [49, 85]. However, not all the reported ARB are clinically significant.

Table 2 Antibiotic-resistant bacteria in different environment matrices

Source	Location	Bacterial group	Type of resistance	Reference
Kshipra River	Ujjain, Madhya Pradesh	*E. coli*	ESBL	[48, 62]
Musi River	Hyderabad, Telangana	Ciprofloxacin resistant bacteria	MDR	[63]
Mula-Mutha River	Pune, Maharashtra	*E. coli*	MDR	[38]
		Thermotolerant fecal coliform	DDR	[43]
		Acinetobacter spp.	MDR	[37]
Yamuna River	Delhi	ESBL and Amp C- producing *E. coli*	MBR	[64]
		ESBL producers	MDR	[65]
		Enterobacteriaceae	Carbapenem resistant	[61]
		ESBL-producing *Klebsiella pneumonia*, *Klebsiella quasipneumoniae*, *Klebsiella variicola*	MDR	[66]
Cauvery River	Karnataka	*E. coli, Enterobacter cloacae*, *Pseudomonas trivialis*, *Shigella sonnei*	MDR	[67]
		Staphylococcus spp. and *Staphylococcus aureus*	Methicillin and vancomycin resistant	
Subarnarekha River, Kharkai River, Dimna Lake, and Hudco Dam	Jamshedpur, Jharkhand	*Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Klebsiella*, *Enterobacter*, and *Proteus* spp.	MDR	[58]
Stream, well water, drinking water, tap water, soil	Wayanad, Kerala	*Vibrio cholerae*	MDR	[68]
Drinking water and wastewater	Ujjain, Madhya Pradesh	*E. coli*	MDR	[69]
5 recreational beaches	Mumbai, Maharashtra	*E. coli* pathotypes (EHEC, ETEC, EPEC, STEC, EAEC, and UPEC)	MDR	[70]
Palk Bay	Tamil Nadu	*Vibrio* spp.	MDR	[71]
Port Blair Bay	Andaman and Nicobar Islands	*Enterococcus faecalis*	DDR	[72]
River, ponds, kunds, hand pumps, piped supply and dug wells	Across India	*Escherichia coli* O157: H7	MDR	[73]

(continued)

Table 2 (continued)

Source	Location	Bacterial group	Type of resistance	Reference
Wetland	Lakhimpur Kheri, Uttar Pradesh	*Citrobacter*, *Aeromonas*, *Curtobacterium*, *Erwinia*, *Providencia*, *Shigella*, *Arthrobacter*, *Chryseobacterium*, *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Janthinobacterium*, *Bacillus*, *Yersinia*, *Rahnella*, *Rheinheimera*, *Sphingobacterium*, *Micrococcus*, *Vogesella*, and *Kluyvera* spp.	MDR	[74]
Karwar coast (Arabian Sea)	Karnataka	*Bacillus toyonensis* PNTB1, *Lysinibacillus sphaericus* PTB	MDR	[75]
Cochin estuary	Cochin, Kerala	*E. coli*	MDR	[41]
Recycled hospital wastewater	Kanchipuram, Tamil Nadu	*E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus mirabilis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*	MDR	[76]
Wastewater outfalls of 12 hospitals	Delhi	Gram-negative pathogens	Carbapenem and ESBL	[77]
Wastewater outlets of a rural and an urban hospital	Ujjain, Madhya Pradesh	ESBL-producing *E. coli*	MDR	[78]
WWTP	Jalandhar, Punjab	*Staphylococcus aureus*	MDR	[79]
WWTP	Haridwar, Uttarakhand	Fluoroquinolone- resistant bacteria	Ciprofloxacin, norfloxacin, and ofloxacin	[54]
STPs	Tamil Nadu	*E. coli*	MDR	[10]
STPs	New Delhi	*E. coli*, *Pseudomonas putida*, *Enterobacter cloacae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Aeromonas caviae*	ESBL	[60]
		E. coli, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* subsp.	Carbapenem resistant	

(continued)

Table 2 (continued)

Source	Location	Bacterial group	Type of resistance	Reference
		pneumoniae, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas putida*, *Shigella dysentery*		
Drain sites, hospital waste outfalls, effluents from STPs	New Delhi	*Enterobacteriaceae*	Carbapenem resistant	[61]
Sewage outfalls from hospital to community drains	New Delhi	*Pseudomonas putida*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Klebsiella pneumoniae* subsp. *pneumoniae*	Carbapenem resistant	[77]
		E. coli, *Pseudomonas putida*	ESBL	
Electronic industrial effluent discharges and CETP	Hyderabad, Telangana	*Pseudomonas*, *Bacillus*, *Halomonas*, and *Kocuria* spp.	Chloramphenicol, streptomycin, and ampicillin	[80]
Shrimp (effluent) pond discharge point, Vellar estuary	Parangipettai, Tamil Nadu	*Bacillus pumilus* and *Bacillus flexus*	MDR	[81]

MDR multidrug resistant, *DDR* dual drug resistant, *MBR* multiple β lactam resistant, *ESBL* extended spectrum β-lactams

8 Antibiotic Resistance Genes in the Environment

Some microbes have intrinsic resistance to antibiotics irrespective of antibiotic use [86]. However, excessive use of antibiotics results in resistance acquisition and transfer. Though ARGs are inherent to microbial community, the intensive use of antibiotics for human, animal, and agriculture accelerates ARG mutation and acquisition of new ARGs [53]. ARGs are widely reported in different environment including hospital wastewater, untreated sewage, STPs, drinking water, river water, and sediments (Table 3). ARGs are considered as emerging pollutants, and dissemination of ARGs in the environment is of concern. ARGs can be of genomic or plasmidic origin. However, antibiotic resistance traits are usually associated with horizontally transferable mobile genetic elements [87]. ARGs in genomic DNA are transferred to progeny, while plasmid DNA may also be transferred to different bacteria species via horizontal gene transfer (HGT) through conjugation, transduction, and transformation mechanisms [59]. ARGs are associated with MGE such as

Table 3 ARGs reported in different environment across India

Source	Location	ARGs	Gene abundance (log gene copy mL^{-1})	Resistance	HGT genes/ RDRs	Reference
Mula- Mutha River	Pune, Maharashtra	*qnr*A, *qnr*B, *qnr*S, *oqx*A	–	Quinolone	*int*I1, *int*I2	[37, 38]
		bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, GES, OXA-1, OXA-2, OXA-10, CAR	–	β-lactam		
		ant(3″)-*la*, *ant*(2″)-*la*, *ant*(6)-*la*, *aph*(3″)-*lb*	–	Aminoglycoside	Integrases ISCR, transposases	[37]
		GES-type, OXA-58, NDM, KPC, IMP, OXA-48 type	–	Carbapenem		
		*cat*A16, *cat*B2, *cat*B3, *cml*A	–	Chloramphenicol		
		ere(A), *ere*(C), *erm*(F)	–	Macrolides		
		*inu*F	–	Lincosamide		
		*sul*1, *sul*2, *sul*3	–	Sulfonamide		
		tet(M), *tet*(Q), *tet*(X), *tet*(32)	–	Tetracycline		
		mcr-1	–	Colistin		
		*dhfr*A1, *dhfr*A15	–	Trimethoprim		
		tet (X)	–	Tigecycline		
Yamuna River	Delhi	$bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, amp_C	–	β-lactams	–	[64, 65]
		bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$	–	ESBL	–	[66]
		$bla_{NDM\text{-}1}$	~6 ± 0.25–8 ± 0.5	β-Lactams	*int*1, *int*2, *int*3	[61]
		bla_{OXA}	–			
		$bla_{NDM\text{-}1}$	5.4 ± 0.4	β-Lactams	–	[90]
		bla_{OXA}	–			
		tet(M), *tet*(Q), *tet*(W)	–	Tetracycline	–	

Upper Ganges River	Rishikesh-Haridwar	$bla_{\text{NDM-1}}$, bla_{OXA}	2.1 ± 0.6	β-Lactams	–	[90]
		tet(M), *tet*(Q), *tet*(W)	–	Tetracycline		
Kshipra River	Ujjain, Madhya Pradesh	$bla_{\text{CTX-M-1}}$	–	β-Lactams	–	[48]
		qnr S, *qnr* B	–	Quinolone		
		*sul*1, *sul*2	–	Sulfonamide		
Ganga River	Lucknow, Uttar Pradesh	bla_{TEM}	0.36 ± 0.15–5.32 ± 3.69	β-Lactams	–	[91]
Gomti River	Kanpur, Uttar Pradesh	bla_{TEM}	1.94 ± 0.5–5.96 ± 1.54	β-Lactams	–	
		*gyr*A, *par*C, *qnr*A, *qnr*B, *qnr*S, *aac(6′)-Ib-cr*, *oqx*AB, *qep*A	–	Quinolones		
Cauvery River	Karnataka	bla_{TEM}	–	β-Lactams	–	[67]
		dhfr		Trimethoprim		
Cochin estuary	Cochin, Kerala	bla_{TEM}, $bla_{\text{CTX-M}}$	–	β-Lactams	*int*1, *int*3	[41]
		tet (A), *tet* (B)		Tetracycline		
		*sul*1, *sul*2, *sul*3		Sulfonamide		
		*aph*A2		Gentamicin		
		*cat*1		Chloramphenicol		
		*dhfr*1, *dhfr*7		Trimethoprim		
Drinking water and wastewater	Ujjain, Madhya Pradesh	$bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-9}}$	–	β-Lactams	–	[69]
		*qnr*A, *qnr*S		Quinolone		
Sewage outfalls from hospital to community drains	New Delhi	$bla_{\text{NDM-1}}$	11.29 ± 2.11–8.87 ± 2.15	ESBL	*int*1	[77]
		bla_{CTX}, bla_{OXA}, bla_{TEM}	–			
STPs	New Delhi	bla_{CTX}	~6 ± 1–9 ± 0.1	ESBL	*int*1, *int*2, *int*3	[60]
		bla_{OXA}	~4 ± 0.1–6.5 ± 0.1			

(continued)

Table 3 (continued)

Source	Location	ARGs	Gene abundance (log gene copy mL^{-1})	Resistance	HGT genes/ RDRs	Reference
		bla_{TEM}	~7 ± 0.1–9.75 ± 0.25			
		bla_{NDM-1}	~5 ± 0.25–8 ± 0.1			
Drain sites, hospital waste outfalls, STPs effluent	Delhi	bla_{NDM-1}	~6.5 ± 0.25–8.25 ± 0.25	β-Lactams	*int*1, *int*2, *int*3	[61]
		bla_{OXA}	–			
WWTP	Haridwar, Uttarakhand	*gyrA*	–	Quinolone	–	[54]

RDR resistance-determining regions

plasmids and integrons, and transposons enable easy dissemination of resistance via HGT [88]. ARGs in plasmids are self-transmissible and capable of transferring and replicating in different organisms [89].

Antibiotics stress not only selects bacteria but also results in gene mutation in resistance genes [54]. ARGs are persistent and may occur in the environment even in the absence of antibiotic selection pressure [43], and the distribution of different ARGs in environment may vary with season [27, 92]. However, ARGs proliferate irrespective of seasons during wastewater treatment [43]. The gut bacteria especially *E. coli* is a human commensal, antibiotic resistance indicator [69] and a reserve for antibiotic resistance genes, which can be horizontally transferred to pathogenic bacteria [93]. *E. coli* could reach environment matrices such as river due to discharge of partially treated and untreated domestic or hospital wastewater. ARGs encoded by plasmids in *E. coli* may result in resistance gene spread/transfer [69] in such environment. Bajaj et al. demonstrated co-transfer of plasmid-mediated quinolone-resistant (PMQR) gene *qnr*S and plasmid-mediated AmpC β-lactamase genes between *E. coli* strains isolated from River Ganga and quinolone susceptible *E. coli* J53. *E. coli* J53 after gene acquisition exhibited co-resistance to quinolone and β-lactams [94]. This study proves that plasmid-mediated HGT of ARG is possible between an environmental *E. coli* resistant to antibiotics and *E. coli* susceptible to antibiotics.

Mutation in RDR also determines the bacteria's ability to resist antibiotics and may confer cross-resistance to other antibiotics [94]. Hence, it is important to understand the relationship between RDR and ARG. Lamba et al. reported correlation between expression levels of mobile genetic elements *int*1 and *int*3 and $bla_{\text{NDM-1}}$ gene abundance in *Enterobacteriaceae* [61]. The study also reported co-carriage of *int*1 and $bla_{\text{NDM-1}}$ gene in carbapenem -resistant *Enterobacteriaceae* (CRE) isolated from different sources. The co-carriage was reported to be 28%, 45%, 52%, and 57% in CRE isolated from STP, hospital, drain, and river samples, respectively, across New Delhi [61]. Co-occurrence of ARGs is quite common in MDR bacteria. In hospital setting, MDR bacterial infections are difficult to treat and sometimes are life-threatening. Chandran et al. reported co-occurrence of cephalosporin and quinolone resistance genes in *E. coli* isolated from hospital wastewater in Ujjain, Central India [78]. Hospital wastewater is also reported to contain MDR *E. coli* that are genetically diverse [78]. ARGs are inherent to resistant bacteria in the environment. However, anthropogenic activities such as discharging partially treated and untreated wastewater, application of livestock waste as soil amendments, open defection, etc. could increase ARG prevalence in the environment [85]. This could also promote resistance transfer to nonresistant/ pathogenic environmental bacteria [59].

9 Indian WWTPs Status in Eliminating Antibiotics, ARB, and ARG

Wastewater is linked to the natural environment by STPs. In India, the treatment facilities exist only for 31.5% of wastewater generated [95]. The commonly employed treatment technologies include ASP, oxidation pond, upflow anaerobic sludge blanket (UASB), sequential batch reactor, fluidized bed reactor, waste stabilization pond, Karnal Technology, rotating biological rope contactor, chrome recovery pilot plant, and aerated lagoon [96]. In India, the total capacity of treatment plants are 23,277 millions of liters per day, of which 5% is nonoperational, and 11% is under construction [95]. There is a large gap between wastewater generated and wastewater treated.

WWTPs/STPs receiving sewage and sewage mixed with hospital effluents contain antibiotics, ARB, and ARGs. The efficiency of the WWTPs in removing the AMR components decides the quality of the effluent. Hence, it is important to evaluate the performance of existing treatment technologies in removing antibiotics, ARB, and ARGs. The activated sludge process showed good removal efficiency for antibiotics like sparfloxacin and cefuroxime with 99% and 94% removal, respectively [40], and is less effective in case of low concentration antibiotics and recalcitrant antibiotics. The antibiotics removal efficiency is high when extended aeration process is employed for domestic wastewater [97]. Mutiyar and Mittal observed high removal of amoxicillin in STP involving an extended aeration ASP [97]. The antibiotic removal efficiency is also dependent on the influent wastewater quality. Prabhasankar et al. reported high antibiotic concentration in the effluent of the STPs receiving mixed wastewater from hospitals and domestic zone than that treating exclusively the hospital wastewater [1]. The high antibiotic removal rate in the STPs treating exclusively the hospital wastewater could be due to quantity of the influent wastewater and residence time. However, the antibiotics were not completely removed in the effluent of STP treating exclusively hospital wastewater [1]. Proper treatment of hospital wastewater can eliminate antibiotics by 80%, but only 40% of health-care facility has proper wastewater treatment facilities [98]. It is reported that an average of 0.53 kg of sulfamethoxazole is discharged annually from a STP in India serving an average population of 325,000 [21]. The concentrations of antibiotics in effluents from Indian STPs are higher when compared to Europe and North American countries [97]. The effluent of a CETP in Patancheru, Hyderabad, which receives wastewater from 90 drug manufacturing companies, after biological treatment is reported to have high concentration of antibiotics [6].

Mixed or not both domestic and hospital wastewater contain ARB and ARGs [99]. A STP employing UASB followed by ASP and chlorine disinfection scheme was ineffective in removing MDR *E. coli* [100]. Similarly, STPs receiving mixed wastewater and exclusively hospital wastewater employing ASP followed by clarification and chlorine disinfection were not effective in removing MDR *E. coli* [97]. The biological treatment employed in a municipal WWTP in Haridwar, removed 60–80% of fluoroquinolones, and disinfection by chlorine eliminated

96% of fluoroquinolone-resistant bacteria [54]. The effluent of a hospital wastewater treatment plant in Delhi was reported to carry carbapenem-resistant bacteria such as *Pseudomonas* spp., *Klebsiella* spp., *Escherichia* spp., and *Acinetobacter* spp., and ARG $bla_{\text{NDM-1}}$ associated with *int*1 [101]. Similarly, effluents from 12 STPs employing aerobic sludge as treatment process across Delhi were also reported to carry ARB such as carbapenem and ESBL-resistant bacteria and ARGs such as NDM-1, CTX, OXA, and TEM. However, including anaerobic digestion and chlorination as tertiary treatment improved the removal rate of ARB and ARGs [60]. There is no complete removal of antibiotics by the existing treatment plants, and the most common removal mechanism is sorption to sludge particulates [40]. Seasonal changes and type of wastewater treatment process do not have an impact on the prevalence of resistant isolates [10]. ARB and ARGs are also not completely removed during the treatment process, and maximum percentage ends up in the sludge [60].

10 India's Action Plans on AMR and Current Status

Antibiotic resistance is not considered a serious threat in majority of the developing countries due to lack of awareness among public. In India, antibiotic resistance made the news pages in 2010 with report on isolation of New Delhi metallo-ß-lactamase-1 (NDM-1). Ever since the NDM-1 reports in 2010 several clinical studies reporting isolation of antibiotic-resistant pathogens, and genes in clinical samples are increasing, indicating the awareness among research community. Several policies addressing effective antibiotic use and AMR containment are adopted in India since 2011 (Table 4). National antimicrobial policy for containment of AMR in India was adopted in 2011 [102], while Global Action Plan on AMR was adopted in 2014 [103].

Even though India's action plan on AMR was adopted in 2011, the plan considered only human and animal consumption and over-the-counter sale of antibiotics as the main reason for AMR spread. This resulted in inclusion of antibiotics in schedule H1 drug, AMR prevention and containment declaration, guidelines for antimicrobial usage in treating infectious disease, and widespread campaign across India. In August 2016, the Prime Minister of India addressed the antibiotic resistance and launched "Red line campaign" to make the public aware of the importance of antibiotic use and misuse [102]. This was followed by India's National Action Plan on AMR in 2017 that focuses on the use of antibiotics and resistance in human, animal, agriculture, food products, and environment [110]. One of the objectives of India's National Action Plan is to reduce environmental contamination with resistance genes, resistant pathogens, and antibiotic residues arising from solid/liquid waste generated from manufacturing, use, waste treatment, and disposal [110]. Even though antibiotics are classified under schedule H1 drug, antibiotics are still sold over the counter without prescription. However, the frequency has significantly dropped compared to the earlier reported studies [113]. Following

Table 4 India's policies on AMR

Year	Authority	Policy	Reference
2011	Ministry of health and family welfare	National policy for containment of AMR	[104]
2011	Ministry of health and family welfare, WHO	National action plan on AMR (2011–2016)	[104]
2011	Ministry of health and family welfare, WHO	Jaipur declaration	[105]
2012	Directorate general of health services/national centre for disease control	Chennai declaration	[106]
2016	Ministry of health and family welfare, WHO	AMR and its containment in India	[107]
2016	Indian council of medical research (ICMR)	National AMR research and surveillance network (AMRSN) and inclusion of antibiotics in schedule H1 drug	[108]
2016	National centre for disease control (NCDC)	National treatment guidelines for antimicrobial use in infectious diseases	[109]
2017	Ministry of health and family welfare, WHO	National action plan on AMR (2017–2021)	[110]
2017	National centre for disease control (NCDC)	Delhi declaration on AMR	[111]
2017	Food safety and standards authority of India (FSSAI)	Food safety and standards (contaminants, toxins, and residues) regulations in food animals	[102]
2017	Indian council of agriculture research (ICAR)- food and agriculture organization (FAO)	Indian network for fisheries and animal antimicrobial resistance (INFAAR)	[112]

National Action Plan on AMR in 2017, research on environmental AMR in India has significantly increased and is gaining attention. About 61.3% of increase in households with toilet is reported with implementation and funding through Swachh Bharat Mission [114], and open defecation has greatly reduced. In January 2020, Indian government has published a draft comprising discharge standard for 121 antibiotics in the treated effluents of bulk drug and formulation industry and CETP treating pharmaceutical wastewater. The draft also suggests incineration of the sludge containing antibiotics residue [115].

11 Conclusion

Implemented rules and regulations for human consumption, animal use, and disposal of antibiotics must be strictly followed and regularly monitored in India. Public awareness about the importance of antibiotics to treat serious infections and AMR

emergence must be created to avoid misuse of antibiotics. Recall and safe disposal of unused antibiotics from households should be implemented. AMR research in India is majorly focused on contribution of WWTPs of domestic sewage, pharmaceutical industries, and hospital effluents in environment AMR spread. There is little to no data on contribution of environmental AMR by other sources such as sewage sludge, solid/liquid waste generated by poultry, aquaculture, dairy and other livestock, agriculture run off, etc. Also a holistic approach to study the dissemination pathways of ARB and ARGs for better understanding and designing of suitable treatment technology is needed. Standards for treatment and discharge of hospital and domestic wastewater must be introduced. Similarly framing antibiotic discharge standards for sectors like poultry, aquaculture, and dairy wastewater, etc. is also necessary considering the fact that emerging contaminants like antibiotics, ARB, and ARG pose serious threat if disposed/discharged untreated. In a developing country like India, wastewater treatment capacity must be increased to treat the waste generated, and advance treatment technologies may be implemented as tertiary treatment option to ensure safe effluent discharge or reuse. Also existing conventional treatment facilities may be retrofitted with advanced tertiary treatment technologies to eliminate antibiotics, ARBs, ARGs, and the like. Alternatively, standard for reuse of reclaimed water for potable and non-potable purpose could be implemented. Quality of such water should be regularly monitored for emerging contaminants like antibiotics, ARB, and ARGs.

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Mitigating Antimicrobial Resistance Risks When Using Reclaimed Municipal Wastewater for Agriculture

Pei-Ying Hong, Changzhi Wang, and David Mantilla-Calderon

Contents

Abstract The global need for food is posing a serious threat to water security. Treated wastewater can be used as an alternative water supply to mitigate our reliance on nonrenewable waters (defined as water that cannot be replenished within our life span). However, concerns related to emerging contaminants such as antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) can impede efforts to push for widespread use of treated wastewater in agricultural irrigation. This chapter aims to provide a better understanding of the potential concerns by first using case studies in two countries that have already practiced water reuse. Second, we collate and analyze data that suggests that wastewater treatment plants able to achieve at least 8-log reduction in microbiological contaminants may suffice as appropriate intervention barriers for ARB dissemination to the environment. This chapter also recognizes that extracellular DNA-carrying ARGs may not be effectively removed even with membrane-based treatment. There is

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therefore a need to assess whether extracellular DNA may accumulate in agricultural soils due to repeated use of treated wastewater and to determine the concentrations of extracellular DNA needed to significantly increase horizontal gene transfer (HGT) in the natural environment. Given the large knowledge gaps that hinder an accurate assessment of the associated risks, it would be prudent to adopt the precautionary principle and to implement appropriate intervention strategies and best management practices that minimize the impacts and concerns arising from the reuse of treated wastewater in agriculture.

Keywords Antibiotic-resistant bacteria, Horizontal gene transfer, Log removal values, Multi-barrier treatment, Membrane bioreactor, Wastewater reuse

1 Introduction

Almost 70% of our global freshwater is used to produce food to feed the seven billion people worldwide [1]. The global population is expected to increase to 9.8 billion by 2050 [2], meaning even more freshwater is needed to produce sufficient food to feed us all. If no alternative sources of water are made available, the long-term consequence will be a depleted water supply.

