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Chapter 6

DETECTION OF MULTIDRUG-RESISTANT *ESCHERICHIA COLI* STRAINS WITH VIRULENCE PATHOTYPES ISOLATED FROM URBAN RIVERS LOCATED AT RIO DE JANEIRO METROPOLITAN AREA, BRAZIL

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ABSTRACT

Escherichia coli are the most common inhabitants of gastrointestinal tract and highly affected by the propagation of resistance and virulence genes in urban rivers. This species are the major cause of community and nosocomial infections especially when are related to resistance and virulence genes associated with biofilm formation. This study demonstrates the presence of MDR *E. coli* isolates presenting virulence pathotypes with ability of biofilm formation in urban rivers located at Rio de Janeiro metropolitan area, Brazil. A total of 48 *E. coli* were isolated and 79% were classified as MDR. *E. coli* strains presented both resistance genes coding for ESBL, AME and PMQR and virulence pathotypes for EAEC, EIEC, ETEC EPEC and EHEC. All strains were able to form biofilm but in different levels. The presence of *E. coli* isolates with these characterisctics is found worldwide and has been considered a health care problem due to the increase of community and nosocomial infections.

Keywords: *Escherichia coli*, aquatic environment, virulence pathotypes, resistance genes, biofilm formation

INTRODUCTION

The use and consumption of water is essential to living beings biological activities. Due to increased world population, aquatic environments have been contaminated, not only with waste and products from human activities but also for antimicrobials and chemical products from hospitals and industries (Canepari, Pruzzo, 2008). Ingestion or use of contaminated water is considered a major public health problem, especially in developing countries where sanitary conditions and water treatment are inadequate. This contaminated environment can lead to infections such as typhoid fever, shigellosis, cholera, salmonellosis, hepatitis A, amoebiasis, giardiasis, and others gastroenteritis (Freitas et al., 2001; Bortoloti et al., 2018).

Microorganism monitoring is an important indicator of drinking water quality (Freitas et al., 2001; Yassin et al., 2006). In many countries around the world, including Brazil, this monitoring occurs through the isolation and quantification of *Enterobacteriaceae* family, including *Klebsiella*

pneumoniae and *Escherichia coli* (Suzuki et al., 2019; Brazilian Ministry of Health, 2004). Aquatic environments are considered as reservoir of acquisition and transferability of resistance and virulence genes in microorganisms of different genera and species (Suzuki et al., 2019; Kittinger et al., 2016).

Escherichia coli strains are the most common inhabitants of gastrointestinal tract and are considered the main indicator of fecal contamination due to their close relationship with human and animal feces representing the basis of fecal contamination tests used in Public Health. *E.coli* is highly affected by the propagation of resistance and virulence genes in urban rivers when compared to others *Enterobacteriacea* due to both genetic and phenotypic diversity (Tortora, Funke, Case, 2008, Kittinger et al., 2016).

The growing spread of multidrug-resistance (MDR) strains associated with the lack of new drugs development is a global concern. It is stipulated that by 2050 antimicrobial resistance will be responsible for 10 million annual deaths with a global economic impact of approximately $ 100 trillion (O'Neil, 2016). However, few studies investigate the microbial resistance profile in aquatic environments, underestimating the presence of multidrug resistant (MDR) microorganisms, and their potential to cause infections (Bortoloti et al., 2018).

Antimicrobial Resistance

Enterobacteriaceae species are one of the most significant causes of nosocomial and community acquired infections, related directly to emergence and dissemination of resistance mechanisms. The presence of emergence drug resistance bacteria is a result of indiscriminate antimicrobials use in human, veterinary medicine and agriculture, becoming a serious worldwide public health concern (Koczura et al., 2012; Stalder, Loncaric, Walzer, 2014).

Escherichia coli are the most common enterobacteria isolated in clinical laboratories and are a major cause of community and nosocomial infections.

They usually inhabit the gut microbiota of humans and other mammalian animals, and can be beneficial or harmful to health host, depending on where they are found in the organism. These isolates are related not only to bloodstream and urinary tract infections but also with others severe infections and some subspecies known as enteropathogenic or enteroinvasive, could cause gut disease (Livermore, 1995; Medeiros, 1997; Mbelle et al, 2019).

Antimicrobials bacterial resistance is widely researched due to the high importance for public health. The indiscriminately use of antimicrobials in therapy leads to increase of bacterial resistance, including the *Escherichia* genus. The spread and emergence of MDR *Enterobacteriaceae* is a big concern worldwide due to their correlation with community and nosocomial acquired infections (Mbelle et al., 2019; Linciano, 2019).

The activity of antimicrobial agents is influenced by site of action, absorption rate, penetration power in the cell and specific metabolism agent. Therefore, their activity can be affected not only by the drug interaction and pathogen but also host and pathogen. MDR pathogens may be inserting into environments by several human activities such as incorrect disposal of medication, release of untreated waste from pharmaceutical industry and indiscriminate use of drugs in nosocomial, community and veterinary sites. Most antimicrobials are absorbed or metabolized poorly and administered dose is excreted after hours leading to antimicrobials resistance propagation (Stoesser, et al., 2016; Aslantas, 2017; Christou et al., 2017; Cristovão et al., 2017).

The antimicrobial resistance analysis in commensal and pathogenic *Escherichia coli* is considered an excellent indicator of antimicrobial selection in the environment. Recent evidence detected resistance genes to all available antimicrobials classes used in animals. However, there are multiple reports of *E. coli* resistant to antimicrobial no long used in animal production, characterizing possible environmental and food contamination. Data revealed the presence of MDR *E. coli* are most commonly found in community rather than healthcare settings, being necessary to implement surveillance measures that apply to public and environmental health in order

to limit the spread of possible pathogens (Schierack et al., 2013; Mbelle et al., 2019).

Antimicrobial resistance genes can spread through mutation in chromosomal DNA or horizontal gene transfer of mobile elements. This generates cross-resistance to same class drugs, as observed at bacteriostats group, which includes sulfonamides, tetracyclines, aminoglycosides and macrolides. Dissemination of MDR can be mediated by several mechanisms including poor bacterium penetration which minimizes the antimicrobial intracellular concentration; antimicrobial target modification by genetic mutation; post-translational target modification and antimicrobial inactivation by hydrolysis or modification. Some examples of these mechanisms are: Beta-Lactamases enzymes against Beta-Lactams antimicrobial, Aminoglycoside Modifying Enzymes (AME) and Plasmid Mediated Quinolone Resistance (PMQR) (Quinn et al., 2005; Blair et al., 2015; Partridge, 2015; Adler et al., 2016; Iredell, Brown, Tagg, 2016).

The Beta-lactam group is the most used to treat infections. They inhibit cell wall formation after bind irreversibly to penicillin binding proteins (PBP), stopping the transpeptidation step on peptidoglycan synthesis. The main beta-lactam resistance mechanisms are beta-lactamases enzymes, coded by *bla* genes that inactivate beta-lactam ring hydrolysis. They are classified according to amino acid sequences, into enzymes class A, C, and D, which use serine for beta-lactam hydrolysis (SBLs) and class B metalloenzymes (MBLs), which requires zinc ions divalent for substrate hydrolysis (Blair et al., 2015; Partridge, 2015; Iredell, Brown, Tagg, 2016; Linciano et al., 2019).

The most important resistance mechanisms among *Enterobacteriaceae* family and in this study are Class A enzymes beta-lactamases production. This class includes narrow spectrum enzymes, such as bla_{TEM} and bla_{SHV} genotypic groups that imprint resistance to penicillins and cephalosporins, extended-spectrum beta-lactamases (ESBL) that have activity against oxyimino-cephalosporins like $bla_{CTX\text{-}M}$ genes and other inhibitor-resistant variants. ESBL enzymes have capacity to hydrolysis third generation cephalosporins antimicrobials, and their resistance worldwide is related to availability, correctly use and disposal of antimicrobial agents, general

standard of living, healthcare and waste/water management (Blair et al., 2015; Stalder, Loncaric, Walzer, 2014; Partridge, 2015; Iredell et al., 2016).

MDR gram-negative bacteria, such ESBL carbapenemases producing are prevalent worldwide and may cause high mortality due to fewer antibiotic options available to treat this kind of infections. In many ESBL-producing bacterial cases, even common infections, such as urinary tract infections, requires a more complex treatment leading to hospitalization and intravenous carbapenems antibiotics. Carbapenems are the remaining antibiotics able to treat ESBL-producing bacteria, however resistance enzymes able to destroy these antibiotics are on rise (Medeiros, 1997; Liu et al., 2019).