Clearly, innovative solutions have to be considered to replenish the rapidly depleting fossil freshwaters we rely on (e.g., through aquifer recharge) or to substitute a portion of the nonrenewable water supplies with recycled or desalinated waters. Desalinated seawater, a process whereby saline waters (either coastal seawaters or inland brackish waters) are converted to freshwaters either by means of thermal or membrane-based processes, can be used as an alternative water source. The advantage of doing so is that high-quality water is produced. However, the energy costs associated with desalination typically range from 3 to 4 kWh/m^3 [3]. Considering the amount of water that is used per day for food production (estimated at 57.1 million m^3 for Saudi Arabia) [4], relying on desalinated waters alone to minimize our reliance on freshwater is going to amount to huge economic burden, even in a country such as Saudi Arabia which is the world's largest producer of fossil fuels (and hence has lower energy costs than nonproducing countries).

In contrast, treating wastewater by converting it from dirty water to a level clean enough for reuse takes less energy than desalination. The conventional wastewater treatment process requires about 0.3–1 kWh/m^3 [5–7], which is three to four times lower than the energy required by desalination. The downside is that this practice is commonly associated with a "yuck" factor. Moreover, municipal wastewaters contain a diverse suite of contaminants that are not completely removed by conventional treatment processes [8, 9]. The problem is further compounded when the list of contaminants that are found in our wastewaters becomes increasingly more complex

too. Consumers are becoming more aware of health impacts arising from bacteria and viruses in our daily environment and in our food. This increased awareness in turn drives up demand for antimicrobial products. Retail companies respond to this demand by offering new products that promise antimicrobial effects through the addition of silver nanoparticles, triclosan, quaternary ammonium salts, and so on, just to list a few. A modern individual is also consuming more pharmaceutical compounds with every passing year [10]. Unmetabolized residues of these pharmaceutical compounds as well as metabolites and transformation products of the parent compounds will find their way down the toilet to our sewage. We do not and cannot know the full extent of detrimental impacts on public health during reuse events.

Just on the topic of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in treated wastewater alone, there has been an ongoing debate about whether their presence can potentially impact public health during reuse events. In the absence of robust antimicrobial risk assessment, there is currently no consensus among the various stakeholders (e.g., researchers, regulators, utilities) about whether there is a need to mandate new regulations or invest in state-of-the-art treatment technologies (e.g., membrane bioreactors, advanced oxidation processes) to enhance removal efficiencies of ARB and ARGs from wastewater prior to reuse for non-potable purposes. The reluctance to implement new treatment technologies like membrane bioreactors to existing WWTPs is in part due to the energy and operational costs associated with running membrane bioreactors. An aerobic membrane bioreactor requires about 2 kWh/m^3 [11], which is ca. two times higher than the energy costs needed to operate a conventional treatment process. Furthermore, fouled membranes have to be routinely cleaned or replaced. Similarly, advanced oxidation processes like UV/H_2O_2 or UV/ozone require additional energy for the operation of UV lamps and incur chemical costs that may be more expensive than the conventional chlorine. Under any scenario, both the operational and capital costs associated with either new or upgraded treatment technologies to enhance removal of ARB and ARGs would be significantly higher than before.

The question therefore arises: does the benefit of excessively treating our wastewaters outweigh the costs involved? This is a valid question considering that antimicrobial resistance threats are especially more acute in many developing countries with a lack of available funds to revamp or upgrade existing treatment infrastructure. There has to be a point where investment is made to an optimal level, and not beyond that needed, so that the invested technology safeguards the risk at the acceptable level without overburdening the financial system.

In this chapter, we note that there has been no strong evidence to suggest a significant risk related to antimicrobial resistance threats in countries that have already actively reused good-quality treated wastewater for both potable and non-potable purposes. Treatment technologies are assessed to determine what level of log removal values (LRVs) is needed to investigate whether remnant levels of ARB and ARGs are likely to pose concerns during agricultural irrigation. These include concerns such as the potential for horizontal gene transfer arising from the presence of extracellular DNA. The objective of this chapter is to provide a

pragmatic outlook on how to facilitate safe and sustainable reuse of treated wastewater, thereby reducing our reliance on nonrenewable water supplies.

2 No Direct Evidence of Increased Antimicrobial Resistance Threats in Singapore

The greatest safety and public health concerns arise in relation to drinking treated wastewater. However, it is important to recognize that multi-barrier technologies are put in place by countries (e.g., Singapore) or cities (e.g., Orange County) opting for indirect potable reuse. To exemplify, Singapore has implemented indirect potable reuse of treated wastewater (referred to as NEWater) since 2003. The country treats wastewater through a membrane bioreactor (MBR)-based wastewater treatment plant (WWTP) prior to reverse osmosis (RO)-based filtration at a water reclamation plant. The treated product is termed NEWater and is subsequently blended with surface water stored in catchment reservoirs and retained in the reservoirs for a certain duration that varies depending on local water usage rates. Eventually, this blended water (comprising up to 40% NEWater) [12] then goes through the conventional drinking water treatment, which includes chemical coagulation, filtration, and disinfection, before being distributed to individual households.

Given the relatively high percentage of NEWater in its drinking water supplies, one can in theory track the population over time to determine if consumption of highly treated recycled wastewater is likely to cause an increase in antimicrobial resistance rates among the local community pre- and post-introduction of NEWater, particularly since Singapore is a small country with full nationwide coverage of piped potable waters provided for only by Singapore Public Utilities Board (the main agency in charge of all water-related matters in Singapore). It is unknown if any epidemiological studies were commissioned since the start of NEWater implementation with this research question in mind. However, in response to the 2017 National Strategic Action Plan on Antimicrobial Resistance, Singapore Public Utilities Board funded a number of projects that include monitoring for ARB and ARGs in the local reservoirs. In Singapore, local surface reservoirs usually contain rainwater, storm water, surface water from Malaysia, as well as reclaimed water (NEWater). Despite local reservoirs receiving reclaimed water, the abundance of most ARG-like sequencing reads detected was comparatively lower than that detected in hospital wastewaters or untreated wastewater [13]. Using quinolone resistance gene (i.e., *qnrS2*, which confers resistance through protection of sites targeted by quinolone) as an example, the relative abundance of reads (per Gb of sequencing data) increased more than five times, from an average 1973 reads per Gb of sequencing data in the local reservoir surface waters to approximately 12,000 reads/Gb of sequencing data derived from untreated wastewaters. Instead, the levels in the local reservoir approximate to that sampled from treated effluents (383–637 reads/Gb), suggesting that those relative abundances detected in the local reservoirs

were most likely baseline environmental concentrations [13]. This meant that even though ARGs are detected in local reservoirs (which are used as a source of drinking water), these positive detections may not be directly due to the mixing of treated wastewater, but instead due to background environmental exposure.

These findings were also in agreement with another independent study conducted by Yi et al. Over a 2-year period, the authors performed fluorescence in situ hybridization on water samples collected throughout Singapore and noted that the percentage of macrolide-lincosamide-streptogramin B (MSLB)-resistant bacteria in total bacterial community of reservoirs was consistently lower than that detected in surface waters collected from urban parks and commercial, residential, and industrial zones of Singapore [14]. Their observations, along with the earlier study [13], reiterate that the multi-barrier approaches put in place by Singapore water utilities are able to effectively minimize the dissemination of ARBs and ARGs through indirect potable reuse.

3 No Direct Evidence of Public Health Outbreak Caused by Reclaimed Water

The above case study in Singapore suggests that properly treated wastewater, particularly that which has gone through a multi-barrier treatment train, may not significantly elevate risks associated with ARB and ARGs if reuse is coupled with a proper management and governance system. In the USA, nurseries and vegetables are irrigated with treated wastewater [15]. California is the leading state in reusing 46% of reclaimed water for agricultural irrigation [16]. The state stipulates at least tertiary treatment and disinfection of treated wastewater before irrigation [15]. The guideline further recommends that not more than one sample in any 30-day period should have >23 most probable number (MPN) of total coliforms per 100 mL of reclaimed water applied onto food crops that are consumed raw [17]. Alternatively, if the level of treatment falls below tertiary treatment, more restrictive agronomic practices are required. For example, if only secondary treatment is available, drip irrigation (where water drips slowly from the soil surface/subsurface to roots of plants) instead of spray irrigation must be used, and only for orchards or animal feed production, not for the production of vegetables [18]. In such instances, the guideline still requires no more than 240 CFU per 100 mL of reclaimed water collected within any 30-day period [17].

Based on the abovementioned guideline, to date there have not been any reports of public health outbreaks tied to food irrigated with treated wastewaters in California. Instead, nonpoint contamination of irrigation waters and unsanitary practices have been more of a concern. Using the April–June 2018 outbreak of *E. coli* O157:H7 in romaine lettuce as a case study, 210 people from 36 states in the USA were infected with the outbreak strain. Among them, 27 people developed kidney failure and five deaths were reported [19]. Following this outbreak, the US Food and Drug

Administration (FDA) performed a trace-back analysis to determine a common point of contamination in the distribution chain. Records collected from restaurants and stores where sick people ate or shopped were reviewed. This led to the identification of 23 farms in the Yuma growing region as the common point source supplying the contaminated romaine lettuce across states. Environmental assessments were then carried out at these farms. Specifically, canal waters at these farms were positive for *E. coli* O157:H7 and, through whole genomic sequencing, identified to be of the same strain causing the outbreak [19]. Since the canal water was used to irrigate the romaine lettuce, it was believed that the *E. coli* strain came into contact with the romaine lettuce either during germination or at various times during growing season, including after a freeze event that may have led to damage of some portion of the crop and facilitated internalization of the pathogenic strain. When trying to account for how this outbreak strain was introduced into the irrigation canal, the environmental assessment team noted that a large concentrated animal feeding operation (CAFO) is located adjacent to the irrigation canal. However, they were unable to identify an obvious route for contamination from the CAFO to canal since the same outbreak strain was not identified at the CAFO by whole genome sequencing [20].

In October to December 2018, a new outbreak involving *E. coli* O157:H7, also in romaine lettuce, from Central Coastal growing regions of northern and central California was reported [21]. There were in total 62 reported illnesses, with 25 hospitalizations and two cases of kidney failure. Using the same approach as illustrated earlier, sediment samples from an on-farm water reservoir tested positive for the outbreak strain in this instance and were likely transferred to the irrigation water when conveyor pumps were activated and perturbed the sediments. However, the reservoir was not connected to other water sources or distribution systems in the growing region, and hence no apparent source of contamination could be identified. In addition, the farm did not have an optimal disinfection system in place for the agricultural water [22].

These two recent outbreaks suggest that (1) open-air surface waters are particularly vulnerable to contamination, (2) it is not easy to pinpoint the exact source of contamination and implement remediation measures, (3) farmers may not be well-trained to carry out appropriate treatment and disinfection of irrigation waters on-site, and (4) both outbreaks were not associated with reusing treated wastewater. This suggests that a safe reuse of treated wastewater is feasible, and perhaps safer than the current practice of using traditional sources of irrigation waters, as long as the appropriate intervention strategies and policies are put in place.

4 Wastewater Treatment Processes Are Important Barriers

A recent multivariate regression analysis using datasets obtained from 73 countries reported that better sanitation and governance were associated with lower antimicrobial resistance indices [23]. This reiterates the absolute need for proper treatment of wastewater prior to reusing the water in an open environment.

Treated wastewater is currently reused in three main ways, namely, managed aquifer recharge, agricultural irrigation, and indirect potable reuse. At the time of writing, there has been no full-scale practice of direct potable reuse although this may happen in the future. Existing regulations pertaining to the treated water quality do not explicitly list the permissible level of ARB or ARGs for each type of reuse activity. Instead, recommendations are based on coliform counts, biochemical oxygen demand (BOD)/chemical oxygen demand (COD) reduction, total nitrogen and total phosphate concentrations, and other parameters related to the chemical constituents of these waters [24–26]. Table 1 lists some of the reuse water quality limits recommended by the Ministry of Environment, Water and Agriculture in Saudi Arabia, US EPA, and International Organization for Standardization (ISO). The list is not exhaustive, and readers can refer to the references for a more complete list.

Given that the permissible concentrations of contaminants can vary depending on the type of reuse purpose, an appropriate treatment train therefore needs to be devised accordingly. This is otherwise termed as fit for purpose. In most instances, it is recommended that wastewater goes through at least secondary treatment if it is intended for aquifer recharge and/or agricultural irrigation [27, 28]. In addition, ISO guidelines also suggest that in order to achieve the restricted irrigation (defined as irrigation in places where public access is controlled or restricted by physical or institutional barriers), a potential corresponding treatment should include activated sludge wastewater treatment process, sand filtration, and disinfection [26]. An earlier study reported that a conventional secondary treatment process in Saudi Arabia, which includes a primary clarifier, an activated sludge tank, a secondary clarifier, and a final disinfection (Fig. 1a), was able to achieve $\leq$1,000 CFU/100 mL of fecal coliforms and hence can be used for restricted irrigation [29].

Collating results from other studies, the anticipated log removal rates for total bacteria or total suspended solids are ca. 1-log for both clarifiers (i.e., 70% removal for primary and secondary clarifier, respectively) [30], 1- to 2-log by the activated sludge tanks depending on the operating conditions [31–34], about 2-log removal (i.e., 99% removal) for slow sand filtration [35], and an additional >3-log (i.e., 99.9% removal) if chlorination and ozonation, the two most commonly used disinfection processes, are effectively carried out [29, 32, 36] (Fig. 1a). If membrane filtration, typically a microfiltration or ultrafiltration membrane, is also retrofitted to the wastewater treatment plant, ca. average 5-log removal of microbiological contaminants is routinely reported [37, 38] (Fig. 1b). However, because different WWTPs use different treatment trains and different operational parameters that may affect the overall efficiency of each individual treatment unit, log removal values can potentially vary over a wide spectrum [31].

Table 1 Recommended contaminant limits in reclaimed water intended for different reuse purposes

Reference	*Regulatory agency*	*Parameter*	*Restricted irrigation*[a]	*Unrestricted irrigation*[b]	
[25]	Saudi Arabia Ministry of Environment, Water and Agriculture	Fecal coliforms (CFU/100 mL)	≤1,000	≤2.2	
		Biochemical oxygen demand, BOD (mg/L)	40	10	
		Total suspended solids, TSS (mg/L)	40	10	
		Turbidity (NTU)	5	5	
Reference	*Regulatory agency*	*Parameter*	*Landscape irrigation, toilet flushing*	*Surface or spray irrigation of any food crops*	*Surface irrigation of orchards and vineyards*
[24]	US EPA	Fecal coliforms	No detectable per 100 mL	No detectable per 100 mL	≤200
		Biochemical oxygen demand, BOD (mg/L)	≤10	≤10	≤30
		Total suspended solids, TSS (mg/L)	Not specified	Not specified	≤30
		Turbidity (NTU)	≤2	≤2	Not specified
Reference	*Regulatory agency*	*Parameter*	*Restricted irrigation and agricultural irrigation of processed food crops*	*Unrestricted irrigation and agricultural irrigation of food crops consumed raw*	*Agricultural irrigation of nonfood crops*
[26]	ISO	Fecal coliforms (CFU/100 mL)	≤200	≤10	≤1,000
		Biochemical oxygen demand, BOD (mg/L)	≤10	≤5	≤20
		Total suspended solids, TSS (mg/L)	≤10	≤5	≤30
		Turbidity (NTU)	Not specified	≤2	Not specified

[a]Restricted irrigation refers to irrigation where public access is controlled or restricted by physical or institutional barriers
[b]Unrestricted irrigation refers to irrigation in settings where public access is not restricted

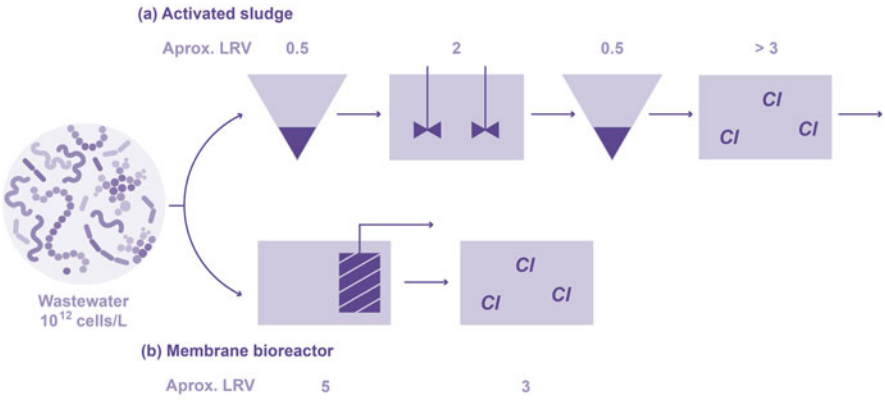

Fig. 1 An illustration of the wastewater treatment processes and the approximate log removal values (LRVs) of total microbiological contaminants reported at each stage. Treatment process can include the use of (**a**) primary clarifier, activated sludge, secondary clarifier, and disinfection or (**b**) membrane bioreactor prior to disinfection

5 Potential Impacts of Treated Wastewater Irrigation on the Indigenous Soil Microbial Community

In the context of this chapter, we argue that most of the treated wastewater should be reused for agricultural irrigation since this is the sector that uses the most water. Hence, emphasis was made to evaluate if ARB in treated wastewater would complicate public health concerns during agricultural irrigation. A similar assessment has been done by Pepper et al., in which they evaluated the abundance of ARBs that were reported by past studies, and compared against that reported in the baseline soil microorganisms [39]. The authors noted that applying conventionally treated effluent (i.e., with no MBR) on agricultural lands during a single application event resulted in ca. 0.001% increment in ARB compared to the baseline ARB concentrations in soils. This increment is lower than the percentage increase arising from single application of biosolids (0.1%) and manure (0.05%) application [39]. A further comparison between treated wastewater-irrigated and freshwater-irrigated soils revealed identical levels in both ARB levels after irrigation [40] and supports the calculations made by Pepper et al [39]. It is therefore likely that effluent or manure-associated ARB would not be able to compete successfully against the resident soil bacteria [40, 41] and would attenuate to baseline levels in between irrigation events.

To further investigate the potential for the accumulation of total bacterial cells in irrigated soils due to long-term irrigation, we took the average cell counts (ca. 10^{11} cells per L) sampled after the primary clarifier and determined by flow cytometry [38] and the known LRVs reported by each stage of the WWTP discussed earlier and estimated that the total cells found in the final reclaimed water would range between 10^4 and 10^6 cells per L, depending on the treatment process (Fig. 1). We assume that

the WWTP provides 4,000 m^3 of treated wastewater per day, therefore discharging in a worst-case scenario 4×10^{12} cells/day into the agricultural soils if all of the reclaimed water were to be used for irrigation. Assuming a gross irrigation depth of 70 mm over 0.01 km^2 irrigated land, this volume of wastewater is sufficient to irrigate over 0.06 km^2 land mass. Using an average soil bacterial cell density of 10^9 cells per g [39], this would equate to 753.9 cells per mm^2 of land [42], and therefore 4.5×10^{13} cells would be exposed to the treated wastewater (4×10^{12} cells/day) (Fig. 2). This abundance of indigenous soil bacterial counts is 1-log higher than that from the treated wastewater, and a single irrigation of the treated wastewater would therefore unlikely to result in a significant increment in cell mass balance.

While a single irrigation event with treated wastewater would be unlikely to result in a significant increment in cell counts, in practice, irrigation is routinely carried out throughout the growing season, with specific irrigation schedules varying depending on the type of crop, stage of crop growth, weather, and soil characteristics. Assuming that irrigation is done once every 10 days in Saudi Arabia for crops like lettuce, cucumbers, and peppers, and with an assumed bacterial decay of 5-log due to solar irradiation [43, 44] in between consecutive irrigation events, the contributed amount of bacteria by conventionally treated wastewater would still be approximately on the same magnitude of abundance than that in the indigenous soil microbiota after 1 month of using treated wastewater (Fig. 2). However, the contributed portion may be comprised of mainly ARB since an earlier study found that the fraction of ARB normalized to the total culturable bacteria increased after the WWTP than in the untreated wastewater [29] and that a pathogenic and antibiotic-resistant strain of *E. coli* survived harsh environmental conditions better than a commensal *E. coli* strain [44].

In contrast, the amount of bacteria in MBR-treated effluent is 3-log lower than that in the baseline soil. Assuming the same irrigation frequencies and decay rates, it would not be possible to accumulate to levels higher than the indigenous bacteria already present in the soil within the entire growing season (Fig. 2). These calculations assume that an agricultural site uses undiluted stream of treated wastewater on-site. However, in most agricultural sites, the demand for irrigation water is substantially higher than the volume provided for by treated wastewater alone. For example, as already mentioned, an estimated 57.1 million m^3 of water per day is needed for agricultural irrigation in Saudi Arabia [4]. A full capture of all wastewater in the kingdom would generate only 8.7 million m^3 (based on an average 260 L of water used per day per capita in 2017 that will be discharged back into sewers as waste by the current 33.4 million population [45]) of treated municipal wastewater, which is not sufficient to meet the demand for agricultural irrigation. Hence, dilution of treated wastewater with groundwater or desalinated water is most likely needed, and this dilution would further lessen the contribution of bacteria from treated wastewater into the natural soils.