Antimicrobials from aminoglycoside family are complex compounds with aminocyclitol nucleus linked to amino sugars through glycosidic bonds, for example, gentamicin, amikacin, tobramycin and streptomycin. Despite their broad spectrum activity, they are especially used to treat MDR gram-negative bacteria infections. They act binding the aminoacyl-tRNA recognition site (A-site), decoding ribosome 16S rRNA centre, interfering at protein synthesis (Blair et al., 2015; Partridge, 2015; Ghotaslou et al., 2017; Castanheira et al., 2018).

Different kind of resistance mechanism can occur when related to aminoglycosides especially when associated to mobile genes encoding aminoglycoside modifying enzymes (AME). More than 50 types are already identified, been a reliably resistant phenotype in *E. coli* isolates. Their action changes DOS nucleus or sugar moieties inactivating the drug. AME works as subset of aminoglycosides, attaching and modifying their structure. Three families are usually analyzed: aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphoryltransferases (APHs) (Partridge, 2015; Iredell, Brown, Tagg, 2016; Lopez-Diaz et al., 2017; Ghotaslou et al., 2017; Fernandez-Martinéz et al., 2018; Castanheira et al., 2019).

Quinolones are synthetic antimicrobials used frequently to treat Gram-negative bacterial infections. Fluoroquinolones and their derivates, has a large spectrum and superior activity when compared to quinolones. Their activation consist to aim DNA gyrase and topoisomerase IV enzymes, the

part that is responsible for modulates the topological DNA state, interfering in nucleic acids processes. These enzymes can double-stranded breaks in DNA and do re-ligation. The quinolones bind to cleavage-ligation active site and intercalate into DNA, blocking ligation and enhancing DNA fragmentation, as well as impairing both enzymes function (Partridge, 2015; Moumouni et al., 2017).

High resistance to fluoroquinolone acquisition could be a multistep process, involving even chromosomal mediated (by mutations altering target enzyme or permeability) and plasmid-mediated quinolone resistance (PMQR) determinants. Three PMQR mechanisms have been recognize until now: target protection mediated by *qnr* genes; quinolone modification through *acc(6')-lb-cr* (aminoglycoside acetyltransferase variant and a ciprofloxacin resistance protein enzyme) and plasmid-mediated quinolone efflux pumps (*QepA* and *OqxAB*). The target protection can be related to *qnr* genes family A, B, C, D, S and VC, they encode pentapeptide repeat proteins reducing the bind of DNA gyrase and topoisomerase IV to DNA and bind them together, inhibiting quinolones from entering cleavage complex. The most regular quinolone gene is *qnrB*, founded by researchers at *Citrobacter* species different chromosomes (Blair et al., 2015; Partridge, 2015; Yassine et al., 2019).

MDR *E. coli* strains including resistance to fluoroquinolones, ESBL, aminoglycosides, carbapenems and others antimicrobial groups are spreading into the community. According to several authors, colistin resistance might be related to genetic elements like chromosomal and mobile elements, including plasmids and transposons which determine *Escherichia coli* resistance to antimicrobials. Colistin has been recognized as last-resort antimicrobial for treatment of intractable infections involving multidrug-resistant Gram-negative bacteria, especially carbapenem-resistant. Neverless, the increasing use of colistin in food-producing animals has resulted in a rising prevalence of colistin-resistant bacteria and their prevalence seems to be dependent to the medium used for bacteria isolation leading to difficult treatment of these infections. Moreover, the mobile colistin-resistant gene *mcr*-1 was recently discovered in *Escherichia coli* isolates from animals and humans in China. A *mcr*-1 gene genetic mutation

allows bacteria to become highly resistant to colistin antimicrobial. This antimicrobial is used to treat very resistant bacteria, usually present only in hospitalized patients. To date, *mcr*-1 - producing *E. coli* strains have been reported in many countries throughout Europe, Asia, and South and North America (Kawahara et al., 2019).

The importance of health care associated infections transcends individual medical aspects, and their endemic and epidemic presentation, gives a global dimension problem. Aggravating this situation, resistance to available antimicrobial treatments, with emphasis on bacteria, has been considered by the World Health Organization (WHO, 2014) a priority problem to all countries. Some authors considered that we are moving into a post-antimicrobial era, where infections and quickly treatable former lesions can now kill, unless all the society fights to prevent infections and change the view of antimicrobial use, production and prescription.