Although MBR-based treatment can lower the contribution of bacterial load from treated wastewater into natural soils, one still needs to consider the possibility of conjugation between the remnant bacteria in the reclaimed water and the indigenous soil microorganisms. This concern stems from a number of recent studies that

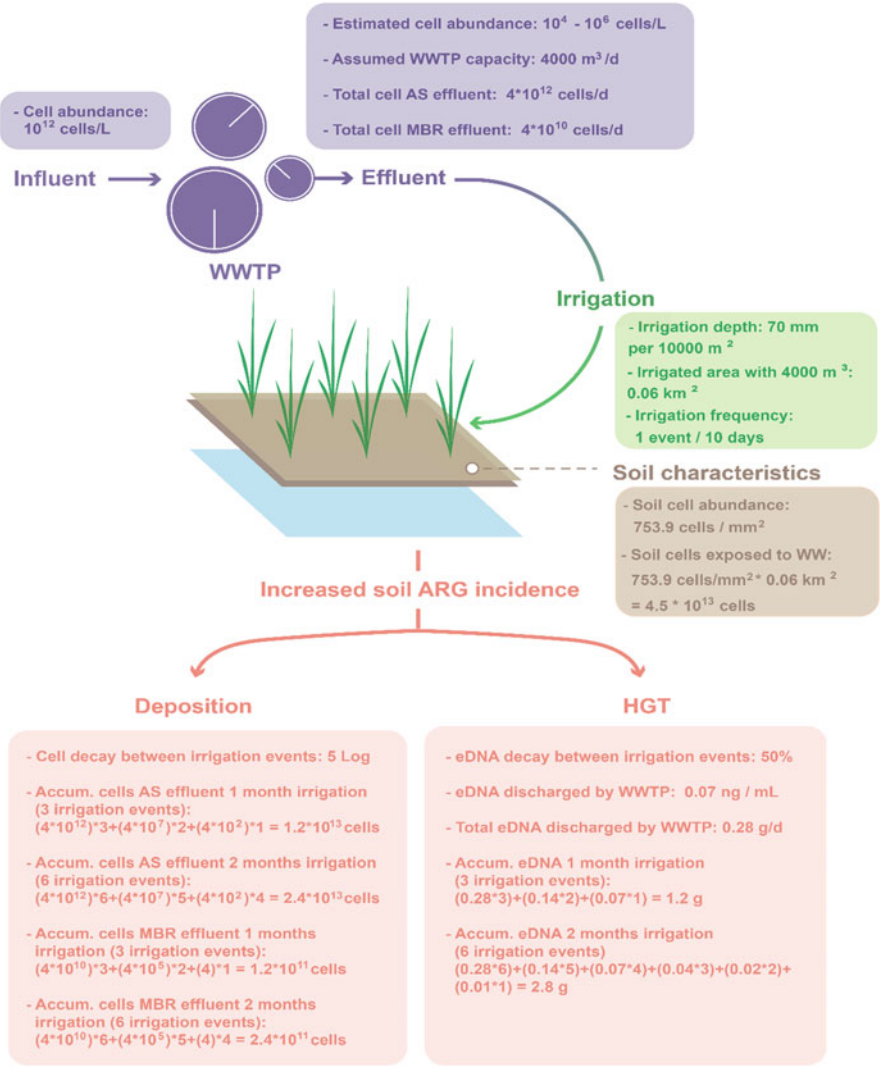

Fig. 2 An estimation of the amount of bacterial cells and extracellular DNA (eDNA) that would be contributed by the effluent treated by either activated sludge (AS) processes or by membrane bioreactor (MBR). The estimation is made given an assumed irrigation scenario, soil characteristics, and decay rates

showed in vitro chemical pollutants prevalent in the wastewater stream (e.g., carbamazepine, triclosan, copper nanoparticles, or copper ions) can trigger intracellular reactive oxygen species (ROS) production and enhance cell membrane permeability [46–48]. This in turn resulted in a higher conjugation rates between *E. coli* strains or between *E. coli* and species of other genera (e.g., *Pseudomonas putida* and *Salmonella enterica* serovar Typhimurium) compared to the baseline spontaneous

Table 2 Collation of studies performed to assess (a) conjugation frequencies and (b) transformation frequencies in the absence and presence of stressors

(a)

Conjugation studies [Ref.]	*Tested donor*	*Tested recipient*	*Donor/recipient ratio (cell density)*	*Test conditions*	*Detected frequency*
Zhang et al. [49]	*E. coli* S17–1	*E. coli* K12	1.5:1 (10^8 CFU/mL)	No stressor (baseline) PBS 4 h reaction 25°C	10^{-5}
				Free chlorine (0.1–1 mg/L) PBS 4 h reaction 25°C	Concentration-dependent increase (three- to sixfold higher than baseline)
				H_2O_2 (0.24–3 mg/L) PBS 4 h reaction 25°C	Concentration-dependent increase (two- to sixfold higher than baseline)
Wang et al. [47]	*E. coli* K12 LE392	*E. coli* K12 MG1655 mutant	1:1 (10^8 CFU/mL)	No stressor (baseline) PBS 8 h 25°C	6.85×10^{-5}
				Carbamazepine (0.05–50 mg/L)	Concentration-dependent increase (4- to 12-fold higher than baseline)
	E. coli K12 LE392	*Pseudomonas putida* KT2440	1:1 (10^8 CFU/mL)	No stressor (baseline) PBS 8 h 25°C	2.67×10^{-6}
				Carbamazepine (0.05 to 50 mg/L)	Concentration-dependent increase (five- to eightfold higher than baseline)
Lu et al. [46]	*E. coli* K12 LE392	*E. coli* K12 MG1655 mutant	1:1 (10^8 CFU/mL)	No stressor (baseline) PBS 8 h 25°C	3.13×10^{-5}
				Triclosan (0.02–2 μg/L)	Concentration-dependent increase (two- to sixfold higher than baseline)
	E. coli K12 LE392	*Pseudomonas putida* KT2440	1:1 (10^8 CFU/mL)	No stressor (baseline) PBS 8 h 25°C	4.91×10^{-6}
				Triclosan (0.02–2 μg/L)	Concentration-dependent increase (1.8- to 2.2-fold higher than baseline)

Zhang et al. [48]	*E. coli* K12 LE392	*Pseudomonas putida* KT2440	1:1 (10^8 CFU/mL)	No stressor (baseline) PBS 24 h 25°C	1.8×10^{-6}
				Copper (0.5–1 μmol/L)	Concentration-dependent increase (2- to 2.2-fold higher than baseline)

(b)

Transformation studies [Ref]	*Tested recipient*	*Cell: DNA ratio (CFU: μg)*	*Test conditions*	*Detected frequency*
Mantilla-Calderon et al. [50]	*Acinetobacter baylyi* ADP1	2×10^8: 2	No stressor (baseline) 24 h 37°C	1.9×10^{-6}
			Bromoacetic acid (100–600 μM)	Concentration-dependent increase (1.1- to 1.8-fold higher than baseline)
			Disinfection by-product cocktail containing trichloromethane (1.4 μM), bromoacetic acid (1.9 μM), formaldehyde (3.8 μM), *N*-nitrosodimethylamine (0.7 nM), tribromoacetamide (0.03 μM)	1.7-fold higher than baseline
Ausburger et al. [51]			No stressor (baseline) 24 h 37°C	3.4×10^{-6}
			Sunlight (70–150 mJ//cm^2)	Concentration-dependent increase (1.5- to 2-fold higher than baseline)

conjugation rates of 10^{-5} (Table 2a). In addition, sublethal concentrations of chlorine and hydrogen peroxide, both of which are disinfectants used in conventional WWTPs, were also able to independently promote conjugation within and across bacterial genera at sublethal concentrations [49]. However, these conjugation experiments were performed in oligotrophic buffer solution matrix and with a ratio of 1 to 1 of donor to recipient bacterial cells, each in cell density of 10^8 CFU/mL (Table 2a), and are not conditions simulating that in the natural environments. Therefore, further studies should look into replicating the same experiments in conditions more similar to that in the natural environment so as to determine whether enhanced conjugation would indeed happen between the microorganisms in soil and reclaimed water.

6 Multi-barrier Intervention Strategies for Low-Resource Countries

Based on the literature gathered for this study, we are leaning toward the opinion that the risks arising from ARB in reclaimed water used for agricultural irrigation may be sufficiently mitigated if (1) the effluent is derived from a treatment process that is cleaning municipal wastewaters containing approximately 10^{12} cells/L and provides 8-log or more reduction in total bacterial cell count or (2) appropriate agronomic practices are put in place (e.g., dilution of treated wastewater, drip irrigation, and non-leafy crops).

Although the general scientific evidence supports the conclusion that MBRs generate treated effluent that would result in low risks during agricultural irrigation, it is important to remember that countries that rely heavily on agriculture for their gross domestic product also include low-resource countries that have less monetary capacity to invest in their sanitary infrastructure [52]. Therefore, instead of trying to push for advanced state-of-the-art technologies (e.g., MBR or RO) which incur high operational and capital investments and are out of reach for these countries, the question to ask is whether low-cost treatment strategies, possibly through a multi-barrier approach, would be equally effective to mitigate risks associated with reuse.

Examples of low-cost multi-barrier approach could be coagulation, flocculation, and sedimentation (typically achieves 1-log reduction [53]), followed by retention of supernatant in evaporation ponds or wetlands (typically achieves 1- to 2-log reduction [54, 55]), and then surface spreading for infiltration of the water through sand gravel layers prior to storage in underground aquifers (typically achieves 1-log reduction for every 8.5 m of infiltration [53, 56]). The stored water can then be pumped up from the aquifer and disinfected (typically achieves 3-log reduction) before using for agricultural irrigation. In such instances, the foremost criteria would be abundance of land and long retention time for natural attenuation to occur effectively. The aim would be to achieve a comparable 8-log reduction in total with this multi-barrier approach using resources that are abundant, low cost, or free.

Feasibility assessment of low-cost multi-barrier approaches would need to be performed – first, by determining the LRV at each stage of the implemented barrier and, second, by evaluating the energy and operational costs associated with the entire treatment train. For successful implementation, different stakeholders (e.g., engineers, regulators, researchers, utility operators, end users) should work together to form a better picture of the best estimated extent of risks involved in using treated wastewater. For example, researchers can lead with regular monitoring of their treatment technologies and in evaluating water for new emerging contaminants. They could also work with engineers to design or implement improved versions of treatment technologies in instances where the existing treatment technologies do not provide sufficient barriers. Relevant utilities can then make use of the data derived by scientists and engineers to design best management practices for farmers and to protect public health.

7 ARGs: Living in the Cloud of Uncertainties

Although the LRVs of bacteria achieved by each individual stage along the WWTP are generally well studied, LRV of extracellular DNA by the same treatment process is not as well characterized. In an earlier study, a lab-scale MBR-based reactor only achieved ca. 2- to 4-log reduction of extracellular DNA [57], suggesting that the final reclaimed water still contains these potential contaminants. Indeed, the presence of extracellular DNA is not unique to treated wastewater; it has been reported to be present in a median concentration of about 0.07 ng/L in Singapore's drinking water [58].

Since there are currently no similar studies performed in estimating the amount of extracellular DNA present in the final reclaimed water, we assume all reclaimed water should have gone through a treatment that would achieve 0.07 ng extracellular DNA per liter of water since it approximates to that present in clean drinking water in Singapore [58]. Subsequently, we used this value to estimate the amount of extracellular DNA that can be disseminated into the environment if the reclaimed water were to be reused. Based on the same parameters used in earlier calculations for ARB, we calculated that approximately 0.3 g of extracellular DNA is disseminated into the environment each day through reuse of reclaimed water based on the assumed irrigation frequencies, land area, etc. stated in Fig. 2.

Earlier studies have found that extracellular DNA can persist in soil environments for longer period of time than newly introduced bacterial cells. For example, DNA introduced by agricultural irrigation and/or manure application could still be detected by PCR-based methods after as many as 16 months [59–61]. The degradation rates of DNA in the soil are well studied, but multiple factors are known to affect the decay rates [62]. A general degradation rate of DNA in soils cannot easily be determined due to contradictory results reported by various studies. Therefore, assuming 50% of extracellular DNA decays by the time of next irrigation event, ca. 1.2 g of DNA would be accumulated by the end of 1 month and 2.8 g of DNA by

the end of 2 months (Fig. 2). Concerns may arise if this extracellular DNA is taken up by naturally competent bacteria cells present in the recipient environment and subsequently integrated into the genome to be expressed into problematic gene products (e.g., virulence traits, antibiotic resistance). However, it is also difficult to know how much of these DNA would contain such problematic gene products, reiterating that the true extent of risks from extracellular DNA is difficult to ascertain.

Recent studies using *Acinetobacter baylyi* ADP1 as model bacterium demonstrated a baseline transformation frequency of about 10^{-6} when extracellular DNA was introduced to the competent bacterium in DNA to cell ratio of 1 μg to 10^{8} cells [50, 51] (Table 2b). Furthermore, it was observed that in lab studies, when these extracellular DNA are present along with sublethal concentrations of disinfection by-products (DBPs) and solar irradiation, transformation frequencies are increased in a concentration-dependent manner by up to twofold [50, 51]. The increase in transformation frequencies was found to be due to the mutagenic but nonlethal effect caused by DBPs and sunlight. In both instances, there was an upregulation of *recA* gene expression, while the upregulation of competency-related genes was only observed in the presence of sunlight. In bacteria, *recA* plays a shared role in both the repair of DNA double-strand breaks [63] and the chromosomal integration of foreign DNA during natural transformation [64]. The increase in transformation frequencies observed in both DBPs and sunlight was therefore likely to be due to increased integration of extracellular DNA into *A. baylyi* ADP1 as the bacteria seeks to repair damage induced by both stressors.

Although there was a concentration-dependent increment in transformation frequencies in the presence of stressors introduced during the treatment or in the natural environment, the fold increment observed in these studies was at most twofold [50, 51]. This would mean that the transformation frequency remains in the same magnitude as that of baseline – 10^{-6}. It remains unknown if multiple stressors, for example, combinations of DBPs and sunlight, would impose an additive or antagonistic effect on transformation frequencies, and if so, whether it would significantly increase or decrease the hazards arising from horizontal gene transfer. There remains a knowledge gap as to whether this extracellular DNA is truly of concern and should constitute a new class of emerging contaminants, e.g., when disseminated into the environment through water reuse.

8 More Questions to Be Addressed

This review only focuses on antibiotic-resistant bacteria and antibiotic resistance genes and argues that the risks arising from them would be low if sufficient intervention measures are put in place. However, these are not the only contaminants present in the treated wastewater matrix. Currently, we can never presume to have tested all of the individual contaminants that may potentially be of concern. However, what we do know now is that pharmaceutical compounds and other chemical

contaminants (e.g., triclosan, heavy metals) are also present alongside microbiological contaminants in the reclaimed water [65–67]. As such, many burning questions remain. Have we evaluated the treatment technologies to determine the log removal values of microbiological and chemical contaminants that are of concern? Are there potential additive or synergistic effects when multiple contaminants of emerging concerns and environmental factors come into confluence, such that the associated risks would be significantly elevated? Or are the interactions going to be antagonistic such that risks would be minimally low? To further compound the problem, climate change is predicted to make it even more challenging to ensure consistently good water quality for potable and reuse purposes. For example, changing rainfall patterns may mean variability in water resources [68] – drought in certain places or extreme rainfall events in other places would mean that existing wastewater treatment plants that are typically designed within a certain safety factor might have to operate under suboptimal conditions. This can potentially lead to partially treated wastewater being released for reuse. How can we then ensure a continuous delivery of good water quality?

Answering these questions will not be straightforward considering that it is technically and logistically challenging to monitor every single contaminant present in the wastewater. Rapid, high-throughput, non-target-based monitoring methods will be needed. Some of these methods include metagenomics for identifying the microbial and functional gene diversity, high-throughput toxicity screening to determine whether treatment processes are able to reduce toxicity levels, and high-resolution mass spectrometry to identify various compounds in wastewater simultaneously. Besides expanding our monitoring capacity, detecting mere presence and/or absence of contaminants will not be beneficial without quantifying the risks. However, the risk models related to antibiotic-resistant bacteria and resistance genes or many of the emerging contaminants are not yet established [69], and gathering information related to dose response or probability of transmission will require animal models to start with. Clearly, a multidisciplinary approach involving chemists, biologists, toxicologists, microbiologists, and experts in other related fields will be needed to address these pressing issues.

In the meantime, it is not practical to steer away entirely from reusing treated wastewater just to avoid any potentially unfounded detrimental consequences, especially when water scarcity is a real and growing global concern. Instead, we recommend adopting the precautionary principle and implementing appropriate intervention strategies and best management practices to minimize the impacts and concerns arising from the reuse of treated wastewater in agriculture.

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Antibiotic Resistance in Soil

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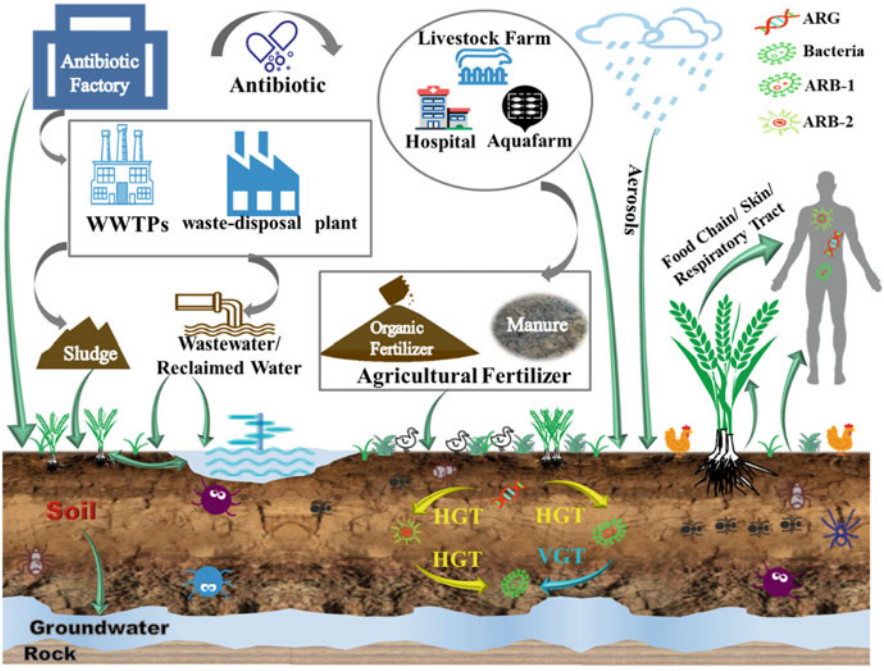
Abstract Exposure to antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) from natural and agricultural ecosystems such as soil can significantly affect the dissemination of resistance determinants to the human microbiome. Soil contains a diverse natural resistome and also serves as an important environmental reservoir for ARB and ARGs derived from water sources, aerosols, and sewage sludge. Soil microbiomes have been impacted worldwide by the use and overuse of antibiotics for anthropogenic activities (clinical use and livestock production) and agricultural practices (manure application and irrigation with wastewater). The dynamics and persistence of ARB and ARGs in soil are affected by soil management and environmental factors. Both abiotic and biotic factors (pH, temperature, organic matter, nutrient availability, and syntrophic, competing or antagonistic organisms) can act as driving forces for ARG fate, evolution, and horizontal gene transfer processes. Meanwhile, ARGs in soil may also be transferred to other environments, such as groundwater and the phytosphere. To tackle the potential threat of ARGs, treatment measures (aerobic composting, anaerobic digestion, and disinfection) have been evaluated to reduce the selective pressure and import of ARGs into soil. Furthermore, the "One Health" approach was put forward to manage the development and dissemination of ARGs in a cross-disciplinary manner, to more holistically reduce human risk to the lowest level.

Graphical Abstract



Keywords Antibiotics, Antibiotic resistance gene, One Health, Persistence, Resistome, Soil, Transmission

1 Introduction

Nowadays, millions of tons of antibiotics are produced each year, and veterinary antibiotics are largely used to promote the growth of animals globally [1, 2]. Worldwide overuse and abuse of antibiotics in human health and livestock production over the last few decades have greatly contributed to the propagation of antibiotic resistance [3].

Antibiotics and antibiotic resistance genes (ARGs) coexist naturally in the environment. The former are universal and multifarious, while the latter can be mobile and transferable [4]. ARGs may not only be transferred via environmental media, they may also be transmitted between parents and offspring or among different species of bacteria by vertical and horizontal gene transfer (HGT), respectively. Bacteria without ARGs may acquire antibiotic resistance by taking up naked DNA released into the environment after the death of microbes carrying ARGs, as extracellular DNA can exist for a long time under the protection of soil minerals and organic colloids [5].

ARGs have been widely identified in diverse environments, including livestock production systems, wastewater and sludge, atmosphere, and soil [6–9]. Soil undoubtedly contains the most diverse and richest DNA sequences and microorganisms [10], which significantly affect the emergence and propagation of antibiotic resistance, mainly facilitated through mobile genetic elements (MGEs) and selected mutations of existing genes exerted by antibiotics and even heavy metals [11]. The prevalence of resistance determinants is highly related to antibiotic-resistant bacteria (ARB) and ARGs in various environments [12, 13]. Widespread antibiotic resistance is identified as the top of the six emerging environmental issues and global challenges humans face in this century [14].

Antibiotic resistance has been described as the quintessential "One Health" issue [15], which requires an interdisciplinary vision, coordinated study and action across three main domains: human health, animal health, and the environment. Soil is a crucial component in the One Health approach since it not only harbors a diverse natural resistome but also receives ARB and ARGs from both human waste and livestock manures which can be further transferred to humans and animals through multiple pathways, including the production and use of vegetable and animal products, the water cycle, and aerosols [1].

2 ARG Distribution in Soil

2.1 Natural ARGs in Soil

In the early twentieth century, it is confirmed that ARGs disseminated long before the antibiotic era was proposed and claimed in many studies [16, 17]. For instance, there is a high diversity of ARGs conferring resistance to β-lactams, tetracyclines,

and glycopeptides, in the DNA from Late Pleistocene organisms in Arctic soil that has been frozen for 30,000 years [17]. A variety of novel β-lactamase genes and chloramphenicol resistance genes were found in frozen Alaskan soils that were largely unaffected by human activities, and the bifunctional β-lactamase gene was also first discovered in this environment [18]. Bacterial strains isolated from sediments below the land surface at the US Department of Energy's Hanford Site in Washington state were far away from sources of anthropogenic antibiotics, but still showed a relatively high frequency of resistance [16]. Microbes obtained from the Lechuguilla Cave in New Mexico, which had been isolated for 4 million years, also showed high resistance to antibiotics in nature, with some strains even tolerating up to 14 different commercially available antibiotics [19]. *Firmicutes*, *Arthrobacter*, *Bacteroidetes*, *γ-proteobacteria*, and *α-proteobacteria* from nine sites of Eastern Siberia permafrost sediments buried and frozen 15,000–3,000,000 years ago showed resistance to aminoglycoside, tetracycline and chloramphenicol antibiotics [20].

Thirteen subtypes of ARGs and MGEs were identified in soil, animal waste, and deposits that were unaffected by human use of antibiotics in Tibet, China, where *bacA, mexB, mexF*, and *mexW* were dominant over other subtypes. Eight major ARG categories consisting of 73 ARGs and MGEs (integrons, transposons, plasmids, and gene cassettes) were detected at the Gondwana Research Station and the new Jang Bogo Research Station in Antarctica, less affected by humans [21]. These studies confirm that ARGs existed in soil long before antibiotics were in widespread use by humans.

2.2 ARGs in Soil with Human Activity

Although there is plenty of evidence that some ARGs occur naturally, new ARB from humans and animals can potentially enter the soil environment and become important environmental contaminants [22]. Notably, a specific subset of clinically relevant ARGs is becoming enriched in the environment [23]. Therefore, the spread of ARGs in diverse environments is mainly due to increasing selection pressure from continuous anthropogenic usage of antibiotics [24].

Intensive animal husbandry has greatly increased the use of antibiotics and stimulated the development and spread of ARGs in agroecosystems [25]. Livestock production practices using antibiotics and the subsequent application of organic fertilizers to land can induce ARG expression in bacteria and/or mutation to produce new ARGs [26]. All major classes of antibiotics except vancomycin were found in manure-amended farm soils from three Chinese provinces (Beijing, Zhejiang, Fujian) after the use of in-feed and therapeutic antibiotics in swine production [25]. Compared with antibiotic-free manure or soil controls, the top 63 ARGs of 149 unique resistance genes, detected in these large-scale swine farm samples via high-capacity quantitative PCR arrays, are enriched up to 28,000-fold [25]. In addition, leachate from sewage and landfills increases the abundance and diversity of ARGs and bacteria in the soil [27]. One study found that as the composting time in

landfill increased, the abundance of target genes *sul1* and *tetO* in solid waste decreased, whereas the abundance of target genes in leachate increased [27]. Therefore, antibiotic residues and ARGs in landfills represent a potential risk to the environment.

In general, the external input has a profound effect on ARG pollution. Moreover, the soil bacteria commonly vary in different locations, and they respond to the environment differently; this is closely related to effects of human activities in those different regions [13]. Table 1 lists the types and abundance of ARB and ARGs in soils from different countries and regions.

3 Anthropogenic Sources of ARGs in Soil

3.1 ARGs from Manure

3.1.1 Manure Production and Antibiotic Use in Animal Production

Livestock feedlots from industrial farms (concentrated animal feeding operations, CAFOs) are the major source of animal manure worldwide, especially in developed countries [37]. In the USA, nearly 500 million pounds of animal feces are produced per year, with industrial farms generating approximately 300 million pounds, 90% of which is periodically disposed of by application as organic fertilizer for agriculture [38]. In China, over 80% of the manures produced in industrial farms are used on agricultural fields [39]. Household farms serve as another crucial source of manure. About 23% and 50% of the manures from integrated livestock farms and individual household farms, respectively, were first composted before field application in China [39]. The proportion of the manure from industrial farms is likely to increase as livestock husbandry transits to more integrated systems, giving rise to greater utilization of antibiotics in livestock production for disease prevention and animal growth enhancement because the administration of antibiotic usage lags behind [40]. Commensal microbiota and pathogenic microorganisms can exchange genes and spread in high-density farms during sub-therapeutic administration of antimicrobials. It is urgent to eliminate and reduce the use of nontherapeutic and sub-therapeutic administration of antimicrobials in poultry production [41]. Information on antibiotic utilization is important for evaluating their effects on the environment. Therefore, it is necessary to carry out market surveys to estimate the production of antibiotics and their utilization in animal and human medicines.

3.1.2 Effect of Manure Application on Soil Resistome

The overuse of antibiotics in livestock increases the risk of transmission of ARGs and MGEs from manures to soil environments [42, 43]. Highly diverse ARGs conferring resistance to tetracyclines, β-lactams, aminoglycosides, macrolides,

Table 1 Distribution of ARGs and ARB in soils and sediments

Country/ region	Agrotype	External source import	Type of ARGs or ARB	Absolute abundance (copies/g)	References
Distribution of ARGs and ARB in soils unaffected by human					
30,000-year-old Beringian	Permafrost sediments	Nature	Genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics	–	[17]
Alaskan	Unpolluted soil	Nature	β-lactam-resistant bacteria	–	[18]
South Carolina and Washington	Sediments	Nature	Resistance to nalidixic acid, mupirocin, or ampicillin	–	[16]
New Mexico	Soil	Nature	Resistance for macrolide antibiotics	–	[19]
Antarctica	Soil	Terra Nova Bay	73 ARGs and MGEs	–	[21]
Arctic permafrost	Sediments	Subsoil	Aminoglycoside-, chloramphenicol-, and tetracycline-resistant bacteria	–	[20]
Distribution of ARGs and ARB in soils affected by human					
China	Ryland (peanut) and paddy (rice) fields	Manure	*folP*, *sul1*, *sul2*, *sul3*, *ermB*, *mexF*, *oprJ*, *tetPA*, *acrA_5*, etc.	–	[9]
America	Farm soil	Cattle farms	bla_{CMY-2}, *tetB*, *tetC*, *tetO*, *tetW*, and *β-macrolide* resistance genes	–	[28]
America	Channel soil	Agricultural activity and sewage treatment plant discharge lake	*sul1*, *sul2*, *tetO*, *tetW*	–	[29]
China	Farm soil	Pig breeding farm	FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes	–	[25]
Denmark	Farm soil	Pig breeding farm	*tetA*, *tetB*, *tetC*, and other tetracycline resistance genes		[30]
Canada	Farm soil	Cow breeding farm	*ermA*, *ermB*, *ermF*, *qnrB*, *sul2*, *tetT*	10^4–10^5	[31]

(continued)

Table 1 (continued)

Country/ region	Agrotype	External source import	Type of ARGs or ARB	Absolute abundance (copies/g)	References
Israel	Farmland soil	Wastewater irrigation	*qnrA*, *tetO*, *sul1*, *sul2*, *ermB*, *ermF*	–	[32]
Poland	Farmland soil	Manure and plant compost	*tetO*, *tetB*, *tetD*, *tetT*, *tetW*, *ermC*, *ermV*, *strA*, *strB*, etc.	1.3×10^5–4.1×10^5	[33]
Switzerland	Organic soil	Pig manure	*tetC*, *tetH, tetQ*, *tetW*, *tetT*, etc.	4.63×10^5–3.74×10^6	[34]
Beijing and Tianjin, China	Farmland soil	Wastewater irrigated soil	*tetA*, *tetB*, *tetC*, *tetD*, *tetM*, *tetO*, *sul1*, *sul2*, *sul3*, etc.	1.9×10^3–5.6×10^7	[35]
Finland	Dairy farms and swine farms	Fresh manure from inside the animal shelter	Genes encoding resistance to aminoglycosides, disinfectants, MLSB, tetracyclines, sulfonamide, trimethoprim, and vancomycin	–	[36]

chloramphenicol, sulfonamides, and multiple drugs exist in manures and manure composts [44]. The highest relative abundance of ARGs (relative to 16S rRNA gene) measured in manures ranged from 10^{-3} to 10^{-1} and 10^{-2} to 10^{-1} in Chinese and Finnish farms, respectively [2]. The transmission of ARGs from manure into soil might also be facilitated when ARGs co-occur with MGEs [45]. Many ARGs in manures are carried on plasmids or integrons [46, 47], implying a high risk of transmission from the manure into the soil ecosystem.

Utilization of manures or manure composts may increase the types and abundance of ARGs in soil via import of manure-borne ARGs and antibiotic residues [25]. After application of manure or compost, the relative abundance of some ARGs may decrease directly in soil, whereas the relative abundance of others may initially be enhanced followed by gradual attenuation [36]. This initial period of increasing relative abundance enhances the risk of transmission of manure-borne ARGs to microorganisms in soil niches, especially in the case of repeated and long-term applications of manures and composts into agricultural soil [2]. Manure-introduced bacteria hosting ARGs might be outcompeted by native soil microorganisms and gradually disappear from the soil microbiome [48]. Nevertheless, some manure-borne ARGs may still persist in the soil via gene transfer to indigenous microorganisms [43], and these ARGs may be further enriched due to the growth enhancement effects of introduced nutrients from the fertilizer [49, 50]. Therefore, applications of manure or compost may affect soil ARGs and MGEs by direct transfer of manure-borne ARGs and selection by manure-introduced antibiotics [2]. It is thus essential to distinguish manure-introduced ARGs and MGEs from the intrinsic soil resistome and indigenous soil ARGs and MGEs enhanced by manure or compost applications,

as well as to identify the ARG hosts selected by antibiotic residues and other co-selection pressures.

3.2 ARGs from Wastewater

It was estimated that global production of wastewater reached 135 and 275 km^3 in the domestic and manufacturing sectors in 2010, respectively [51]. Approximately 167 km^3 of global manufacturing wastewater comes from Russia, China, the USA, India, Indonesia, Brazil, and Japan [52]. According to the AQUASTAT data, only 60% of municipal wastewater is treated effectively [52].

In the past 20 years, the concentration of antibiotics has increased in untreated wastewater, which is rich in nutrients and bacteria [53]. The wastewater treatment system is thus considered to be a high-risk environment for the selection and transfer of microbial genetic material and is also one of the most important sources of ARGs in other water environments. Studies in irrigated soil in the Pearl River Delta Region in Southern China have suggested that wastewater irrigation can significantly increase the concentration of tetracycline and sulfamethazine and the relative abundance of ARGs associated with those drugs [54]. Compared with nonirrigated soil, the absolute abundance of ARGs other than *tetQ*, the *aadA*, *intI1*, *qacE+qacE*Δ1, and *IncP*-1 plasmids (linked to multi-antibiotic resistance) increased by two orders of magnitude in soils irrigated with untreated wastewater for 100 years in Mexican soil [55]. In 12 urban park soils with reclaimed irrigation water in Victoria, Australia, a total of 40 unique ARGs were identified, with β-lactam resistance genes being most prevalent by high-throughput qPCR and terminal restriction fragment length polymorphism techniques. Compared with domestic wastewater-irrigated soils, there are higher concentrations of antibiotics and abundance of ARGs in soils irrigated by fishpond water, indicating that different types of wastewater exert different impacts on ARGs in the soil [54]. Generally, the increase of ARGs may be attributed to the accumulation rather than the in situ enrichment of ARGs in soil, and short-term wastewater irrigation cannot attenuate the accumulation of ARGs [55].

3.3 ARGs from Sludge

In the European Union, a total of 10 million tons (dry matter) of sewage sludge was produced in 2010 [56, 57], with 76% from Germany, the UK, Italy, France, and others. In the USA, over 8 million tons of solids is produced annually and 55% is applied to arable land [57]. In China, 6.25 million tons of dry solids were produced in 2013, with an average annual increase of 13% [58].

Antibiotics and ARGs might be absorbed to and enriched in sludge during sewage treatment. The abundance of ARGs significantly increased by an average of 947-fold in activated sludge compared to influent samples, which might be due to the increase in the total number of bacteria [59]. Various optimization measures have

been tried to reduce the abundance of ARGs in treated sludge/biosolids, such as the addition of natural zeolite to the sludge [60] and bio-drying [61]. Regardless of the disposal method of sludge, the process of treatment, or the use of recycled products, ARGs and ARB can be easily transferred into the soil. Bacteria can share genetic information through HGT with MGEs allowing ARGs to transfer from microorganisms in the sludge to indigenous microbes in the soil [62]. Furthermore, it indicates that sludge serves as a hot spot for the enrichment of ARGs and MGEs among bacteria, and its application may increase the HGT activities of ARGs in soil regardless of the difference in bacterial populations between sludge and soil [60].

Land application of sewage sludge is the key management approach of sludge disposal and an important channel for ARG spread to farmland [63], leading to the dissemination of ARGs into the soil [64]. Compared with ARGs in unamended soil, there was a short-term increase within 20 days in the number of ARGs in amended soils, but there was no significant difference in the relative abundance of ARGs [65]. The sludge can be mixed with straw or bark to produce organic fertilizer through microbial fermentation. The changing composition of the bacterial community is responsible for the reduction of ARGs upon composting, mainly because the high temperature produced by composting is conducive to the suppression or reduction of resistant bacteria and hence their resistance genes [66]. This was found to be driven by several key effects: (1) most of the pathogenic microorganisms were killed when temperatures exceeded 50°C for at least 5 days during composting; (2) high temperatures affect microbial enzyme activity, changing the rate of enzymatic reactions and ultimately affecting cell synthesis; and (3) the extracellular DNA in the sludge contains some ARGs whose rate of hydrolysis and biodegradation increases with increasing temperature.

3.4 ARGs from the Atmosphere

ARGs in soil can also be exchanged and transmitted through air [67]. Especially in heavily polluted air, the increased concentration of airborne particles is conducive to microbial transport as it provides more adhesion sites [6]. Studies have indicated that farms produce a large number of microorganisms, such as ARB, pathogenic bacteria, and opportunistic pathogens, which are easily aerosolized during animal production activities and fecal disposal, hence becoming a potential repository and carrier of ARGs [68].

ARGs can enter soil through either dry deposition or wet deposition. Dry deposition is caused by turbulent diffusion, gravity sedimentation, molecular diffusion, etc. and can transport aerosol particles carrying ARGs to the surface of the earth, where molecular forces enable them to adhere to soils [69]. Wet deposition refers to the process in which aerosol particles, participating in the formation of cloud droplets, are removed from the atmosphere by rainfall and snowfall. Cloud removal makes the aerosol particles with ARGs themselves become part of the cloud droplets as a result of condensation nuclei, and subcloud removal refers to the formation of a droplet of rain, snow, ice, etc., which can adsorb and contain aerosol

particles with ARGs [70]. The ARGs in air may disseminate across different regions, as the pollutants can be transported and settled to soil in remote areas [13]. Therefore, the ARGs coming from the air also increase in the soil [71]. In return, a series of soil pollutants and microbes may also be aerosolized and further exacerbate ARG spread via air.

4 The Persistence of ARGs in Soil

Although there are a number of reports focused on transmission of ARGs from soil to plants, and the effects of anthropogenic activities and environmental factors on transmission of ARGs, the persistence of ARGs in soil is still unclear. The potential factors that influence the dynamics and persistence of ARGs in soil are thus attracting more attention.

In a field study, the relative abundance of *sul1*, *sul2*, and *ermF* increased rapidly in soil after slurry application and dissipation rate of *ermF* in slurry-treated soils was higher than *sul1* and *sul2* in dry-stack-amended soils [72]. The relative abundance of ARGs increased rapidly at day 1 after application of manure, followed by a decrease to the background levels by day 60, whereas a slight increase of the relative abundance of ARGs, followed by a decrease to the background levels by day 32 after application of compost [73]. The half-lives of *ermB*, *sul1*, *tetA*, *tetW*, and *tetX* in soil range from 13 days to 81 days after the application of different ratios of the biosolids from a wastewater treatment plant [74]. Half-lives of ARGs (0.40–3.87 d) and specific genes (*ermF*, 1.42–3.51 d; *tetG*, 0.43–2.86 d; *tetX*, 1.35–8.79 d) differed in the treated soils according to the addition of different sludge composts [75]. However, long-term application of manure compost in a red soil did not greatly increase the relative abundance of ARGs and MGEs in dryland soil or paddy soil, compared to the soil without any fertilization over 26 years [9]. Therefore, the persistence of ARGs varies considerably depending on fertilizer treatments and a series of factors, such as cropping systems, soil oxygen status, the introduction of nutrients, and other factors related to microbial community structure. Therefore, the removal rates of ARGs in soil are highly related to several factors: transport of extracellular DNA containing ARGs and cells carrying ARGs [76], binding of ARGs to soil or organic matter [77], decay of extracellular ARGs [78], and death of host bacteria [79].

5 The Transmission of ARGs in Soil to Groundwater and Underground Water

There is an increasing concern that the resistant bacteria generated in farm activities may migrate into soil and groundwater with the land application of manure. Antibiotic resistance contaminants in soil (especially large animal facilities) may be carried

by runoff and erosion to secondary reception systems (surface water and groundwater) or leach directly to groundwater [80]. Several ARGs directly impacted by animal agriculture were characterized in natural groundwater [81]. Furthermore, seven tetracycline resistance genes (*tetM*, *tetO*, *tetQ*, *tetW*, *tetC*, *tetH*, and *tetZ*) in lagoons and groundwater adjacent to swine production facilities were monitored and source tracked from 2000 through 2003 [82]. Results showed that gene sequences in the impacted groundwater are highly identical to those in the lagoon. Additionally, novel sequence clusters and unique indigenous resistance gene pools were also found in the groundwater. Thus, swine manure seriously affected the ARGs in groundwater, as well as part of the indigenous gene pool [82].

6 The Transmission of ARGs from Soil to Plant

The enhanced water fluxes and nutrient input in the rhizosphere can stimulate bacterial metabolic activities and the transfer of conjugative plasmids in their inhabitants [83]. These conditions establish "hot spots" for microorganism gene transfer activity in the phytosphere, including both the rhizosphere and the phyllosphere [50]. ARGs in manure-amended soil can be potentially transferred to vegetables, including those that are eaten raw or with minimal processing [31]. It has been shown that organic lettuce may be apt to carry more diverse ARGs. For example, one recent study reported up to eightfold higher absolute abundance of 134 ARGs in organic lettuce than conventionally produced lettuce [84]. Furthermore, long-term exposure of plants to antibiotics and ARGs in soil has increased the risk that ARGs carried by pathogens can enter the food chain via contaminated crops, indicating that consumption of raw leafy vegetables may potentially increase direct human exposure to ARGs [85, 86].

More and more studies show that endophytic bacteria (root endophytes, leaf endophytes, and phyllosphere microorganisms) can acquire antibiotic resistance from manure-amended soil, and sulfonamide and tetracycline resistance genes, such as *sul1*, *sul2*, *tetC*, and *tetG*, have been detected in harvested vegetable samples [87]. Yang et al. [88] showed that nine subclasses of ARGs were shared among soil, roots, and shoots of lettuce and that ARGs were mainly located in endophytes within the lettuce [88]. These endophytic bacteria are the main carriers and disseminators of drug-resistant genomes and mobile genetic elements, and they can acquire drug resistance through various ways [89]. Such bacteria may be directly transmitted through the chain of soil vegetables from animal feces and act as an important driver of the change of multi-antibiotic-resistant bacteria in vegetables [90]. Therefore, microorganisms and secretions (sugars, organic acids, and amino acids) in the rhizosphere may affect the migration and attenuation of ARGs in soil [91, 92].

Additionally, the potentially transferable gene pool in the phytosphere was found to be highly mobile and directly correlated with host fitness. The conditions in these hot spots fluctuate temporally as they are heterogeneous and dynamic environments [50]. Therefore, it is difficult to evaluate the extent to which these factors affect HGT processes of ARGs under different conditions [93].

7 Effect of Anthropogenic Activities and Natural Factors on Transmission of ARGs

The development of bacterial resistance to antibiotics poses a major threat to human health [94]. The application of antibiotics in human medicines and livestock production and the use of manures and municipal wastes in agriculture are regarded as pivotal selective pressures on the dissemination of ARGs [25, 95]. In addition, resistance of bacteria to antibiotics co-occurs with other environmental factors, such as lack of nutrition, extreme temperature stress, and oxidative conditions [94, 96]. The current state of knowledge on the dissemination of ARGs induced by anthropological activities and natural factors can be described as follows.

7.1 Agricultural Regulation

7.1.1 Soil Management

Common soil management practices like cropping systems, irrigation, and fertilization are known to influence both the introduced and intrinsic soil resistome. Manure is rich in available carbon for bacterial growth and may also carry co-selectants such as antibiotics and metals [25]. Additionally, all these fertilizers also contain organic and inorganic contaminants, which have been related to a series of negative environmental impacts on the dissemination of antibiotic resistance [97]. Irrigation with treated wastewater may increase ARG levels in soil bacteria, potentially adding to the global dissemination of clinically relevant antibiotic resistance. It is also evident that ARG and ARB risks could be impacted differently under different cropping systems, e.g., dryland vs. paddy soils. Wang et al. [9] showed that the relative abundance of *tetPA*, *oprJ*, *mexF*, and *acrA_5* increased, whereas other genes decreased in paddy soil compared to the dryland soil, indicating that the overall pattern of ARGs in the soil varies according to cropping system and may have been driven by the differences between aerobic and anaerobic conditions [9]. Therefore, the origin, dynamics, and propagation of ARGs in soils are closely related to different agricultural practices and the physicochemical properties of soils. It is important to understand comprehensively the dynamics of intrinsic and introduced ARGs in soil and therefore develop strategies for risk mitigation.

7.1.2 Biochar Amendment

Biochar, a soil amendment produced via pyrolysis/carbonization of plant- and animal-based biomass, is widely used in agriculture to increase the soil water holding capacity and improve soil fertility [98]. π-π interactions play an important role between the aromatic rings on antibiotics and biochar [99, 100], probably driving selection or co-selection of ARGs among soil microorganisms.

Biochar amendment may significantly decrease the abundance of ARGs in non-planted soil [101]. The underlying mechanisms could be attributed to two factors: (1) the mobility of antibiotics and heavy metals being decreased through adsorption and (2) the bacterial community structure being influenced by the addition of biochar, with corresponding effects on the resistome [102, 103]. In another study, mushroom and rice straw biochar were both produced at 500°C for 4 h under oxygen-limited conditions using a muffle furnace. Whereas the application of mushroom biochar effectively removed ARGs and pathogenic bacteria, this is not the case with the rice straw biochar [104]. Therefore, further systematic and integrated study is needed to unveil the relationship between biochar physicochemical properties and their influence on the persistence and/or propagation of ARGs in soil.

7.2 Soil Properties

Soil physical, chemical, and biological parameters have been identified as environmental stresses that induce or maintain antibiotic resistance evolution and transmission [77]. Pearson correlation analysis indicated that there is a correlation between the abundance of ARGs and antibiotics or soil properties (pH and soil organic matter). The concentrations of tetracyclines and abundance of ARGs decreased with increasing soil depth [54, 105]. Relative studies showed that the abundance of ARGs in soil was increased after the long-term application of sewage sludge [11], whereas another study found inconsistent levels of ARGs in different sites of amended soil [106].

Soil microbial phylogenetic structure is commonly regarded as the dominant biotic determinant of ARG compositions [107], as soil microbes are not only the producers of many antibiotic compounds [4] but also hosts to various ARGs. Many studies have shown that soil physicochemical properties, plant species, and climate, as well as chemical substances, can significantly influence the diversity, structure, and function of microbial communities in soil [108, 109]. Moreover, it has been demonstrated that microbial community structures revealed great differences among the paddy soil samples, which were consistent with differences in pH values [110]. In addition, the total *tet* gene copies were highly related to soil organic matter near swine feedlots [111]. All the results verified that soil type and physicochemical properties exert an impact on the microbial community, which was correlated with the fate of ARGs.

7.3 Environment Pollutants

7.3.1 Heavy Metals

It is a common practice to add certain heavy metals into animal feeds to promote growth and control disease, and these metals are subsequently accumulated by soils

after the application of manure and sewage sludge [112, 113]. There is a link between the use of Zn in feed additives and the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) in animal production [114]. Positive correlations exist between some ARGs and heavy metals in manures and manure-amended soils [115]. The positive correlation between ARGs and heavy metals is conducive to the survival of bacteria in a polluted environment, which promotes the spread of ARGs in soil to some extent [116].

It is vital to recognize that metals can co-select antibiotic resistance and drive strong selection of bacterial assemblages in various environments [117]. The types, abundance, and mobility potential of ARGs were significantly influenced by long-term heavy metal contamination and result in the unpredicted risk of the ARG dissemination in environments contaminated with heavy metals. Furthermore, the co-selection for antibiotic resistance induced by heavy metals has been highlighted, particularly when the same gene confers resistance to multiple types of metals and antibiotics and when diverse genes encoding metal and antibiotic resistance coexist on the same MGE.

7.3.2 Nanomaterials

Recently, it was estimated that the global market requirement for metal oxide nanoparticles will increase from 270,041 tons in 2012 to 1,663,168 tons by 2020 [118]. Although nanoparticles exist in various environments, such as wastewater effluent and sludge, knowledge about their influence on ARB and ARGs is lacking. The application of nanoalumina significantly promoted the HGT of multidrug resistance genes encoded by the plasmids of RP4, RK2, and pCF10 [119]. It is also reported that AgNP exposure reduced the occurrence of ARGs in collembolan gut microbiota [120]. The conjugative transfer of the RP4 plasmid from *E. coli* to *Salmonella* spp. treated with nanoalumina was enhanced 200-fold, indicating that the environmental and health risks from nanomaterials could include promoting sensitive bacteria to obtain antibiotic resistance [119].

7.3.3 Microplastics

Microplastics in aquatic environments may adsorb antibiotics on their surfaces, resulting in their long-range dissemination and entry into the food chain [121]. Sophorolipid can stimulate ARG dispersion mediated by bacteria/phage in soil co-contaminated with microplastic and tetracycline [122]. Compared to free-living bacteria, the transfer rate of plasmid DNA in phylogenetically diverse bacteria was increased, and this was associated with microplastics [123]. These reports indicate that microplastics could be the hot spot for HGT between phylogenetically distinct pathogens in the environment.

8 Direct Relationships to Human Health

Most clinically relevant ARGs may originate from environmental bacteria [124]. ARGs carried by soil bacteria that are also found in human clinical pathogens [125] and novel ones conferring high-level resistance against sulfonamides, aminoglycosides, and a broad range of β-lactams have been discovered [126]. Functional metagenomic analysis of soil-inhabiting bacteria revealed a high nucleotide identity (>99%) when comparing the multidrug-resistant resistome of soil bacteria with those in clinical human pathogens, inferring possible HGT among bacteria from these different environments [127]. It was also revealed that the contiguous nature of the *intI1* with the ARGs carried by the soil bacteria and clinical pathogens facilitated the HGT process [127]. The *intI*1, belonging to class 1 integrons, is closely related to the integration of multiple ARGs at the same genetic site. In natural and anthropogenically influenced environments, integrons have been acknowledged to be the prevalent carriers of multiple ARGs [46]. A substantially increased abundance of *intI*1 was reported when manure was applied to archived soils, inferring the transfers of multiple resistances from soil environments to clinical activities. This resistance could be intensified by the applications of excessive antibiotic-contaminated manures to land.