Virulence Path Types

E. coli is known as an important pathogen infecting millions of humans each year in both industrialized and developing countries. Some *E. coli* strains present virulence factors with an enhanced potential to cause diseases. These pathogens have been classified in two major groups: enteric pathogens and extraintestinal pathogens (Kuhnert et al., 2000).

Extraintestinal pathogenic *E. coli* (ExPEC) have a wide range of virulence factors including adhesins, toxins, iron acquisition factors, lipopolysaccharides, polysaccharide capsules, and invasions. ExPEC are facultative pathogens and constitute a separate group mainly causing infections not only in urinary tract in all age categories, sepsis and meningitis in small children but also their presence may be responsible for causing uncomplicated UTIs and bacteremia, with UTI being the most prevalent (Sarowska et al., 2019).

There are six *E. coli* pathotypes recognized and have been broadly divided into: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli*

(EAEC), enterotoxigenic *E. coli* (ETEC) and diffusely adhering *E. coli* (DAEC) (Kuhnert et al., 2000).

ETEC is responsible for several diarrheal episodes worldwide in people visiting endemic countries and in infant under five years old living in low-income countries due to their poor sanitation. After ingestion, ETEC colonize the small bowel and attach to its mucosa causing a dysfunction of the electrolyte and water transport in enterocytes leading to diarrhea, low grade fever, vomiting, cramps and nausea. The production of heat-labile (*Lt* gene) and/or heat-sable (*St* gene) enterotoxins leads to hypersecretion of H2O and Cl- at the root of diarrhea (Nataro, Kaper, 1998; Roussel et al., 2018).

EPEC represents another cause of diarrhea in third world countries normally affecting small children. EPEC are usually transmitted by contaminated food and may colonize the small intestine leading not only to diarrhea, but also vomiting and fever. EPEC carries the site to enterocyte effacement (LEE) pathogenicity island which contains the *eae* gene coding for an outer membrane protein called intimin responsible for EPEC adhesion to epithelial cells (Roussel et al., 2018; Scaletsky, 2019).

A Shiga-like toxin-producing *E. coli* (STEC) infection represents a typical disease of industrialized countries and the most severe forms of infections are observed in young children and elderly. Fecal contamination of food represents the main source of infection causing a range of symptoms including diarrhea, hemorrhagic colitis and hemorrhagic uremic syndrome. The pathogenicity of STEC depends on the toxin type, virulence genes and serotype. STEC genes are subdivided into *stx*1 and *stx*2 (Griffn, 1999; Frank et al., 2019).

EIEC shares similar biochemical and pathogenesis features with *Shigella* and it has been suggested to be grouped into a single pathovar *Shigella*/EIEC. This pathotype cause watery diarrhea and, in severe cases, dysentery, which may lead to severe life-threatening complications like hemolytic uremic syndrome (HUS) especially in developing countries. Their epidemiology is poorly understood however, is known that *ial* gene is involved in intestinal cells invasion and its infection seems to be mainly

through contaminated food and water causing either outbreaks or sporadic cases (Griggn, 1999; Bona et al., 2019).

EAEC present a unique aggregative adherence to HEp-2 cell in culture and their heterogeneity and inconsistence of profile genes makes establishing its pathogenicity difficult. EAEC infections occur as sporadic cases or outbreaks mainly in developing countries, especially in infants and young children. The clinical signs of its infections are watery mucoid (sometimes bloody) diarrhea with fever and no vomiting. EAEC produces a number of virulent genes such as *Eagg*, an aggregative adhesion plasmid (Nataro & Kaper, 1998; Bamidele et al., 2019; Vasques-Garcia et al., 2019).

The epidemiology and exact pathogenic potential of DAEC strains are less well understood. However, literature describes these strains as an etiological agent of diarrhea in children and adults (Ochoa et al., 2009; Meraz et al., 2008). Another factor that may have contributed to the controversy over DAEC pathogenicity is their ability to adhere diffusely to Hep-2 cell, which are typical of EPEC (Sidhu et al., 2013).

The majority of *E. coli* strains are commensal but some strains have acquired specific virulence attributes that allow them to cause a wide spectrum of intestinal and extraintestinal infections. Disease outbreaks related to exposure to contaminated freshwater are well documents however, the prevalence of *E. coli* pathotypes in the urban aquatic environment is not well characterized.