To date, several limitations for confirming ARG transfer from environmental microbiomes to human pathogens have been identified although credible evidence of this process is yet to be well or fully documented [128, 129]. In any case many ARG hosts exist on the surfaces of leafy vegetables (arugula, cilantro) grown in manure-amended soil that are eaten raw [93, 130]. This represents a direct food chain link between the environment and humans. After incubation of lettuce or cilantro leaves, the total DNA of the bacterial community was obtained from the enrichment, and multiple resistance plasmids were found in *E. coli* isolates, indicating that bacteria colonizing leafy vegetables are the original source of transposons and integrons [130, 131]. As multiple ARGs in the bacterial genomes are being found in the plasmids, it is obvious that antibiotic resistance pathogens or "superbugs" may propagate on a global scale. This is supported by the occurrence of plasmid-carrying *mcr-1* resistant to colistin (polymyxin E) in various pathogens worldwide [132]. Colistin has been revived as a last resort drug to tackle the threat of multidrug-resistant bacterial outbreaks [133]. The plasmid-carrying *mcr-1* was first reported in animal and human sources in Shanghai, China. Nowadays, *mcr-1* has been identified in samples from various sources including human pathogens, swine, poultry, pork, and environmental sources worldwide [132]. The consumption of pork has also been reported to significantly promote the spread of the ARGs in Dutch travelers [134].

9 Technology for Reducing the Introduction of ARGs into Soil

Much effort is being spent to mitigate the threat of antibiotic resistance, namely, developing new or alternative antibiotics and reducing antibiotic usage [135, 136]. An alternative and complementary stewardship initiative consists of

monitoring and removing ARGs as environmental pollutants [29]. Various treatment processes, such as aerobic composting, anaerobic digestion, and disinfection, have been applied and tested for their efficacy in removing ARGs and antibiotics (Table 2). The intention is to reduce the selection pressure and introduction of ARGs into soil through manure and compost application and wastewater irrigation.

9.1 Aerobic Compost

Aerobic composting is widely used to treat organic wastes and manure. The effects of aerobic composting on the variation of ARGs are based on different processing technologies. For example, continuous thermophilic composting can significantly reduce ARGs and integrons in animal manure [140]. Superabsorbent polymers are also considered suitable amendments for reducing ARGs in swine aerobic compost [143]. Conversely, several types of ARGs cannot be effectively removed by aerobic composting, with compost products, thus remaining an important reservoir of ARGs [49]. Temperature was viewed as an important factor to influence the fate of ARGs in aerobic digested sludge where the shift in temperature would largely change the bacterial community [137]. The ARGs and MGEs from sewage sludge were significantly reduced by hyperthermophilic composting (temperatures up to 90°C), as were other indicators during the composting process (mushroom biochar, rhamnolipid, Tween 80 and other surfactants, etc.) [144, 145]. Additionally, the temperatures under elevated thermophilic composting up to 70–80°C without exogenous heating and additives (zeolite, superphosphate, or zeolite and ferrous sulfate) on the removal rate of ARGs during chicken manure composting were investigated. The removal of ARGs in manure was 86.5%, 68.6%, and 72.2% in zeolite, superphosphate, and zeolite and ferrous sulfate, respectively, which is higher than the control [146].

9.2 Anaerobic Digestion

Anaerobic digestion of manure from livestock and poultry can produce methane, while the digested residue can be applied as organic fertilizer [147]. Additionally, there is some evidence that anaerobic digestion can reduce populations of ARB in manure and biosolids, as well as genes coding for resistance to antibiotics, and may thus be an effective approach for recycling agricultural waste and removing ARGs [148]. Several treatment strategies including pretreatment, thermophilic digestion, two-stage digestion, additives, and solid-state digestion were employed to compare the removal of ARGs by anaerobic digestion [149]. Operating parameters played crucial roles in the reduction efficiency of ARGs. For example, longer solids retention time showed a higher level of ARG removal rate in anaerobic digestion of residual sludge [66]. Therefore, it is necessary to critically evaluate existing

Table 2 Technologies tested to reduce the introduction of ARGs into soil

Targeting antibiotics	Gene	Jang et al. [137]	Burch et al. [79]	Ghosh et al. [138]	Zhang et al. [139]	Qian et al. [140])		Zhang et al. [141]	Zhang et al. [142]	
		Aerobic (55°C)	Aerobic (25°C)	Anaerobic (35°C)	Anaerobic (35°C)	Aerobic (<45°C)	Aerobic (55°C)	Ultraviolet disinfection (80 mJ cm^{-2})	Thermophilic anaerobic	Mesophilic anaerobic
Tetracycline	*tetA*	−[a], +[b]	−[a]	+[b]					+[a]	+[a]
	tetB	−[a, b]						−[b]		
	tetC					+[b]	−[b]			
	tetD	−[a, b]								
	tetE	−[a, b]								
	tetG	−[a, b]			+[a], −[b]	−[b]	−[b]			
	tetH	−[a, b]								
	tetJ									
	tetL									
	tetM	−[a, b]			+[a, b]	−[b]	−[b]			
	tetO									
	tetQ	−[a, b]				+[b]	ND			
	tetS									
	tetT									
	tetW									
	tetX	−[a, b]	−[a]	+[b]	+[a, b]	−[b]	−[b]			
	tetZ	−[a, b]								
	tetBP	−[a, b]								
Sulfonamide	*sul1*	−[a, b]	−[a]		+[a], −[b]	±	−[b]		+[a]	+[a]
	sul2	−[a, b]			+[a, b]	±	−[b]	−[b]		
	sulA									
	dfrA1									
	dfrA7									

(continued)

Table 2 (continued)

Targeting antibiotics	Gene	Jang et al. [137]	Burch et al. [79]	Ghosh et al. [138]	Zhang et al. [139]	Qian et al. [140])		Zhang et al. [141]	Zhang et al. [142]	
		Aerobic (55°C)	Aerobic (25°C)	Anaerobic (35°C)	Anaerobic (35°C)	Aerobic (<45°C)	Aerobic (55°C)	Ultraviolet disinfection (80 mJ cm^{-2})	Thermophilic anaerobic	Mesophilic anaerobic
Quinolone	*gyrA*								−[a]	−[a]
	parC									
	qnrA									
	qnrC									
	qnrD	−[a], +[b]								
	qnrS	−[a, b]								
	Aac(6')−ib−cr	−[a, b]	−[a]			−[b]	−[b]			
Macrolide	*ermB*	−[a], +[b]			+[a, b]	−[b]	−[b]		+[b]	+[b]
	ermC	−[a, b]								
	ermF									
	ermQ					−[b]	−[b]			
β-Lactam	bla_{TEM}	−[a, b]			+[a, b]				−[b]	−[b]
	bla_{CTX}	−[a, b]								
	bla_{CMY}									
	bla_{SHV}	−[a, b]								
MDR	*oqxA*	−[a, b]							−[a]	−[a]
	floR	−[a, b]								
MGEs	*IntI1*	−[a, b]	−[a]	−[b]	+[a, b]	+[b]	−[b]			
	IntI2					−[b]	−[b]			

+, increase; −, decrease; ±, non-change; *ND* not detected

[a]Absolute abundance

[b]Relative abundance

anaerobic treatment processes for their potential in mitigating ARGs and their propagation in the environment.

9.3 Disinfection

Various techniques are used in wastewater treatment, such as UV, chlorine, and ozone disinfection. To induce bacterial damage, these techniques employ different mechanisms. Comparative studies of disinfection methods on ARG removal are widely reported in the literature [141, 150]. Disinfection process of ARB cells is significantly related to the disinfectants' relative activities and consumption by important cell constituents, including amino acids, saccharides, lipids, and nucleic acids [151]. The frequency of the transfers of ARGs was determined under different rates of UV or chlorine, indicating that UV disinfection and chlorination display different influences on conjugative transfer and that the dose of both UV and chlorine would be important factor in removing ARGs [152]. Additionally, when the ozone dose was 2 mg/L, the abundance of ARGs had higher removal efficiency, but there was no significant correlation between the concentration of ozone and ARG removal efficiency. Although inactivation of ARB via destroying bacterial DNA or the cellular structure could occur during disinfection processes, ARGs may still exist in the cell debris, constituting a potential downstream threat to human health.

10 Conclusions

Although the relevance of ARB and ARGs as environmental contaminants is widely recognized, there is currently limited evidence to assess the related human health risks quantitatively and objectively. Therefore, it is difficult to define threshold values for the maximum admissible levels of ARB and ARGs in treated wastewater and in sludge and manure used in agriculture. Increasing water scarcity and soil degradation will drive increased need for water reuse, and beneficial reuse of municipal sludge and manures, likely increasing inputs of ARGs and ARB into soil. Thus far, several primary strategies have been proposed to mitigate the propagation of antibiotic resistance, namely, the restriction of antibiotic use in clinical activities and veterinary applications, and to promote new drug design.

Given the urgency of the problem, secondary or follow-on strategies are also needed to help preserve the effectiveness of existing antibiotics. These include establishment of a global systematic and publicly available surveillance network, including regular, consecutive measurement of antibiotic application and the diversity of antibiotic resistance from agriculture and clinical activities. Continuous surveillance of antibiotic resistance is conductive to disease diagnosis, effective antimicrobial stewardship, and policy setting, thus the overriding importance of undertaking surveillance across the One Health framework. Governments should

pay more attention to ARG pollution with investment in both basic and applied research to provide a strong scientific basis for formulating effective mitigation practices and a standardized assessment system to document risk reduction of soil and water contaminated by antibiotics and ARGs. This will provide reference data for prevention, reduction, and removal of these important environmental pollutants.

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Religious Mass Gathering (Hajj) and Antimicrobial Resistance: From Challenges to Opportunities

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Abstract Hajj pilgrimage, a form of mass gathering (MG), may facilitate rapid multinational spread of antimicrobial resistance (AMR). Hajj has traditionally been linked as an event, which favors the dissemination of various infectious outbreaks. Despite best effort in minimizing such outbreaks through longtime investment in education and medical care, respiratory infection and gastrointestinal diseases still see high occurrences during Hajj. Such diseases have to be treated by antibiotics, and improper use of antibiotics can result in secondary concerns such as that of AMR dissemination. In this chapter, we identify factors that promote AMR dissemination within the Hajj context. This includes socioeconomic and demographic factors of pilgrims, their general health status including vaccine coverage, high antibiotic use, and/or misuse in the home countries of those pilgrims. Using Hajj as a local example, we exemplified strategy plans to mitigate AMR transmission. First, medical services should be coupled with elements of AMR control strategies. Clinicians can be encouraged to use decision tools to rationally prescribe antibiotics. Second, vaccination requirements for Hajj participation can also help reduce the burden of relevant vaccine-preventable disease symptomatology and related antibiotic prescriptions. Third, priority antibiotic-resistant bacteria can be added to the surveillance agenda to facilitate AMR control. Standardized diagnostic and surveillance systems can help institutionalize these efforts. However, due to inadequate or nonexistent surveillance during MGs on AMR, resistance prevalence and trends are largely understudied, and baseline data for evaluation of potential interventions need more development.

Keywords Demographic factors, Dissemination, Electrophoresis, Gastrointestinal, Hajj pilgrimage

1 Introduction

Antibiotics became commercially available in the1930s and have since become a game-changer in human health and survival. However, with prevalent usage, antimicrobial resistance (AMR) has also emerged over the past 70 years, with the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) of particular relevance to clinical infections [1]. AMR-associated

Table 1 Characteristics of select types, frequencies, and scope of MG events

MG event	Number of persons (millions)	Interval of occurrence	Geographic scope	Type of event
Kumbh Mela, India	73	12 years	National	Religious
Arbaeen, Iraq	9–60	Annual	Regional	Religious
Carnival, Brazil	5	Annual	Regional	Cultural
Olympics	8	4 years	International	Sports
FIFA World Cup	3.4	4 years	International	Sports
Hajj, Saudi Arabia	2.7	Annual	International	Religious
Namugongo Martyr's day, Uganda	2	Annual	Regional	Religious

deaths are projected to rise to ten million by 2050. AMR has therefore become a major theme in international leadership platforms [2–4]. AMR was featured at the 2013 World Economic Forum, resulting in the roadmap to combat AMR by leading pharmaceutical companies announced at the 2016 World Economic Forum. Indeed, the release of the respective strategies at the 2014 World Health Organization (WHO) 67th World Health Assembly and the US White House helped raise the profile of AMR globally. AMR had the distinction of being the third health topic ever discussed at the United Nations General Assembly in 2016. Global data triangulation of anthropological and socioeconomic factors contributing to AMR indicated that the spread of resistant strains and genes rather than reduction in antibiotic consumption would be the dominant drivers of AMR [5].

From this perspective, mass gatherings (MG), defined by the World Health Organization (WHO) as an occasion, either organized or spontaneous where the "number of people attending is sufficient to strain the planning and response resources of the community, city, or nation hosting the event" (WHO, 2008), pose significant challenge to AMR as MGs can facilitate faster and broader transmission of AMR on a global scale. MGs include open-air music and cultural events, religious events, political gatherings, and large migrations (Table 1). However, the relationship between AMR and MGs is far removed from these high-level discourses or relevant academic and scientific discourse. In this chapter, we reflect on the challenges posed by MGs, specifically on the Hajj event that occurs yearly in Saudi Arabia, to AMR. We also discuss opportunities for intervention.

2 Hajj as a Mass Gathering and the Link to Infectious Diseases

The most extensively reported epidemiological data on infectious diseases at MG emerged from the Hajj [6–21]. Due to the close contacts between pilgrims as they perform the rituals, the Hajj promotes transmission of respiratory viruses [22]. It was also observed in an earlier study that Hajj promotes the acquisition of *Neisseria*

Table 2 Examples of infectious disease to which Hajj pilgrims are exposed to

Infectious agent	Associated disease	References
Influenza A and B, parainfluenza, respiratory syncytial virus (RSV), adenovirus, herpes simplex virus (HSV)	Upper respiratory tract infection	[80]
Streptococcus pneumoniae	Pneumococcal disease	[17]
Mycobacterium tuberculosis	Tuberculosis	[81]
Neisseria meningitides	Meningococcal sepsis	[26]
Salmonella spp., *Shigella* spp., *E. coli*	Gastrointestinal infections, gastroenteritis	[37]
Astrovirus, rotavirus, norovirus	Viral diarrhea	[82]
Hep A, B, C, E	Hepatitis	[83]

meningitidis among pilgrims [23]. Over the past decade, a significant number of publications from different countries based on both syndromic surveillance and PCR-based investigation of respiratory pathogen carriage at the Hajj were made available. Table 2 provides a list of infectious disease pilgrims which can potentially be exposed to at the Hajj.

In the past, Hajj-related cholera has been a public health problem and the main cause of morbidity and mortality among pilgrims, leading to major epidemics and international spread. Due to improved sanitation in Saudi Arabia over the years, large-scale cholera outbreaks have not been recorded during the last decades (except for a recent outbreak in Yemen border from 2016–2020) [24]. Similarly, invasive meningococcal disease has been a Hajj-related public health concern with its last outbreaks (serogroup W-135) in the 2000s [25]. However, with the strengthening of prevention through mandatory vaccination, no case of meningococcal disease has been reported in Mecca since 2006 [26]. The Saudi Ministry of Health now requires proof of vaccination to obtain visas for entry to Saudi Arabia, and supplementary vaccination is offered to pilgrims originating from (WHO determined) polio risk and endemic countries. Despite these preventive measures, respiratory tract infections (RTI) and gastrointestinal diseases continue to predominate during Hajj [7, 27].

3 Predominant Disease at Hajj: Respiratory Tract Infections (RTI)

Hajj-related studies were conducted in out- and inpatients at health structures in Saudi Arabia or on return in pilgrim's country of origin and in cohorts of pilgrims regardless of symptoms [7, 15, 17, 28–31]. These studies revealed that RTIs are among the leading causes of admission to hospitals in Mina, Mecca, and Medina during the Hajj period. Most cases are upper respiratory tract infections, but severe respiratory tract infections and pneumonia are not uncommon among pilgrims. Respiratory diseases were the second cause of mortality in Indonesian pilgrims during the Hajj (following cardiovascular diseases). Commonly acquired viruses

detected by means of either monoclonal antibodies, ELISA, viral cultures, or RT-qPCR include influenza virus, respiratory syncytial viruses, and herpes simplex [22]. In another study, clinically suspected pneumonia cases were caused by *Candida albicans*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Klebsiella pneumoniae* [32].

Tuberculosis (TB) transmission is another concern at the Hajj, but there are no large-scale, specific studies to determine its prevalence among pilgrims. A prospective cross-sectional study was conducted in Mecca, during the Hajj period in September 2015. One thousand one hundred sixty-four pilgrims with cough were selected from five countries in Africa and South Asia that are endemic for TB, and it was found that 1.4% of the enrolled individuals (mainly from Afghanistan, Pakistan, and Nigeria) had previously undiagnosed active pulmonary TB [33]. This in turn poses a transmission risk to other pilgrims.

4 Predominant Disease at Hajj: Gastrointestinal Diseases

Gastrointestinal diseases are the second leading cause of consultations at MG, notably at the Hajj [19–21, 28, 34]. Reviews on diarrheal disease at the Hajj showed a prevalence of diarrhea ranging from 1.1 to 23.3% in 14 cohort studies including 262,999 pilgrims from various countries between 2002 and 2013 [35]. Five percent of pilgrims from Riyadh developed diarrheal symptoms during the 2009 Hajj. Twenty-one percent of Iranian female pilgrims suffered from gastroenteritis during the 2011 Hajj. In 2013, 23.3% pilgrims from Marseille, France, had diarrhea during the Hajj, while a 13.7% prevalence was recorded in 2016 [28]. In a latter study, *Escherichia coli* was the predominant pathogen isolated from pilgrims. Enteropathogenic *E. coli*, enteroaggregative *E. coli*, and Shiga-like toxin-producing *E. coli* were acquired by 29.9%, 10.2%, and 6.5% pilgrims, respectively [36]. Among persons infected during the 2011–2013 Hajj and hospitalized in Saudi hospitals, the pathogens responsible for enteric infection were mostly bacteria, with a prevalence of *Salmonella* spp. of 11.4%, while that of diarrhea-associated *E. coli* ranged between 1.3 and 8.8% according to pathotypes [37]. Furthermore, it was determined that genes associated with resistance to third-generation cephalosporins were associated with these *Salmonella* and *E. coli* isolates [37], which can detrimentally impact clinical treatment efficacy.

5 AMR Risk Factors at Hajj

The control of infectious disease, and, therefore, global health security, is seriously threatened by both the emergence of novel pathogens and the steady increase in the number of microorganisms that are resistant to antimicrobial agents. Infections in humans are generally treated with chemicals (e.g., antibiotics) that neutralize the

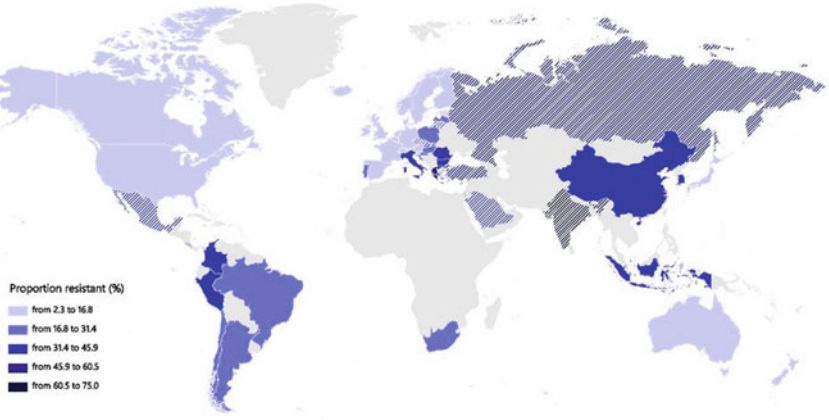

Fig. 1 Average proportions of *E.coli* and *K. pneumoniae* resistant to third-generation cephalosporins and carbapenems (only *K. pneumoniae*), surveyed in 2015. This figure illustrates that almost no countries are spared from AMR. Those that did not show color coding likely suggests lack of testing. Source: OECD [84]

infectious agent. While antibiotic use precipitates the emergence of resistance, the spread of resistant strains and horizontal gene transfer enhances the transmission of AMR. No region globally is free from AMR, as is illustrated in Fig. 1, which shows the average proportions of *E. coli* and *K. pneumoniae* that are resistant to third-generation cephalosporins and carbapenems (only *K. pneumoniae*), surveyed in 2015. This figure illustrates that almost no countries are spared from AMR. There are multiple factors that can contribute to AMR during Hajj. These include the general health status of the pilgrims, vaccine coverage, high antibiotic use, and/or misuse in the home countries of those pilgrims and so on.

First, the most common health problems reported during or after an MG are respiratory tract infections and enteric toxi-infections. Given that Hajj is an intense activity held in a short period of time and as most participants would be unfamiliar with medical care in host country (Saudi Arabia), antibiotic self-use may be the first option chosen by most Hajj pilgrims. Resistance spurred by antimicrobial misuse and shortfalls in infection control and public health in developing countries sustain the endemicity or reservoirs of common infections in human and animal populations. Resistance reservoirs are likely to be maintained without detection. This ecology of endemic conditions is hence transported to host country (Saudi Arabia) during Hajj and back to the countries of origin by the pilgrims after Hajj.

Second, antibiotic prescriptions are common during Hajj and unlikely to be accompanied by on-site resistance testing or assessment of etiology. The frequency of infectious diseases during the Hajj results in a significant demand for antibiotic use. The rate of antibiotic use among pilgrims varied according to their nationality and year, with 61.8% in Malay pilgrims in 2013 [38], 53.8% in French pilgrims in

Table 3 Resistance (%) for eight priority antibiotic-bacterial pairs[a] in 2015[b] and predicted % change between 2015 and 2030[c] in select high Hajj pilgrim volume countries

Country	Pilgrim volume (numbers) 2018	Average % resistance for eight priority antibiotic-bacterium pairs, 2015	Predicted % change between 2015 and 2030[c]
India	183,040	57.1	−1.7
Indonesia	210,984	39.5	7.1
Saudi Arabia	612,953	37.7	1.2
Turkey	116,551	38.8	1.2

[a]Denotes the eight priority antibiotic-bacterium pairs, namely, third-generation cephalosporin-resistant *E. coli*, fluoroquinolone-resistant *E. coli*, penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *S. aureus* (MRSA), carbapenem-resistant *K. pneumoniae*, third-generation cephalosporin-resistant *K. pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa*, and vancomycin-resistant *Enterococcus faecalis* and *E. faecium*
[b]OECD
[c]Official Data, Saudi Arabia

2012 [39], 45–48.3% in Indian pilgrims in 2016 [40, 41], and 58.5% in Iranian pilgrims in 2012 [42].

Third, population demographics, socioeconomics of MG attendees, and development status of the home countries of Hajj pilgrims may favor AMR introduction. It has been shown that high gross domestic product (GDP) per person, high educational level, and higher proportion of healthcare spending were negatively associated with prevalence of AMR [5]. As two-thirds of Hajj pilgrims originate from low-to-middle-income countries where AMR surveillance is suboptimal and poor sanitation is prevalent, it is likely that interactions among pilgrims from different demographics and socioeconomics background would provide an opportunity for the transmission of resistant strains to resistance-naïve populations [43]. Official data from Saudi Arabia listed the percentage resistance for eight priority antibiotic-bacterium pairs in 2015 among pilgrims originating from India, Indonesia, Turkey, and from host country (Saudi Arabia) (Table 3). These eight combinations include third-generation cephalosporin-resistant *E. coli*, fluoroquinolone-resistant *E. coli*, penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *S. aureus* (MRSA), carbapenem-resistant *K. pneumoniae*, third-generation cephalosporin-resistant *K. pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa*, and vancomycin-resistant *Enterococcus faecalis* and *E. faecium*. It was noted that this percentage is relatively higher in India compared to the other three countries, likely due to the lack of sanitation infrastructure in India. Although the predicted percentage of resistance may decrease with time for India, other countries may see an increase in percentage resistance, implying that AMR will continue to be a cause of concern during Hajj.

Fourth, the combination of old age (two-thirds of Hajj pilgrims are aged >60 years old) and other underlying diseases (50%) that predispose them to disease acquisition among some Hajj participants. In fact, while Hajj and other religious mass gatherings are lifetime obligation of devotees, many postpone it to older age due to financial or other constraints to an age when they may be already suffering

from chronic disease such as metabolic, respiratory, or cardiovascular conditions among whom antibiotic consumption may be routine.

Finally, it is worth noting that animal sacrifice is an integral part of the Hajj, with over one million animals sacrificed during Hajj [44]. Animals (sheep, camels) originate from various countries (Africa, Europe, Australia) with varying levels of veterinary use of antibiotics [45]. Global antibiotic consumption by livestock is projected to increase by 67% between 2010 and 2030 [46]. The Islamic form of slaughtering animals involves killing through a cut to the jugular vein, carotid artery, and windpipe, and then all blood is drained from the carcass. Given the exposure to blood streams, there is a potential for accidental transmission of blood-borne pathogens from animals to the slaughterhouse workers. Although the contribution of AMR dissemination from sacrificed animals to exposed individuals is unknown, one can draw precedence from an earlier study which showed *E. coli* isolates recovered from turkeys and a farmer to share the same pulsed-field gel electrophoresis pattern, suggesting potential transmission of resistant clones and resistance plasmids [47].

Given the absence of a routine AMR and medical surveillance system and specifically for returning Hajj pilgrims to resource-poor countries, AMR acquired during Hajj are not always detected. Investments for tackling AMR by most countries in Asia, Africa, Eastern Europe, and the Middle East have remained scant, and funding for research and surveillance has not been forthcoming. The lack of detection does not imply absence of AMR dissemination arising from Hajj but simply due to low testing rates. AMR surveillance continues to be based on routine laboratory samples taken from patients with suspected infection, healthcare-associated infections, or patients for whom first-line antibiotic treatment was unsuccessful. Major data gaps remain on the global burden of AMR and asymptomatic carriage; on the community, animal, and environmental burden and acquisition of antibiotic-resistant bacteria; and on clinical presentations, management, outcome effects, transmission patterns, evolution, genotypic characterization of antibiotic resistance mechanisms, and clonal spread.

6 Studies on Hajj and AMR

The date of the Hajj changes from year to year. Depending on the seasons, certain conditions (e.g., high humid and warm temperatures) may provide a favorable environment for AMR bacteria and the spread of infectious diseases. Studies conducted at the Hajj offers the most comprehensive real-world evidence on AMR from a mass gathering perspective [15, 20, 48–57].

Community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) has been associated with closed settings involving lots of people and travelers. For example, *E. coli* isolated from infected pilgrims during the 2014–2015 Hajj had the same widespread sequence type of *E. coli* ST131 and ST648 [58]. The plasmid-mediated colistin resistance gene *mcr-1* was screened and the prevalences of *mcr-1* positive isolates determined by PCR in rectal swabs of pilgrims to be similar in 2013

and 2014. The prevalence of such isolates was also significantly higher upon return to home country from Hajj compared to before the mass gathering [49]. These studies may suggest an identical source of bacterial transmission among pilgrims during the Hajj season. The spread of clone and specific types of AMR gene might be related to travel destination and food vehicles contaminated by multidrug bacteria. Thus, the detection of AMR genes in locations where the pilgrims visit may be a useful way of investigating the source of AMR transmission.

Reviews showed a growing rate of resistance among Gram-positive bacteria, including MRSA and third-generation cephalosporin-resistant *Enterobacteriaceae* in Saudi Arabia, whereas vancomycin-resistant *S. aureus*, vancomycin-resistant enterococci, and carbapenem- and colistin-resistant bacteria prevalence is still low [59]. However, carbapenem resistance emergence in *A. baumannii* [60] and *Pseudomonas aeruginosa* [61] is of increasing concern in Saudi Arabia. Part of the reason may be because antibiotics are easily obtained from over the counter without legislation or restrictions on their use in Saudi Arabia, which may have led to an increase in antibiotic-resistant bacteria prevalence. High rates of AR bacterial infection in patients hospitalized in Saudi Arabia are worrying, and physicians attending patients in this area should be aware of the situation and undertake adapted isolation measures. Therefore, controlling inappropriate use of antibiotics is the key to reducing antibiotic resistance. Moreover, public educational campaigns to discourage the use of antibiotics should be promoted. This may include country or global-wide to monitor antibiotic consumption and resistance trends among local population and international travelers, including Hajj pilgrims.

7 Status of AMR in Selected Countries with Consistent Hajj Participant Numbers

To provide the topography of AMR in resource-poor countries that participate in Hajj, we first summarize the situation in Nigeria [62, 63], one of the participant countries for the Hajj throughout 2012–2019 [64]. The Federal Ministries of Agriculture, Environment and Health of Nigeria commissioned a report in 2017, which listed marked resistance among clinical isolates to all drugs commonly prescribed for urinary tract infections in the country. To exemplify, clinical isolates were resistant to ceftriaxone, ampicillin, and cotrimoxazole. Most organisms demonstrated 100% resistance to ampicillin and cotrimoxazole, which have long been used as first-line drugs in the treatment of urinary tract infection [65]. The documentation of problematic antimicrobial-resistant organisms such as carbapenem-resistant *Enterobacteriaceae* (CRE) [66], vancomycin-resistant enterococci (VRE) [67], and extended-spectrum beta-lactamase-producing (ESBL) Gram-negative bacteria [68] is alarming in a country where antibacterial alternatives are not available in the event of resistance to the last-line drugs.

Such observations of antibiotic resistant bacteria in infected patients are not restricted to Nigeria alone. Egypt which account for the highest volume of pilgrims in Hajj 2019 [64] also reported an exceeding high rates of carbapenem-resistant *Enterobacteriaceae* in healthcare-associated infections in Egyptian hospitals [69]. In addition, $bla_{OXA\text{-}48}$ carbapenem-resistant *Enterobacteriaceae* was also recovered from community-acquired patients [70].

These observations suggest that antibiotic-resistant bacteria can potentially be disseminated into the wastewater streams when pilgrims perform Hajj at Saudi Arabia. Considering that Saudi Arabia hosts in total 98,814 foreign pilgrims in Hajj 2019, the cumulative impact and dissemination of antibiotic-resistant bacteria into the untreated wastewater streams can potentially be significant. In fact, one study recovered a pathogenic *E. coli* strain with $bla_{NDM\text{-}1}$ gene from wastewaters collected in Jeddah during the Hajj period [71], and such observations meant that unintentional discharge of antibiotic-resistant bacteria by pilgrims can potentially escalate concerns for environmental dissemination within Saudi Arabia since more than 50% of Jeddah city relies on septic tanks for sewage management [72].

8 The Five-Four-Eight-Four Approach to AMR Prevention at Mass Gatherings

To alleviate AMR concerns during Hajj, the 5-4-8-4 approach, which combines various measures proposed by different stakeholders including the World Health Organization (WHO), can be undertaken (Table 4). The first 5 refers to the five pillars of WHO strategy to strengthen evidence base of AMR, namely, through improving awareness and surveillance, reduce infection incidence, optimize

Table 4 Control strategies for antimicrobial resistance threats at Hajj

Five pillars of WHO strategy to strengthen evidence base • Improve awareness • Surveillance • Reduce the infection incidence • Optimize antibiotic use • Develop an economic case for sustainable investment
Four moments of antibiotic prescription Moment 1. “Does this patient have an infection that requires antibiotics”? Moment 2. “Have I ordered appropriate cultures before starting antibiotics? What empiric therapy should I initiate?” Moment 3. “A day or more has passed. Can I stop antibiotics? Can I narrow therapy? Can I change from intravenous to oral therapy?” Moment 4. “What duration of antibiotic therapy is needed for this patient’s diagnosis?”
Eight priority pathogens for monitoring *Acinetobacter* spp., *E. coli*, *K. pneumoniae*, *N. gonorrhoeae*, *Salmonella* spp., *Shigella* spp., *S. aureus*, *Streptococcus pneumoniae*
Four specimens to collect blood, urine, stool, urethral and cervical swabs

antibiotic use, and develop an economic case for sustainable investment in areas related to AMR [73]. The first 4 refers to clinical judgment process, urging doctors to implement "4 Moments of Antibiotic Decision Making Process" into regular practice so as to minimize the prescription use of antibiotics in cases that would not benefit from antibiotics [74]. The next 8 refers to pathogen characterization of the eight priority bacteria, namely, *Acinetobacter* spp., *E. coli*, *K. pneumoniae*, *N. gonorrhoeae*, *Salmonella* spp., *Shigella* spp., *S. aureus*, and *Streptococcus pneumoniae*, detected in surveillance programs, proposed in the WHO initiative GLASS [75] (https://www.who.int/glass/en/). Lastly, the 4 refers to the four priority specimens (i.e., blood, urine, stool, urethral-cervical swabs) that should be collected in order to isolate for the priority pathogens [75].

Mass gathering events are ideal situations and provide unique opportunities for obtaining an accurate evidence base needed for the above 5-4-8-4 approach. With molecular-based techniques, including whole-genome sequencing, widely available now, high-quality surveillance and research studies should be done with a large cohort of pilgrims, from wide geographical regions of the world, traveling to perform the annual Hajj. These longitudinal cohort studies could provide the ideal platforms for establishing coordinated global collaborations and yield a scientifically rigorous and informed evidence base, richer insights on the clonal disseminations of some species, and the genotypic characterization of antibiotic resistance mechanisms. The AMR challenges faced during Hajj are a strong argument to inform countries in the Middle East, Asia, and Africa to show unity of propose and to take forward the goals of the WHO AMR plan, through a multidisciplinary One Health animal, human, environmental, genetic, and societal approach, involving healthcare workers, researchers, epidemiologists, social scientists, and policy makers.

9 Opportunities for AMR Surveillance at Mass Gatherings

Hajj provides a one-stop global surveillance opportunity for all aspects of disease epidemiology [10, 76, 77]. One of the specific characters of Hajj is the ability to access diverse population groups from diverse regions of the globe congregated in a single venue within a specified timeframe. Due to the extreme variation in disease surveillance, control strategies, and availability of resources among home countries of pilgrims, there is no strategy in place to predict what condition, circulating pathogen, or AMR may find an appropriate environment for occurrence or transmission during Hajj. If AMR emerges in any one country that participates in Hajj, travel and trade activities increase mixing of pilgrims with the global community, and hence no country is safe from Hajj-related AMR transmission.

Therefore, Hajj pilgrims are at increased risk for disease acquisition or AMR transmission. When compared to the general population, the risk of adverse public health events among Hajj attendees is greater due to the lack of social distancing, high population density (e.g., airborne diseases), mixing of susceptible (e.g., unvaccinated) or immunocompromised persons with potential carriers of infections,

rituals or activities that increase disease transmission opportunities, unexpected extremes of weather, and demand for facilities exceeding establishment leading to mass injuries and deaths. These predisposing factors and event-specific attributes have highlighted the importance of passive surveillance of AMR at Hajj so as to help predict and prepare for adverse infectious disease outcomes.

To facilitate surveillance, the opportunities for enhanced large dataset collection during Hajj are emerging. Innovative rapid testing laboratory capacities (e.g., whole-genome sequencing), technological advances in data collection (e.g., machine learning), and electronic data on transportation and movements of people available for ticketed or visa-controlled events (e.g., tracking of movements through cell phone usage) are opportunities for surveillance that can allow tracking of movement of pilgrims to predict potential hotspots for infectious diseases outbreak. With a better understanding of the baseline AMR prevalence pre-Hajj and by subsequently monitoring for the same data during Hajj, one can potentially determine if AMR outbreak occurs and the source of such outbreaks. Such knowledge can help implement better control strategies to alleviate detrimental impacts associated with AMR.

10 Opportunities to Increase AMR Awareness Among Hajj Pilgrims

Some infections can be prevented by vaccines, which therefore would minimize the need for further treatment by antibiotics [78]. Hence the facilitation of vaccine uptake by Hajj pilgrims can reduce the potential for symptom development that leads to antibiotic consumption. Pilgrims should be educated on the risk of AMR and prudent use of antibiotics during travel medicine visits to receive vaccines or during pre-departure group seminars [79]. In addition, most of these pilgrims have to travel long distances to reach Saudi Arabia for Hajj. This long journey may yet provide another opportunity to further enhance their awareness on AMR, for instance, through brochures or related public health messages.

11 Conclusions

Antimicrobial resistance (AMR) has transitioned from being a medical challenge to a global development challenge, and its magnitude and intensity may be amplified by mass gatherings (MG), such as Hajj, to epidemic proportions. Multiple factors including demographics of Hajj pilgrims and sanitation infrastructure, among others, can potentially favor AMR dissemination. Several steps can be taken to minimize the burden of AMR at MGs. They include education of healthcare providers to minimize antibiotic usage, improve surveillance, and control effort and increase education among Hajj participants on appropriate use of antibiotics. International stakeholders

can advance the dialogue on these opportunities and develop relevant standards and guidelines to facilitate in fighting AMR globally.

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Human Movement and Transmission of Antimicrobial-Resistant Bacteria

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Abstract Antimicrobial resistance (AMR) is a major issue that poses a serious threat to global health. Recent reports from the World Health Organization (WHO) highlighted the increased threat from continuously emerging AMR organisms, accompanied by the paucity of development of new antimicrobial drugs. Low- and middle-income countries are likely to be the most affected, both in terms of the impact on public health and economic burden.

Recently there has been increased evidence that the global transmission of AMR pathogens is fueled by AMR carriage associated with human movement, including international travel and forcible displacement. This is alarming, with the United Nations World Tourism Organization (UNWTO) reporting that 1.4 billion people, accounting for 19% of the world's population, traveled across international borders in 2018. The number of tourists traveling to different destinations across the globe is predicted to increase to 1.8 billion by 2025. However, traveler population is not entirely formed of tourists, but it comprises different categories including forcibly displaced people and participants of mass gathering events.

In this chapter, we will discuss the contribution of traveler populations to the emergence and global dissemination of AMR bacteria. Specifically, we will highlight the contribution of special traveler populations, such as forcibly displaced refugees and attendees of mass gathering events, on the dissemination of AMR globally.

Keywords Antimicrobial resistance, Colonization, Enteric infections, Immunologically naïve, Mass gathering, Planed international travelers, Refugees, Vector-borne epidemics

1 Introduction

Antimicrobial resistance (AMR) is a major global challenge that is negatively impacting progress towards the sustainable development goals [1], including in health, food safety, poverty, and inequality. A recent report from the World Health Organization's (WHO) Global Antimicrobial Surveillance System (GLASS) demonstrated high levels of resistance to a number of serious and common bacterial infections (e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Salmonella* spp.) in both high- and low-income countries. Currently 700,000 deaths are associated with resistant infections every

year [2], with low- and middle-income countries likely to be the most affected, both in terms of the impact on public health and economic burden [2, 3]. The global resistance-associated mortality is estimated to top ten million people per year in 2050 if prevalence remains at today's levels, at a cost of US$300 million due to premature deaths and up to US$100 trillion lost from the global economy [2, 3].

The challenges of AMR are complex and multifaceted, particularly because the drivers of AMR exist and are interlinked between different ecologies, including humans, animals, plants, food, and the environment [4–7]. The use of antimicrobial drugs within one of these ecologies can promote the emergence of organisms that are resistant to these drugs elsewhere, which promotes the establishment of a resistance network [8, 9]. This network is driven by the complex interactions between clinical (human health, animal husbandry, and veterinary medicine) and other components including human activities (e.g., displacement, misuse, and/or overuse of antibiotics) and environmental factors (e.g., survival of AMR genes [ARGs] in water [including sewage and disposal] and soil) [10]. These multiple links between human, animal, and environmental components allow for the movement of residual of antimicrobial drugs, AMR bacteria, and mobile genetic elements and ARGs [5] (the resistance can move among distinct microbial species via ARGs [11]) among these ecologies, which enhance the dissemination of AMR.

At the same time, the continuous movement of people seems to play an integral role in the development and dissemination of AMR pathogens. Globalization and human migration have profoundly contributed to the dissemination of AMR infections as it allows for the rapid transportation of AMR bacteria and ARGs between different geographical regions [12–15]. The global transmission of AMR pathogens is fueled by AMR carriage associated with human displacement and international travel [12, 16].

Recently, there has been an exponential increase in the number of international travelers, driven mainly by tourists traveling to low- and middle-income destinations [17]. The United Nations World Tourism Organization (UNWTO) has reported that 1.4 billion people (19% of the world's population) traveled across international borders in 2018 [17], and this number is predicted to increase to 1.8 billion by 2025 [17]. According to World Tourism Organization (UNWTO, https://www.unwto.org/) data, 64, 67, 217, 343, and 713 million people have traveled to the Middle East, Africa, Americas, Asia and Pacific, and Europe, respectively, during 2018 [18]. This is challenging, with a recent WHO report on global surveillance of AMR warning that high levels of resistance are now found in all regions of the world [19].

Importantly, the traveling population is not entirely formed of tourists but can instead be categorized according to the purpose of traveling into various groups (subpopulations) that include medical tourists, migrant workers, immigrants, refugees, asylum seekers, and participants of mass gathering events (travelers who gather at specific locations for specific purposes and over a specific period of time [pilgrimages and sporting events such as Hajj and Olympic Games]). This consequently reflects variations in the demographic structure of each of these groups, medical status, and the traveling circumstances (i.e., before, during, and post-travel)

Table 1 Factors linking human movement and AMR transmission

Factor	Implication
Variations among individuals of migrating populations	Human gut microbiota community structure (commensal flora[a]) Human gut resistome[b] Socioeconomic standards[c] Health status of the traveler (e.g., vaccination coverage)
Purpose of the travel	Nature of the activities performed (e.g., tourism, mass gathering) Duration Exposure to particular health risks (e.g., medical tourism[d]) Preventive measures (e.g., vaccination, face mask) Travel medicine advice
Geographical region (origin and destination)	The prevalence of infectious diseases (e.g., colonization with pathogens and acquisition of infections) Rates of AMR in community and clinical settings (e.g., health-care facilities)[e] Level of residual antibiotics in food chain (e.g., farm animals and agriculture)[f] Level of circulation of AMR pathogens and existence of ARGs in environment (e.g., fresh water, soil[g], and sewage water) Community vaccination coverage Level of consumption of antimicrobial drugs and exposure to antibiotics[8] AMR detection (e.g., surveillance)
Living conditions during travel	Access to clean water and sanitation facilities (e.g., traveler diarrhea) Access to healthcare facilities (e.g., diagnosis and treatment of AMR infections) Hygiene and environmental health (e.g., hand wash) Accommodation infrastructure (e.g., proper housing, emergency accommodation)

[a]Gut commensals can also be MDR and could be a potential source of resistance functions of other enteric pathogens [20]
[b]Cultured and uncultured components of human gut flora contain many resistance genes [21]
[c]Recent study has highlighted the contribution of socioeconomic factors in the global dissemination of AMR [22]
[d]Close proximity to infectious agents
[e]Prevalence of drug-resistant pathogens varies greatly across geographical regions [12]
[f]Few studies suggested the horizontal transfer of AMR genes between soil bacteria and clinical isolates [23]
[g]The selection pressure through antibiotic exposure enhances the emergence of new AMR variants and the development of novel resistance mechanisms [4]

that they are exposed to and therefore their contribution to the development and the dynamic of the dissemination of AMR pathogens. The key demographic elements and travel-associated factors and their implications in promoting the acquisition and dissemination of AMR among international travelers are listed in Table 1.

Here we will discuss the contribution of international travel and human displacement to the emergence and dissemination of AMR bacteria globally. We will discuss the dynamics of transmission of AMR bacteria associated with special traveler groups (subpopulations) that are at high risks of exposure to high rates of AMR and those who can transmit highly resistant (multidrug resistant) strains.

2 Travel Promotes the Transmission of Infectious Diseases

Human movement can promote the introduction of pathogens into susceptible populations (immunologically naïve) or increase the contact between susceptible and infected populations, promoting the occurrence of outbreaks or increased disease prevalence. Human movements have been shown to play a key role in the transmission of viral (e.g., influenza, [24, 25] measles, [26] dengue, [27, 28] polio [29], Ebola [30]), bacterial (e.g., cholera [31]), and vector-borne (e.g., malaria [32]) diseases. Therefore many researchers have developed models that directly incorporate travel data into disease models to comprehensively and precisely determine the transmission dynamics of infectious diseases [33–35].

The transmission of pathogens associated with human movements seems to be controlled by many factors, including the structure and density of mobile populations, mobility patterns, time and duration of travel, nature of activities performed, and nature of infrastructures during transportation and at the destination (please review [36–39] for detailed discussion).

3 Transmission Patterns of AMR Bacteria Associated with Planned International Traveling

Recently we have systematically reviewed the literature to identify the impact of planned and desired international traveling on the global dissemination of AMR [40]. Studies conducted among forcibly displaced populations (e.g., refugees, immigrants, asylum seekers) and travelers participating in special mass gathering events (e.g., Hajj and other mass gatherings) were excluded from the analysis. A total of ~30,000 AMR bacterial isolates associated with ~17,000 instances of planned international travel (at least one AMR organism documented per instance) have been detected in traveler populations in the period between 1990 and 2019 [40]. *Enterobacteriaceae* members including carbapenem-resistant and extended-spectrum ß-lactamase (ESBL)-producing *E. coli* and *Shigella* spp., carbapenem-resistant *Acinetobacter baumannii*, and fluoroquinolone-resistant *Salmonella* spp. and *Campylobacter* spp. constituted 99% of the travel-related AMR isolates. These have been mainly associated with individuals from Europe and the USA who travel to Africa, Asia, and South America. Methicillin-resistant *Staphylococcus aureus*

(MRSA), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* constituted the majority of the AMR species transmitted by/associated with medical travelers [40]. This is alarming, with all of the resistant bacterial species detected being on the WHO lists of critically important or highly prioritized AMR bacteria (due to their impact on human health and the urgency for the development of new antimicrobial drugs to treat resistant infections) [19, 41].

The risk of travelers returning colonized or infected by AMR organisms varies by region visited, type of organism, and traveler population [12, 42]. There is increasing evidence that international travelers from high-income countries in Europe, North America, and Australia to low- and middle-income countries (Africa, Asia, and South America) are at a high risk of acquiring drug-resistant bacteria, particularly carbapenemase- and ESBL producing *Enterobacteriaceae*, and MRSA [16], and that travel therefore contributes to the dissemination of AMR bacteria globally [16].

The risk of travel-associated acquisition of enteric drug-resistant bacteria has substantially increased during the past two decades, with the magnitude of risk varying depending on the locations of travel [16]. Low- and middle-income countries located in Asia, particularly the Indian subcontinent, Africa, and South and Central America, have been associated with the highest risks of acquiring drug-resistant enteric infections (fecal-oral route) [16]. Based on a traveler population consisting of 29,000 individuals, the global ESBL colonization rate is estimated to be 14%, ranging from 2% in America to 46% in Asia and Africa [43]. Additionally, 60% and 0.4% of returning travelers from the Indian subcontinent have been found to be colonized with ESBL- and carbapenemease-producing *Enterobacteriaceae*, respectively [16]. Another study identified a 70% risk of ESBL colonization when travelers receive a combination of antibiotics and loperamide [44, 45]. This is alarming, with a number of studies demonstrating a long duration of colonization with ESBL producers (e.g., CTX-M9) for up to 12 months following travel [46]. Approximately 80% and 60% of the travelers returning from the Indian subcontinent with typhoid and non-typhoidal *Salmonella* infections, respectively, have been found to have reduced susceptibility to ciprofloxacin [47, 48]. Moreover, the development of traveler's diarrhea (TD) and the use of antibiotics and other drugs that have been widely used in the treatment of diarrhea during travel have been suggested to be among the key factors that promote colonization by drug-resistant enteric bacteria [45].