Biofilm Formation

A biofilm is defined as an aggregate of microorganisms that lives together as community and are often found attached to solid surfaces in environment. They are composed by polysaccharides, extracellular DNA and proteins. Biofilm formation is a common bacterial coping of antimicrobial agent mechanism to resist in environmental stresses and adverse conditions and is considered a multifactorial process. Biofilms can be regarded as universal strategy for bacterial survival which positions them to effectively use the available nutrients. Multiple biofilm mechanisms

operate simultaneously and make it difficult to completely kill cells in a biofilm, especially those situated in deeper layers (Sharma et al., 2016; Cattò et al., 2017; Di Luca et al., 2017).

It is becoming increasingly clear that biofilms are involved in a large amount of infections and may be formed on host tissues/mucosa or surface of several medical devices including central vascular catheters, urinary catheters and others. Thus, biofilm-associated infections commonly do not resolve easily, requiring the mechanical or surgical removal of infected tissue or colonized implant (Di Luca et al., 2017).

E. coli biofilms is highly variable. Consist of a complex surface-associated a community of microorganisms embedded in a self-produced polymeric matrix (EPS) and typically requires the production of adhesive curli fimbriae and exopolysaccharide cellulose. This biofilm formation is considered one of the most important causes of a high number of intestinal infections. The diversity in structural components of biofilm hampers the penetration of conventional antibiotics doing the cells more resistant to antibiotics when compared to their planktonic bacteria. *E. coli* biofilms are also responsible for morbidity and mortality in case of medical device associated infections (Sharma et al., 2016; Uhlich et al., 2014; Sun et al., 2019).

Some surface structures of bacterial cells are involved in biofilm formation, such as flagella, curli, type I fimbriae, conjugative pili, extracellular DNA and Ag43 (antigen 43 protein) - promotes cell-to-cell adhesion and aggregation at the initial stages of biofilm formation. Auto transporter adhesins are members of the type V secretion system and associated with auto aggregation and biofilms. One major component of EPS in *E. coli* is colanic acid, an exopolysaccharide, which forms a protective capsule surrounding the bacterial cell and also sustains the biofilm architecture, but its contribution to biofilm formation is not well defined (Nakao et al., 2012; Rossi et al., 2018; Król et al., 2019).

Several genes have been described in active roles of *E. coli* biofilm formation. The *pga* operon encodes proteins involved in synthesis, export and localization of PGA (poly-beta-1,6-N-acetyl-D-glucosamine) polymer,

which is necessary for biofilm formation mediating cell-to-cell adhesion and attaching to surfaces (Kang et al., 2018).

Although biofilm formation is a well-defined process, > 20% genome variability suggest a hardly explored variability in biofilm formation within the different *E. coli* species. Thereby, in some pathovars biofilm formation is integrally included in virulence phenotype. Biofilms containing *E. coli* have been detected in fresh water streams and drinking water sources (Rossi et al., 2018).

The aim of this present chapter is demonstrate the presence of resistance and virulence genes found in *E. coli* strains isolated from rivers located at Rio de Janeiro metropolitan area and their ability of biofilm formation.

METHODS

Study Area and Water Sample Collection

The study site is located in Rio de Janeiro city. Water samples were collected in three different rivers: Joana River, Maracanã River and both river junction. All three sites receive water from different sources including precipitation, surface runoff, domestic and hospital sewage.

E. coli Strains Isolation

These strains were collected in each one of the rivers cited above. They were analyzed at Medical Science College - Rio de Janeiro State University (UERJ). The water sample was inoculated in 100mL of Brain Hearth Infusion broth – BHI (2x), containing 8 μg/mL of imipenem, as previously described (Nogueira et al., 2015) and incubated during 24 h at 37ºC. Subsequently, Gram-negative strains were identified using MALDI-TOF (matrix-assisted laser desorption/ionization - time of flight) mass spectrometry (Pereira-Ribeiro et al., 2019).

Analysis of Antimicrobial Resistance Profiles

Antimicrobial susceptibility test was carried out by Kirby Bauer disc diffusion method, using the following antimicrobial drugs: cephalothin, cefazolin, cefoxitin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, gentamicin, amikacin, kanamycin, tobramycin, ampicillin, piperacillin/taxobactam, amoxicillin/clavulanic acid, ampicillin/sulbactam, ciprofloxacin, norfloxacin, imipenem, ertapenem, meropenem, aztreonam, chloramphenicol, tetracycline and cotrimoxazole. The results were interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). *E. coli* ATCC 25922 strains was used as control. MDR profiles were defined as resistance to three or more antimicrobials classes (Magiorakos et al., 2012).

Investigation of Resistance Genes by PCR

Beta-lactam-resistant strains were analyzed for bla_{TEM}, bla_{SHV}, bla_{TOHO}, $bla_{CTX\text{-}M}$ genes (Arlet, Philippon, 1991; Pitout et al., 2004) and carbapenems resistant strains were analyzed for bla_{KPC} gene (Yigti et al., 2001). Aminoglycoside-resistant strains were analyzed for *accC2* and *accC3* genes (Van de Klundert, 1993), while fluorquinolone-resistant strains were investigated for *qnr*A and *qnr*B genes (Jiang et al., 2008).

Template DNA was prepared by mixing an overnight culture with 500µL of destiled water in a 2 mL eppendorf tube (Eppendorf AG, Hamburg, Germany) and heating it to boiling for 15 minutes. The mixture was cooled on ice for 5 minutes and centrifuged 5000 rpm for 2 minutes. The supernatant was collected and stored at -20°C and used as template DNA in all subsequent tests. For the PCR reaction, 2 µL of template DNA was added to a 1.5 mM MgCl2, 0.2 mM of a DNTP mixture, 20 pmol of each primer, 1x concentrated PCR buffer and 1.25 U DNA Taq Polymerase. The reactions were subjected to temperature cycling in the DNA Thermal Cycler thermal cycler. About 10 µL of amplified material was deposited along with 10 µL of milliq water into 2% Invitrogen agarose e-gel slots. The

run and visualization of the samples were performed on a Life Technology LTDA E-gel safe imagerTM Real-time transilluminator.

Multiplex-PCR for Detection of Virulence Genes

PCR was performed in 20 μl reactions in PCR tubes according to Omar and Bernard (2010) with modifications. The following genes were used: *eaeA* (917 bp), *Stx1* (614 bp), *Stx2* (779 bp), *ial* (630 bp), *Lt* (450 bp), *St* (160 bp) and *eagg* (194 bp) for enteropathogenic, enterohemorrhagic, enteroinvasive, enterotoxiginenic and enteroadherent respectively.

Biofilm Formation on Abiotic Surfaces

Biofilm assay on polystyrene surfaces were performed for all strains (Merrit et al., 2005). The optical density (OD) of the stained attached bacteria and control wells were read at $\lambda = 570$ nm. The cut-off OD (ODc) was defined as the mean OD of the negative control (TSB only). Based on the ODs of the bacterial films, all strains were classified into the following categories: non-adherent (-: OD ≤ ODc), weakly adherent (+: ODc < OD ≤ 2x ODc), moderately adherent (++: 2x ODc < OD ≤ 4x ODc), or strongly adherent (+++: OD ≤ 4x ODc). Each assay was performed in triplicate and repeated three times. *Staphylococcus epidermidis* strain ATCC 35984 was used as positive control (Sued et al., 2017).

RESULTS

Identification and Antimicrobial Resistance

A total of 48 strains were isolated from collection sites and identified as *E. coli* strains. Joana River presented 39.6% (n=19) of isolates followed by

Maracanã river, with 35.42% (n=17) of isolates and 25% (n=12) *E. coli* strains were isolated from the junction of both rivers.

MDR parameter associated to *Enterobacteriaceae* family is a resistance profile to three or more groups of chosen antimicrobials (Magiorakos et al., 2012). From the 48 isolates, 38 (79%) were considered as MDR presenting resistance to third and fourth generation of cephalosporins, aminoglycosides, fluorquinolones and carbapenems. Joana River presents 68.4% (n=13) of MDR *E. coli* isolates while Maracanã River has 94% (n=16) of MDR isolates and the junction of both rivers presented 75% (n= 9) of MDR *E. coli* strains.

Resistance related to third and fourth generation of cephalosporins groups are usually related to ESBL and was observed in 75% (n=36) of the isolates while carbapenems resistance was found in 9 isolates (18.7%). The aminoglycoside group presented resistance to 81.2% (n=39) of isolates and 77% (n=37) *E. coli* strains were resistance to fluoroquinolones.