International travel and medical care while traveling have contributed to the global dissemination of MRSA, which is one of the most common healthcare-associated infections. However, the risk of any individual traveler acquiring MRSA is substantially lower than the risk of resistant infections associated with *Enterobacteriaceae*. The overall risk of acquiring MRSA is estimated to be 5.8 cases per 1,000,000 travelers, which ranged from 0.1 per 1,000,000 travelers to Nordic countries to a higher acquisition rate of 60 cases per 1,000,000 travelers to African and Middle East countries [49].

4 Forcibly Displaced Populations: Figures at a Glance

Forcible displacement refers to people who have been forced to move from their locality or environment and occupational activities because of different reasons, including armed and civil conflicts, political strife, or human rights abuses. The United Nations High Commissioner for Refugees (UNHCR) (https://www.unhcr.org/en-au/about-us.html) categorizes forcibly displaced people into asylum seekers (people who are waiting for the processing of their request for sanctuary), internally displaced (people who are forced to move but still remain within their own country), and refugees (people who have crossed an international border and cannot return home safely).

During the past two decades, there has been a substantial increase in the global population of forcibly displaced people from 34 million in 1997 to 69 million in 2018 [50]. This rise has been mainly driven by conflicts occurring in a number of countries in Asia (e.g., Syria, Yemen, and Iraq) and Africa (e.g., Burundi, the Democratic Republic of Congo, and South Sudan). The United Nations Refugees Agency (UNHCR, https://www.unhcr.org/) has found that armed conflicts in different regions of the world have been associated with an increase in the forcibly displaced population from 1 in 160 people a decade ago to 1 in 110 in 2018, the highest number seen since the aftermath of the Second World War [50].

In mid-2018, the global refugee population was 25.4 million people, including 19.9 million under UNHCR's mandate as well as 5.4 million Palestinian refugees under the United Nations Relief and Works Agency for Palestine's (UNRWA) mandate (https://www.unrwa.org) [50]. This is in addition to ten million stateless people who have been denied a nationality and access to basic rights such as education, health care, employment, and freedom of movement [50].

5 The Global Distribution of the Refugee Population

The most recent global distribution of the refugee population is shown in Fig. 1. At the end of 2017, 9.3, 5.8, and 1.2 million refugees are distributed across Asia, Africa, and Europe, respectively. These 16.3 million people represent 82% of the total refugee population under UNHCR's mandate, mostly originating from and hosted in low-income (developing) countries. The majority of refugees originated from ten countries, including Syria (6.3 million), Afghanistan (2.6 million), South Sudan (2.4 million), Myanmar (1.2 million), Somalia (986,400), Sudan (694,600), Democratic Republic of Congo (620,800), Central African Republic (545,500), Eritrea (486,200), and Burundi (439,300) [51]. In 2018, it has been estimated that 57% of the global refugee population worldwide originates from Syria, Afghanistan, and South Sudan [50].

According to the United Nations Statistics Division classification, the majority of refugee populations are hosted in low-income settings in developing countries.

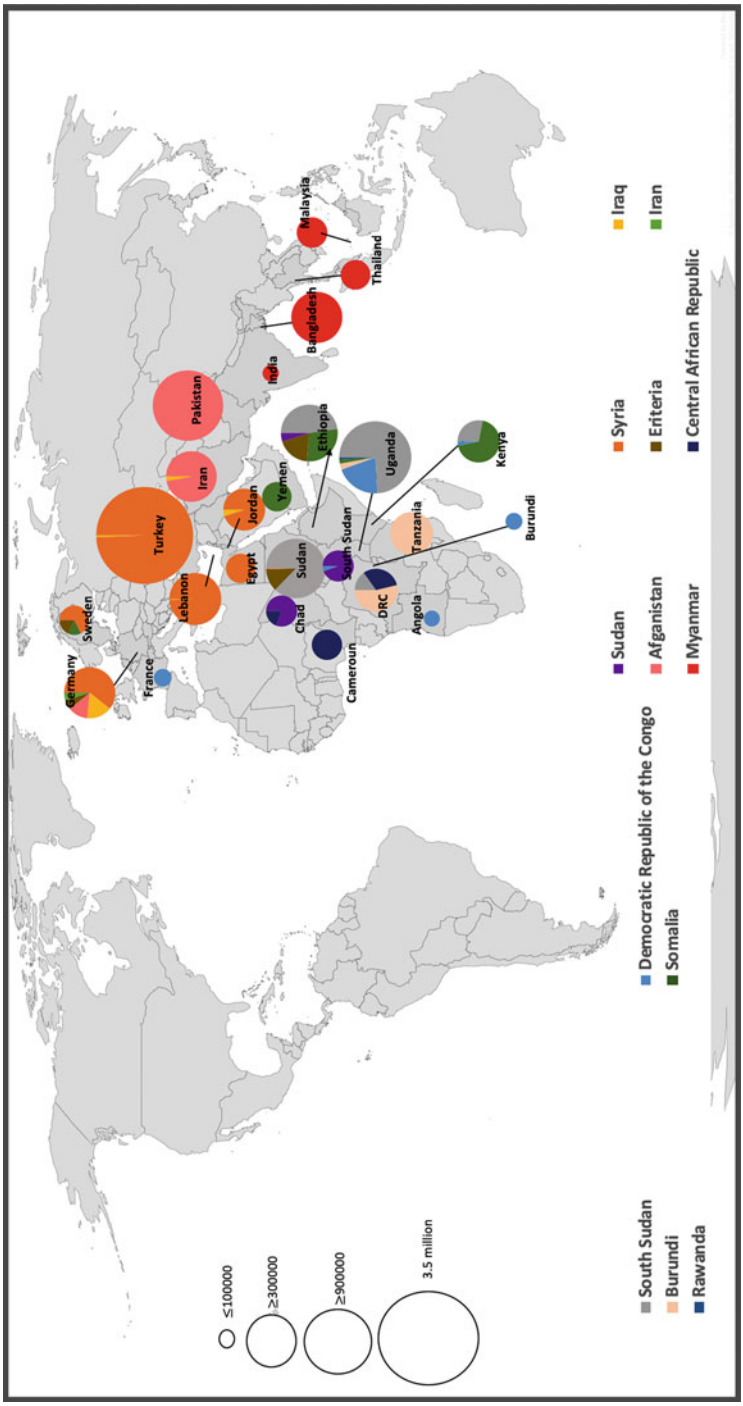

Fig. 1 The global distribution of refugee populations. The majority of refugees are originating from and hosted in low-income countries. The size of the pie charts correlates with the number of the refugees hosted in a particular country. The coloring of pie charts shows the distribution of refugee population in the host countries. The data displayed on the global map were extracted from the United Nations High Commissioner for Refugees (UNHCR), UNHCR's Statistics Database, and the United Nations Relief and Work Agency for Palestine's (UNRWA)

Moreover, some African countries (e.g., Ethiopia and Uganda) that have been classified among the least developed countries in the world are facing the challenges of large refugee flows. The top refugee-hosting countries include Turkey (3.5 million), Pakistan (1.4 million), Uganda (1.4 million), Lebanon (998,900), Iran (979,400), Germany (970,400), Bangladesh (932,200), Sudan (906,600), Ethiopia (742,700), Jordan (691,800), Kenya (523,500), and Chad (386,100).

While these countries are the main hosts, the refugee population is distributed globally. The distribution of the largest population groups, by country of origin, is discussed in the following section.

Syrian refugees, the largest population group by country of origin, have found asylum in 125 countries throughout the world, with the majority being hosted in Turkey (3,424,200), Lebanon (992,100), Jordan (653,000), Germany (496,700), Iraq (247,100), Egypt (126,700), Sweden (103,600), Austria (43,900), and the Netherlands (30,900) [51]. It has been reported that the second largest population of refugees is from Afghanistan and are distributed in 93 countries globally, with the majority currently being settled in Iran (951,100), Germany (104,400), and Austria (26,900) [51].

The majority of refugee population (or refugees) from South Sudan have settled in neighboring countries, including Uganda (one million), Sudan (772,700), Ethiopia (421,400), Kenya (111,500), and the DRC (89,000).

The majority of refugees from Myanmar have been hosted in Bangladesh (932,200), with sizeable populations also settled in Asian countries, including Thailand (100,000), Malaysia (98,000), and India (18,100) [51]. Kenya (281,700), Yemen (255,900), and Ethiopia (253,800) were the main host countries of Somalian refugees, while smaller groups resided in South Africa (27,000), Uganda (25,000), and Sweden (22,000) [51].

6 Refugee Living Conditions and the Transmission of Infectious Diseases

A discussed in the previous sections, many factors are associated with the transmission of infectious diseases among traveler populations. These include the baseline of endemic and epidemic diseases at the origin and in destination countries, living conditions of the traveling population (e.g., number, size, location, and density of settlements), availability of safe water and adequate sanitation facilities, medical status of the traveling population (e.g., nutritional status and immunization coverage among the population), and degree of access to health care and to effective case management. Many studies have highlighted the impact of poor living conditions in refugees' accommodations (on and post traveling) on human health and well-being, particularly the transmission of infectious diseases. In this section, the key factors that promote the transmission of infectious diseases (therefore increasing the

probability of the spread of AMR infections/pathogens) among refugee populations are discussed.

6.1 Adequate and Proper Housing

Adequate and proper housing directly and indirectly affects human health and well-being. Many studies have reported on the inadequate and improper housing and poor accommodation conditions in refugee camps [52, 53]. This includes high levels of overcrowding, with some studies finding that at least two children are sharing the same bed in up to 38% of families, with an average of 3.56 children per bedroom [54]. Other poor housing and accommodation conditions include poor ventilation, a lack of natural light, the presence of bugs and mold, exposure to insects (e.g., mosquitos) and other zoonotic carriers (e.g., rats), and a lack of or inadequate access to clean water and sewage systems [52]. These conditions promote the transmission of communicable diseases, particularly respiratory, enteric, and zoonotic infections. There have been significant associations between overcrowding and flooding and rainwater leakage (due to housing units with poor structural integrity) in refugee camps with the likelihood of reporting multiple health problems [55, 56]. For instance, few studies have demonstrated a link between the overcrowded accommodation in refugee camps and a number of illnesses, including the common cold, coughs, tonsillitis, and ear infections [57]. Acute respiratory infections, promoted mainly by crowding, smoking, and poor indoor air quality, have been associated with mortality rates of 28% and 38% in adults and children (<5 years) of Rohingya refugees living in refugee camp in Bangladesh, respectively [58].

6.2 Water and Sanitation

The availability of clean drinking water is one of the major challenges during emergencies and crisis settings. The international water accessibility limit of 500 m has not been achievable in most refugee camps [59]. Instead, refugees are relying on water sources of poor quality, including underground wells and rivers [53, 60]. Recently, quality testing of the water accessible by Rohingya in refugee camps in different settlements in Bangladesh showed that 92% of water samples have been highly contaminated with *E. coli*, with 48% of these samples containing at least 100 colony-forming units/100 ml, which indicates water contamination with human feces, probably due to the practice of open defecation near to the drinking water sources. These circumstances promote the transmission of waterborne diseases, such as cholera, and other fecal-oral viral (hepatitis A and E viruses) and bacterial (e.g., invasive non-typhoidal *Salmonella*, *Shigella* spp., and other diarrheagenic *E. coli*, (including the long water surviving enterotoxigenic *E. coli*) infections. Importantly this raises a high risk for the dissemination of AMR

organisms, with many studies highlighting water as being a major environmental reservoir of AMR genes [6, 61–66]. There have been increasing concerns regarding the availability of adequate sanitation systems and wastewater treatment in refugee camps [53, 60, 67].

6.3 Food Safety and Nutrition

Food safety and nutrition stress add further burdens to refugee populations hosted in transit camps, particularly to children aged less than 5 years, who are the highest risk age group for the development of diarrheal infections [68, 69]. Diarrheal infections caused by a variety of bacterial, viral, and parasitic agents are life-threatening among this age group of children and cause substantial morbidity, including malnutrition complications [70, 71]. Data from Rohingya refugee camps indicate that 52–57% of children aged 6–59 months have suffered from global acute malnutrition (GAM) [72]. Moreover, the proportion of children who require hospitalization due to severe acute malnutrition (SAM) complications have been estimated to be 7.5% [73].

Of particular concern is that a significant proportion of the refugee populations comprise children (<18 years old), who are among the most susceptible age groups at increased risk of acquiring infectious disease and other health risks. The distribution of refugees according to age and gender among the largest refugee populations in 2018 (60% of the global refugee population originating from Syria, Afghanistan, South Sudan, Myanmar, and Somalia) indicates that more than half of the population were children.

6.4 Access to Health Services

The refugee population comprises individuals at high health risks, including children, pregnant women, and the elderly, and those suffering from communicable and non-communicable diseases. The most frequent health problems identified in newly arrived refugees and migrants include accidental injuries, hypothermia, burns, gastrointestinal illnesses, cardiovascular conditions, pregnancy- and delivery-related complications, diabetes, and hypertension [74]. A recent study found that refugees self-rated health falls below the resident population, with 20% and 40% of male and female refugees, respectively, having unmet health needs [75]. However, sufficient attention has still not been paid to the health needs required for the increasing number of refugees worldwide [76]. Asylum seekers and those without documentation often fall between the cracks of health service providers and humanitarian relief programs [76]. Moreover, the access of forcibly displaced individuals to appropriate health services is affected by many factors, including poverty, stigma, discrimination, social exclusion, language, and cultural barriers, and these exclusions accumulate [77].

6.5 *Environmental Factors*

Refugee settlements often occur in environmentally sensitive areas (e.g., semiarid locations with minimal vegetation and limited access to sufficient water, or agriculturally marginal areas) that add more challenges to refugees' circumstances, particularly with respect to health status. Although environmental problems can exist anywhere, many reach an exaggerated scale where large numbers of people are forced together through a common sense of survival.

Many studies have highlighted the role of surface fresh and aquatic water, rural groundwater, and sewage in the deamination of AMR pathogens. The emergence of AMR is part of a complicated ecological and evolutionary network, with the use of antimicrobial drugs anywhere within the system potentially selecting for resistance to that drug elsewhere in the network [8]. Gram-negative bacterial resistance, in particular, is promoted through horizontal gene transfer by the acquisition of mobile elements [78–80]. There is also increasing evidence that ARGs found in human microbial communities are likely to have been acquired from an environmental source [6, 61]. These reservoirs include sewage systems, raw septic tanks, and marine and groundwater sources. The processing of human, farm, and industrial waste together has a significant impact on the emergence of AMR to a wide range of the most clinically effective antibiotics [62, 81]. In addition, even treated sewage samples discharged into rivers or lakes from treatment plants may contain significant concentrations of ARGs that enhance the development of AMR bacteria and raise major public health concerns [9, 63, 64, 82].

7 Natural Disasters and Transmission of Infectious Diseases

In 2018, it has been estimated that ~62 million people have been affected by natural disasters [83]. Access to clean water, sanitation, nutrition, shelter, and nonfood items are the most crucial domains for securing the health, and therefore the survival, of populations affected by crisis and emergency settings [84]. Natural disasters such as earthquakes, volcanic eruptions, tsunamis, and floods can have serious health consequences, particularly with the eruption of outbreaks and transmission of communicable diseases [85, 86]. The risk factors associated with population displacement (as a consequence of natural disasters) have been highlighted as the key determinants for the transmission of communicable diseases with outbreaks being rarely reported following natural disasters that do not result in population displacement [85, 87]. Substantial population displacements are usually associated with changes in the environment (particularly following natural disasters), human conditions, and the susceptibility of the affected population to the pathogens circulating in the environment [86]. Therefore risk factors including access to clean water and sanitation, crowding, health status of the displaced population, and access to health-care

services determine the dynamics of the transmission of communicable diseases in the affected population [85].

A recent systematic review found that natural disasters occurring in the period between 2000 and 2011 have been associated with the transmission of many infectious diseases, including diarrhea, acute respiratory infections, malaria, leptospirosis, measles, dengue fever, viral hepatitis, typhoid fever, meningitis, tetanus, and cutaneous mucormycosis [86].

Diarrheal diseases with a mortality rate of up to 40% have been identified as the leading cause of death in emergency sittings after disasters [86, 88]. The lack of access to clean water favors the emergence and spread of enteric infections (e.g., diarrhea and hepatitis) and waterborne diseases (e.g., cholera). For instance, the emergence of cholera epidemics in India and Bangladesh [89, 90] has been attributed to floods. In the United States, enteric pathogens including *Salmonella*, *Vibrio cholerae*, and norovirus have been detected among Hurricane Katrina evacuees [91–93]. A recent systematic review demonstrated that food- and waterborne diseases (including *E. coli*, *Campylobacter jejuni*, *Salmonella enterica*, and other pathogens (*Cryptosporidium hominis*, *Giardia lamblia*, and norovirus) have been the dominant infectious disease outbreaks associated with natural disasters in Europe [83]. However, a few studies demonstrated that the risk of the emergence of diarrheal disease outbreaks following natural disasters is higher in developing than developed countries [87, 94].

In addition, overcrowding, common in various displaced populations, can facilitate the transmission of respiratory illness (e.g., measles, influenza, and pneumonia) and person-to-person infections (e.g., *Neisseria meningitidis* meningitis). Meningitis outbreaks were documented after the tsunami in Aceh, Indonesia [95]. However, large meningitis outbreaks have been more common in forcibly displaced populations than disaster-affected populations, probably due to the variations in immunization coverage of the affected population [96]. Similarly, few measles outbreaks have been associated with natural disasters, mostly among susceptible populations living in crowded accommodation with low community vaccination coverage [95].

Additionally the transmission of vector-borne diseases (e.g., malaria and dengue fever) can be promoted by meteorological natural disasters (e.g., cyclones, hurricanes, and flooding) [97–99]. The occurrences of vector-borne epidemics after disasters are mainly associated with environmental factors including the disruption in water supply, waste management, and sanitary services [86].

Disaster-affected populations and refugees seem to face similar challenges, particularly due to substandard living conditions in refugee camps (in transit and at the destination) and short-term emergency accommodation established after crises. However, the contribution of disaster-affected populations to the transmission of infectious disease is localized (i.e., usually restricted to limited geographical regions). Environmental factors and community vaccination rates are key determinants of the transmission of infectious diseases in disaster-affected populations. Importantly, environmental factors have been reported to be the main drivers of outbreaks following natural disasters in Europe [83]. For example, exposure to

contaminated flood water and the contamination of rivers, lakes, springs, and water supplies due to heavy rainfall or flooding have been associated with several outbreaks [83].

8 The Impact of Refugees on the Global Transmission of AMR

As indicated above, refugees are a very special population of travelers. The refugee population is heterogeneous in nature (in terms of ethnicity, social grouping, age, and gender) comprising individuals originating from low-income settings who are suffering from challenging life circumstances that significantly increase the associated health risks. We have annotated the current traveling (migration) routes of the largest refugee population groups on a global map (adopted from a recently published study [100]) with all countries and territories color coded according to the predictions of AMR abundance (Fig. 2). It has been demonstrated that the majority of refugees have traveled from high to low AMR abundance regions, which is different from the migration (traveling routes) of tourists (travel from low- to high AMR abundance regions).

Previously we have systematically reviewed the literature to identify the potential impact of refugees and asylum seekers on the dissemination of AMR [101]. The analysis of the few relevant articles available in the literature [102–125] has suggested a relationship between AMR transmission and the displacement of refugees, with high percentages of AMR organisms being detected among different refugee populations [101]. We have updated the search to include recent relevant articles that have been published before January 1, 2019. Specifically, we searched the medical research databases including MEDLINE, PubMed, Embase, Scopus, and Web of Science Core Collection to identify primary research and observational studies reporting on the detection and transmission of AMR organisms among different refugee populations. In total, 24 articles describing observational and primary research studies and reporting on AMR carriage and/or infections among refugees were identified and reviewed. The characteristics of these studies and the main findings for the studied populations, geographical locations, types of AMR bacteria detected among different refugee populations, and methods used in the detection of AMR profiles are summarized in Table 2.

The AMR bacteria identified in the studied refugee populations included multidrug-resistant (MDR) *Mycobacterium tuberculosis* (with complex and variable AMR patterns), MRSA, and MDR *Enterobacteriaceae* including ESBL- and carbapenemase-producing strains. The AMR profiles associated with Gram-negative bacteria included ESBL-producers (*E. coli*, *Shigella* spp., and *K. pneumoniae*), carbapenemase producers (*K. pneumoniae*, *Acinetobacter baumannii*, *E. coli*, and *Pseudomonas* spp.), and MDR species (*A. baumannii*, *E. coli*, and *Pseudomonas* spp.).

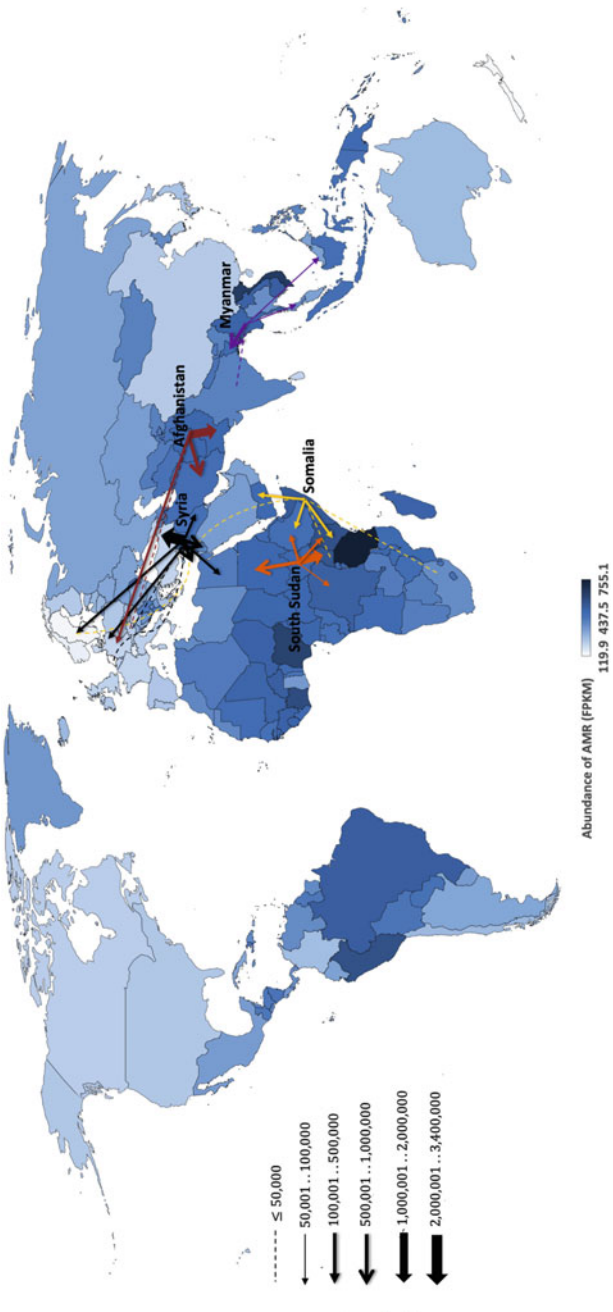

Fig. 2 Refugees displacement and the global transmission of AMR. The migration trends of refugees by largest population groups, by country of origin (Syria [6.3 million], Afghanistan [2.6 million], South Sudan [2.4 million], Myanmar [1.2 million], and Somalia [986,400]), are shown (arrows) on a global map. The arrow thickness correlates with the number of forcibly displaced individuals. The map is colored from light blue (low AMR abundance) to dark blue (high AMR abundance) according to the recently published [100] prediction of AMR abundance in all countries and territories

Table 2 Summary of studies detecting AMR bacteria in refugee populations

Study		Refugee population		AMR detection			AMR profile/organism					Study country
Period	Type	Total	Distribution (%)	Asymptomatic (%)	Infections (%)	Method	VE (%)	ESBL (%)	MR (%)	CPE (%)	MDR (%)	
2010–2017	Hospital	447[a]	Iraq (46.5) Afghanistan (10.3) Syria (9.6) Somalia (6.9)	201 (45)	10 (5)	Phenotyping and PCR	0	147 (32.9)/*E. coli*, *K. pneumoniae*	95 (21.3)	3(0.7)/ND	201(45)/ND 2(0.4)/*Pseudomonas aeruginosa* 2(0.4)/*A. baumannii*	Finland [102]
2017	Community	35	Middle East	2 (6)	–	Phenotyping	ND	ND	2 (6)	ND	ND	Germany [103]
2015–2016	Hospital	325	NN	32 (9.8)	–	Phenotyping			32 (9.8)			Germany [104]
2015–2016	Hospital	290	NN		–	Phenotyping		67(23.3)/ND		6 (2.1)/ND	30(10.4)/ *Acinetobacter* spp. and *Pseudomonas* spp.	Germany [104]
2015	Hospital	143[a]	Syria (50), Iraq,(20) Afghanistan (20) Somalia (10)	87 (60.8)	–	Phenotyping PCR	ND	34(23.8)/ *E. coli* 6(4.2)/ *K. pneumoniae*	ND	1(0.7)/ *K. pneumoniae 2(1.4%)* *A. baumannii*	38(26)/*E. coli* 6(4.2)/ *K. pneumoniae*	Germany [105]
2015–2016	Hospital	325[a]	Syria (51.8) Afghanistan (22.9) Iraq (21.7) Iran (1.2) Bosnia (1.2%) Nigeria (1.2)	110 (34)	11 (3.4)	Phenotyping	1(0.7)/*Enterococcus faecium*	68(50)/ *E. coli* 18(13.2)/ *K. pneumoniae*	22 (16)	ND	110 (99.9%)/ND	Germany [106]
2014–2016	Community	2091	Syria, Eritrea, Ethiopia, Iraq, Afghanistan, Middle East and South America	154–522 (7.4–25)	–	Phenotyping		240(11.4)/*E. coli* 26(1.2%)/ *K. pneumoniae*	185 (9.5)	1(0.4)/*E. coli*	*331 (18.5)/ Enterobacteriaceae*	Netherlands [107]

2013–2016	Hospital	107[a]	Syria	89 (83)	62 (58)	Phenotyping and PCR	3(3)/*Enterococcus*	83(78%)/*Enterobacteriaceae*	5 (5)	*10(9)/E. coli*	8(7)/*A. baumannii*	Israel [108]
2011–2013	Community	48	Syria	15–39(33–83)	–	Phenotyping	ND	1(2%)/*E. coli* 2 (4.1%)/*K. pneumoniae*	6 (12.5)	*4(8.3)/Pseudomonas*	ND	Italy [109]
2015	Community	119[a]	Afghani (67), Eretria (7.5), Somalia (5.8), Syria (5.8), Ethiopia (4.2)Iraq (3.3), Pakistani (2.5), Yemen (1.1).	42 (35)	–	Phenotyping	ND	42(35)/*Enterobacteriaceae*	ND	ND	9 (8)/ND	Germany [110]
2016	Environmental	44[b]	Sewage samples – Al-Qaa refugee camp			Molecular typing[c]	21(49)/*E. coli*	ND	ND	ND	22 (53.1)/*Pseudomonas* spp., *Acinetobacter* spp., *Pasteurella spp.*, *Aeromonas* spp., *Proteus* spp., *Klebsiella* spp., *Enterobacter* spp., and *Alcaligenes* spp.	Lebanon [111]
2015	Community	16[a]	Afghanistan (81) Iraq (12.5) Iran (6.2)	–	13 (81)	Phenotyping	ND	7(43)/*Shigella* spp.	ND	ND	ND	Greece [112]
2015	Community	21[a]	Afghanistan (66.6) Syria (23.8) Iraq (8.6)	–	21 (100)	Phenotyping	ND	11 (52.3)/*Shigella* spp.	ND	ND	5 (23)/*Shigella* spp.	Austria [113]
2011–2013	Community	27714[a]	Tibet	–	1,441 (5.2)	Phenotyping	ND	ND	ND	ND	1,441(5.2)/*M. tuberculosis*	India [114]
2011–2013	Community	345	Syria	–	61 (18)	Phenotyping	ND	ND	8 (42)	11(36.7%)/*A. baumannii* *E. coli*	20(66)/*A. baumannii* *E. coli*, *Pseudomonas* spp.	Jordan [115]
2010–2011	Hospital	307	Tibet	–	264 (85.9)	Phenotyping	ND	ND	ND	ND	51(46.9)/*M. tuberculosis*	India [116]

(continued)

Table 2 (continued)

Study		Refugee population		AMR detection			AMR profile/organism					Study country
Period	Type	Total	Distribution (%)	Asymptomatic (%)	Infections (%)	Method	VE (%)	ESBL (%)	MR (%)	CPE (%)	MDR (%)	
2011	Hospital	1	Libya	–	1(100)	Molecular typing[d]	ND	1(100)/ *K. pneumoniae*	ND	1(100)/ *K. pneumoniae*	1(100)/ *K. pneumoniae*	Italy [117]
2008–2010	Hospital	25	Sub-Saharan	–	23 (92)	Phenotyping	ND	ND	ND	ND	2(8.6)/ *M. tuberculosis*	Israel [118]
2005–2006	Hospital	1	Chechnya	–	1 (100)	Molecular typing	ND	ND	ND	ND	1(100)/ *M. tuberculosis*	Austria [119]
2004–2005	Community	15,455	East & Southeast Asia (Hmong)	–	272(1.7)	Phenotyping	ND	ND	ND	ND	24(8.8)/ *M. tuberculosis*	Thailand [120]
1999–2006	Community	7,722	North Korea	–	78 (1.01)	Phenotyping	ND	ND	ND	ND	33(42.3)/ *M. tuberculosis*	South Korea [121]
1999	Community	76	East Timor	–	6 (8.6)	Phenotyping	ND	ND	ND	ND	6(8.6)/ *M. tuberculosis*	Australia [122]
1995–1999	Community	100	Cambodia, Iraq, Lithuania and Somalia	–	53(22.6)	Phenotyping	ND	ND	ND	ND	4 (7.5)/ *M. tuberculosis*	UK [123]
1995–1996	Community	241	Somali Kenyans, Sudanese, Ethiopians	–	7(2.9)	Molecular typing	ND	ND	ND	ND	7(2.9)/ *M. tuberculosis*	Kenya [124]
1980–1981	Community		Southeast Asia	–		Phenotyping	ND	ND	ND	ND	12/*M. tuberculosis*	US [125]

[a]Study population includes children
[b]Total number of sewage samples collected
[c]MLST and detection of AMR genes by PCR
[d]Sanger sequencing of AMR genes, *NN* not provided and *ND* not determined

9 The Dynamics of AMR Transmission Associated with Refugee Migration

The available data summarized in Table 2 demonstrate relatively elevated rates of colonization/infection with AMR organisms among different populations of refugees. However, these data do not fill all of the existing knowledge gaps regarding the contribution of refugee displacement to the transmission of AMR bacteria. All of these studies contain several key limitations (please review detailed discussion below). Together, they raise significant questions and concerns that highlight the need for further evidence to guide policies.

Importantly, the actual impact of refugee migration on the global AMR burden and the dynamicity of transmission have not been fully identified yet. The role of refugee displacement on the circulation of AMR includes the importation of AMR to the refugee hosting countries and the exposure of refugees to increased risk of colonization of, or infection with, AMR organisms during their journey or even at the destination. Although most of the relevant data attribute the increasing role of forcibly displaced populations (including refugees) in the dissemination of AMR organisms to their potential to import AMR infections/organisms from refugee countries of origin to their destinations, recent studies have also highlighted the important role that refugee community settings (e.g., refugee camps, transit centers, detention facilities in transit and in host countries) may play in promoting AMR transmission though increasing the rates of AMR carriage and infections [126]. Poor conditions in refugee settings include inadequate sanitation, overcrowding, and restricted access to health services, which all provide an ideal environment for the emergence and transmission of AMR organisms [88, 127, 128] (please see Table 1). A few studies have suggested that these conditions may influence AMR dissemination even more than the direct importation of resistance by refugees from their country of origin [126].

10 The Impact of Refugees on the Transmission of Drug-Resistant Tuberculosis

Recently, the WHO framework for TB elimination highlighted the importance of implementing targeted interventions to control the transmission of MDR and extensively drug-resistant (XDR) TB infections among highly susceptible populations, including immigrants, asylum seekers, and refugees [129, 130]. These forcibly displaced populations are often exposed to major challenges before, during, and after travel that promote the transmission of TB [131, 132]. These include poor living conditions in migration camps, discrimination, and financial issues. Importantly, these vulnerable populations of refugees are migrating from low-income settings of high TB incidence to high-income settings in Europe that are also characterized by moderate to high rates of TB incidence.

The overall flow of the refugee population during 2015 demonstrated that the majority of people were migrating from high TB incidence countries, such as Nigeria, Afghanistan, and Somalia. TB incidence rates ranged from 125 to 332 per 100,000, with MDR-TB incidence rates ranging from 6% (Syria) to 17% (Nigeria) of new cases. The majority of the migrating population in 2015 were hosted by eight European countries, including France, Germany, Italy, Russia, Spain, Ukraine, and the United Kingdom [133]. This raises major concerns as migrants from high TB incidence countries usually have higher rates of TB than native European populations [134] and have a higher probability of developing TB due to latent TB acquired in the country of origin or en route to their destination [135]. The 2015 TB notification data showed that 21% (120,000 out of 580,000) of the currently existing MDR-TB cases worldwide occurred in the WHO European region [131, 132, 136]. Moreover, the European Centre for Disease Prevention and Control (ECDC) has recently reported that 25% of new TB cases have been identified in foreign-born individuals in France, Germany, Spain, and the United Kingdom, contributing for ~75% of all migrant cases [129].

11 Refugees and Transmission of AMR *Enterobacteriaceae* Strains

The available data indicate that refugees admitted to health-care facilities, both during transit or in host countries, frequently carry MDR pathogens, particularly Gram-negative bacteria including *Enterobacteriaceae* strains [137]. High rates of MDR carriage have been detected among Syrian refugees undergoing surgery and/or medical treatment in Israeli hospitals, with 47% of the 60 adults being found to carry at least one MDR pathogen (these MDR profiles included carbapenem- and ESBL-producing *Enterobacteriaceae* isolates and *A. baumannii*) [138]. The rates of MDR carriage have been even higher among children, with ESBL-producing *Enterobacteriaceae* detected in 66% of 29 wounded Syrian children upon admission screening [138]. In another study, 83% of 107 wounded Syrian children were found to be colonized with an MDR pathogen (included NDM- and ESBL-producing *Enterobacteriaceae* isolates) upon admission [108]. A recent study that was conducted among 459 refugee patients from Syria detected carbapenemase-producing *Enterobacteriaceae* in 6.5% of the patients (30 out of 459) [139], with 53% and 31% of the detected isolates identified as *Klebsiella pneumoniae* and *E. coli*, respectively [139]. In another study conducted in Jordan on wounded Syrian refugees, MDR isolates were detected in 69% of patients (31 out of 45), with Gram-negative bacteria being the causative agents in 56% of cases [115].

MDR infections have been also detected in refugee camps. MDR *Shigella* isolates (ESBL producers and resistant to ciprofloxacin and azithromycin) have been detected in children hosted in refugee camps in Austria and Greece [112, 113]. Some studies suggested that the rates of ESBL-producing *E. coli*,

ESBL-producing fluoroquinolone-resistant *E. coli*, and ESBL-producing *K. pneumoniae* and MRSA among refugees were more than fourfold the respective rates among non-refugee populations hospitalized during the same period [105]. German authorities have recommended preventive isolation and MDR organism screening of refugees upon admission to health-care facilities. However, recommendations have not been made yet for the screening of MDR organism during the initial registration of refugees [140].

This high rate of MDR carriage reflects the high rate of circulation of resistant pathogens among healthy populations and also in health-care systems in the refugees' countries of origin. However, the acquisition of MDR pathogens (via human-to-human transmission, poor hygiene, limited access to health-care service, and living conditions) during the forcibly displaced journey cannot be ruled out.

12 Knowledge Gaps on AMR Transmission Trend Associated with Refugee Migration

Studies addressing AMR in refugee populations have been quite limited to date, and significant knowledge gaps remain. Studies have been restricted with respect to the structure of refugee populations (in terms of size and diversity) enrolled in these studies, living conditions and itinerary of travel, and the methodology used in diagnosis/detection of AMR profiles.

Although the majority of refugees have been hosted in developing countries, the majority of the studies in the literature have been conducted and completed at single sites in tertiary health-care facilities in high-income settings in European countries. This results in a clear knowledge gap with respect to the trend of AMR associated with refugees hosted in low-income countries. Additionally, the majority of the studies were conducted in hospitals with only a few studies, which have been entirely focused on *M. tuberculosis*, being conducted in refugee-associated community settings. All studies have been conducted in clinical settings except for one environmental study that was conducted to investigate the circulation of ESBL-producing *E. coli* in wastewaters collected from a refugee camp in Lebanon [111]. The lack of accurate and comprehensive figures for the AMR trend in refugee community settings is another large information gap, with recent studies highlighting the potential benefits of screening for AMR colonization and infections in community settings rather than only in health-care facilities [105, 109, 141].

Traditional microbiology approaches and phenotyping (bacterial isolation followed by antimicrobial susceptibility testing) have been the most common diagnostic methodology used. These culture-dependent methods allow for the determination of resistance of an isolated pathogen to a given antimicrobial. Therefore they are selective and time-consuming methods (from 12 h at their fastest to days). Also these methods have very limited capabilities in characterizing the associated mechanism of resistance. In addition, it was noted that the etiologic agents and complete

AMR profiles have not been comprehensively identified in the majority of these studies (please see Table 2).

Although the numbers of subjects enrolled in most of these studies were relatively small, they were diverse in terms of age, gender, and country of origin (diverse background and different socioeconomic living conditions). Importantly, the lack of any information on refugees' itineraries (detailed information on the route taken during the journey trajectories, including the length of time spent in transit and at destination) was a common limitation of most of the studies addressing this issue to date [142].

The ECDC has called for improved prevention efforts through the implementation of hygiene and targeted interventions in refugee community settings to prevent the transmission of AMR [143, 144]. The targeted initiatives enabling the timely detection and treatment of AMR organisms in refugee community settings align with existing global frameworks, including the WHO Global Antimicrobial Resistance Surveillance System [145], the WHO draft framework of priorities and guiding principles to promote the health of refugees and migrants [76] and calls for explicit migrant health policies to address inequalities [146].

13 Hajj-Associated AMR Enteric Infections Pose Serious Threats

Recent studies have demonstrated that pilgrims are at high risk for acquiring and transmitting drug-resistant enteric pathogens [147], including multidrug-resistant *Acinetobacter* spp., carbapenemase-producing *E. coli* [148], and extended-spectrum cephalosporin- and colistin-resistant non-typhoidal *Salmonella* [149]. For example, there are concerns that Hajj, the annual Muslim pilgrimage to Mecca, Saudi Arabia, may be a focal point for the acquisition, emergence, and global dissemination of drug-resistant infections.

Hajj is a recurrent planned mass gathering event that attracts ~3 million pilgrims from 188 countries around the globe. This enormously diverse population of pilgrims and the nature of Hajj activities, including the use of crowded accommodation, prolonged stays in tents, using shared toilets, eating food that may be prepared to low hygienic standards, and potential contact with asymptomatic carriers of infectious agents, may facilitate the transmission of infectious agents within susceptible populations. This could subsequently increase the emergence and dissemination of airborne, foodborne, and zoonotic infectious diseases within the host country and globally [150].

Both pharmaceutical and non-pharmaceutical preventive measures have been used to help control the transmission of infectious diseases in Hajj settings. Specifically, vaccination has been used successfully to control the transmission of meningococcal disease during Hajj. The current Hajj vaccination policy includes mandatory vaccination for all pilgrims against meningococcal disease [151]. The

Saudi health authority also strongly recommends seasonal influenza vaccination for all pilgrims, particularly those at high risk of infection complications [151]. However, with the exception of the cholera vaccination that is mandatory for the pilgrims from cholera-affected countries [151], no other vaccines for enteric infectious diseases are either mandatory or recommended for Hajj pilgrims, and few are available in any case.

Despite substantial advances in food and water hygiene in Hajj settings, mass gatherings still represent the ideal environment for the transmission of enteric infections [152]. We have conducted the first large-scale epidemiological study to identify the etiologic agents associated with Hajj-diarrheal infections [147]. In this study, we used integrated antigenic and molecular approaches to screen 544 fecal samples from symptomatic pilgrims during three consecutive Hajj seasons for 16 pathogens associated with diarrheal infections. Our data demonstrate that Hajj-associated diarrheal disease is associated with mild illnesses caused mainly by single bacterial agents (bacteria were the main agents detected in 83% of the positive samples), with enterotoxigenic *E. coli*, *Salmonella* spp., and *Shigella*/enteroinvasive *E. coli* (EIEC) being the major contributors. *E. coli* was the most frequent species detected; it was identified in 43% of the bacteria-positive samples. Enterotoxigenic *E. coli* was the most common serovar (detected in 25% of the positive samples), followed by enteropathogenic *E. coli* (4%), diffusely adherent *E. coli* (4%), and enterohemorrhagic *E. coli* (2%). The study demonstrated a relationship between the severity of diarrheal disease and the etiologic agents. The percentage of ETEC-positive samples was significantly higher in patients with severe cases as compared with those of mild cases (odds ratio 5.49; $p < 0.01$).

The most commonly identified bacteria in this study have all been identified by the WHO as being among the bacterial species of most serious impact on public health. Of particular concern was the presence of ESBL and carbapenemase genes in ~40% of both *Salmonella* spp. and *E. coli*-positive samples collected [147].

14 The Advantage of the Use of One Health Genomics to Tackle the AMR Dissemination Associated with the Refugees' Displacement

Resistant bacteria in humans, animals, or circulating in the environment can spread from one reservoir to the other and between geographical regions through human movements. Therefore the WHO, the Food and Agriculture Organization of the United Nations (FAO), and the World Organisation for Animal Health (OIE) recommend the "One Health" approach as a holistic and multisectoral approach to tackle the increasing global threat of AMR. This, backed by recent advances in genomics technologies, is proving very effective for comprehensively investigating the emergence and transmission of AMR bacteria in different environments. Advances in whole-genome sequencing technologies and bioinformatics analyses

have shifted the paradigm of diagnosis of infectious diseases from time-consuming and limited microbiology approaches to sequence-based methodologies. These approaches are characterized by high discriminatory power due to a high resolution up to single nucleotide level, which distinguishes between closely related strains and therefore helps in the establishment of accurate epidemiological links [153, 154].

Whole-genome sequencing (WGS) is increasing our understanding of the evolutionary dynamics of AMR, dissemination within bacterial populations, and the specific dynamics by which AMR organisms are emerging, persisting, and transmitting within and between hosts and other environments [154]. WGS has been used successfully to track the geographic dissemination of AMR clones and to identify the associated AMR genetic determinants in both health care and community settings. For instance, WGS has identified the link between the acquisition of SCC*mec* determinants and CTX-M-15 carrying plasmids and the emergence of MRSA and MDR *E. coli* infections, respectively [155, 156]. Moreover, genomics has been used to identify the origin and expansion of MDR Gram-negative bacterial clones, including dysentery caused by a *Shigella sonnei* clone with a chromosomal insertion of a mobile genetic element encoding resistance to streptomycin, trimethoprim-sulfamethoxazole, and tetracycline [154]; clone H58 of *Salmonella* Typhi with a plasmid encoding resistance to chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole, streptomycin, and tetracycline [157]; and health care-associated *Klebsiella pneumoniae* clone ST258, carrying the KPC carbapenemase encoding resistance to all ESBL, including carbapenems and third-generation cephalosporins [154]. Curated ARG databases such as the Comprehensive Antibiotic Resistance Database (CARD) [158] and ARG-Annotation (ARG-ANNOT) [159] have become valuable resources for the fast identification of resistant determinants from whole-genome sequencing data. Recently, genomics have been used to characterize an NDM-1 positive ESBL-producing *K. pneumoniae* isolate that was recovered from a urine sample collected from asymptomatic (for urinary tract illness) adult refugee from Syria [160]. New approaches are needed for the comprehensive characterization of multidrug-resistant communities (in terms of microbial diversity, population structure, and potential impact on infection establishment and transmission), to detect non-cultivable AMR bacteria and explore their potential impact on disease dissemination.

In a recent study, the resistomes and microbiomes of refugees from Syria, Iraq, and Afghanistan were characterized and compared to those of German residents (controls) [161]. Briefly stool samples collected from refugees (400 samples) and controls (100 German residents) were subjected to PCR-based quantification (TaqMan-based quantification of ARGs) of 42 of the most relevant ARGs and 16S ribosomal RNA gene. Culture-based validations of MDR bacteria were also conducted. The analyses demonstrated increased prevalence of most ARGs in refugees compared with controls. The majority of refugees carried five or more ARGs, while the majority of controls carried three ARGs or fewer. Interestingly, high prevalence rates of beta-lactamase genes including bla_{TEM} (88%), $bla_{\text{CTX-M-1}}$(43%), bla_{SHV} (35%), and $bla_{\text{OXA-1}}$ (19%), the quinolone-resistant gene *qnrB* (29%), and glycol-peptide resistance gene variant *vanC1* (15%) were detected in

refugee samples [161]. 16S rRNA gene amplicon sequencing analysis demonstrated that the gut microbiome of refugees contained significantly higher abundances of *Bacteroidetes* and *Proteobacteria*, while *Firmicutes* and *Actinobacteria* were significantly more abundant in German controls [161].

Shotgun metagenomics allows the identification of taxonomic composition, structural variations, and metabolic potential of the sampled microbial communities [162]. The use of standardized metagenomic approaches will allow for the assessment of the abundance, distribution, and persistence of AMR microbial populations in refugee populations and identify the factors that impact upon AMR persistence in associated environments. Additionally, the use of a metagenomic approach in monitoring environmental persistence of ARGs will ultimately allow for better understanding of the molecular basis and the dynamics of AMR transmission and therefore the development of real-time detection of novel emerging AMR variants.

Many studies have highlighted the role of sewage as a major environmental reservoir of AMR, as it represents an ideal environment for AMR bacteria and ARGs to persist [163]. Recently, a pioneer study proposed the metagenomic analysis of untreated sewage samples as an effective approach to comprehensively track and predict the global dissemination of AMR bacteria [100]. The authors of this study have developed a standardized metagenomic protocol that has been used to characterize the bacterial resistome contents and the differences in abundance and diversity of AMR genes in untreated sewage samples collected from 79 sites in 60 countries [100]. This study demonstrated variations in the AMR gene abundances in high-income settings in Europe, North America, and Oceania and low- and middle-income settings in Africa, Asia, and South America. The variations in AMR gene abundance were found to strongly correlate with socioeconomic, health, and environmental factors. This approach can be applied in challenging settings, such as refugee communities and mass gathering accommodations, to study the dynamics of the emergence and dissemination of AMR bacteria and, therefore, help in developing management strategies [163].

15 Conclusion

There is growing evidence for an association between human movement (migration) and the dissemination of AMR pathogens and ARGs globally. The magnitude of transmission is mainly determined by multiple interacting factors that include the demographic structure of migrating populations, the baseline of endemic and epidemic diseases and rates of AMR at the origin and destination, the living conditions of the migrating populations, the nature of activities performed while traveling, and the travelers' access to health-care services. However, the dynamics of AMR transmission seem to vary according to the migrating population, with recent data demonstrating that special traveler populations, such as refugees and mass gathering

attendees, are at a particularly high risk of exposure to and transmission of AMR. Emerging methodologies such as One Health genomics are fundamental for holistically tackling the rising threat of AMR.

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