A total of 37 (77.1%) strains presented at least one genes coding resistance for several antimicrobial groups. It was possible to observe the presence of 22 (59.4%) isolates with ESBL resistance gene, including bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV}. There was no presence of bla_{TOHO} gene in neither collection site. Fluorquinolone resistance genes *qnrA* and *qnrB* were found in 18 (48.6%) *E. coli* isolates while AME were identified in 12 (32.4%) isolates coding *aacC2* and *aacC3* genes. Despite resistance to carbapenems, no isolates presented bla_{KPC} resistance gene.

It's important to emphasize that one strain (Ec12G/13), isolated at Maracanã River, presented bla_{TEM} and $bla_{CTX\text{-}M}$ gene coding for ESBL resistance, aminoglycoside resistance *aacC3* gene and *qnrA* gene for fluorquinolones. A total of 10 (27%) *E. coli* isolates presented both resistance genes coding for ESBL and fluorquinolones, two isolates (5.4%) presented resistance genes for ESBL and aminoglycosides followed by one isolate (2.7%) that has aminoglycoside and fluorquinolones resistance genes concomitantly.

Despite several isolates presenting genes coding for two or more antimicrobial groups, nine isolates presented resistance genes only to ESBL,

followed by eight *E. coli* isolates with aminoglycoside resistance genes while six isolates have fluorquinolones resistance genes.

Virulence Pathotypes

From 48 isolates, 27% (n=13) presented virulence pathotypes. EAEC, EIEC and ETEC were found in three isolates each, followed by EPEC and EHEC, who were present in two isolates each. ETEC pathotypes were found in all three collection sites while EAEC isolates were at Maracanã and Joana Rivers. Both EIEC and EHEC isolates were found at Joana River and the junction of both rivers. EPEC isolates were found only at Joana River.

A total of 19 *E. coli* strains were found at Joana River and 36.8% (n=7) presented different pathotypes. Two isolates (Ec5/05 and Ec7CFL/13) presented *eaeA* gene and others two isolates (Ec5G/13 and Ec7CFL/13) has *iaL* gene, coding for EPEC and EIEC pathotypes respectively. EAEC (Ec1G/13), EHEC (Ec11G/13) and ETEC (Ec27CFL/13) pathotypes were also found in one isolate each coding for *eagg*, *stx2* and *Lt* Genes respectively.

Maracanã River presented 17 *E. coli* strains, however, 17.6% (n=3) strains presented virulence pathotypes. The *eagg* gene, coding for EAEC pathotypes were found in two isolates (Ec4G/13 and Ec12G/13) and *Lt* Gene who code for ETEC pathotypes, was at one strain (Ec1/05).

Were analyzed 12 *E. coli* isolates at the junction of both Rivers and 25% (n=3) of them presented virulence pathotypes. Each strain analyzed has one gene coding for a different pathotypes: Ec8/07 presented *stx1* gene, Ec2G/13 has *iaL* gene and Ec10CFL/13 has *st* gene, all coding for EHEC, EIEC and ETEC respectively.

Biofilm Formation

Biofilm formation on polystyrene surfaces was observed at mostly *E. coli* strains (n = 45; 93.75%) but at different levels. The majority of these *E.*

coli strains (n = 21; 43.75%) were classified as strongly (+++) adherent to polystyrene surfaces. Were classified as moderately (++) adherent, 18 (37.5%) isolates and 06 (12.5%) isolates as weakly (+) adherent to polystyrene surfaces. Only three isolates (6.25%) were classified as non-adherent (-).

DISCUSSION

Despite water been the most important natural source for life, there are several factors that have affected their quality, including the influence of organic materials discharged into the environment, fecal pollution drived from human settlements and untreated hospital sewage that can carry a high number of pathogens (Canizalez-Roman et al., 2019). According to World Health Organization (2017), there are 2.3 billion people worldwide who do not have basic sanitation facilities and, at least, 1.8 billion people are estimated to drink water contaminated with feces. Thus, an important aspect to consider is to analyze microorganisms present in water, in which the most common infectious pathogens includes *Salmonella*, *Shigella*, *Vibrio cholera* and pathogenic *E. coli* carrying both resistance and virulence genes (Canizalez-Roman et al., 2019).

Despite advances in water management and sanitation, waterborne diseases continue to occur in both developing and developed countries. Since many *E. coli* isolates with both resistance and virulence genes can be transported by river waters and deposited into water bodies that could be used for irrigation, recreation or even drinking, it's of vital importance to monitor the quality of fresh water sources in order to decrease waterborne infections and thereby controlling morbidity and mortality caused by contaminated water (Leclerc et al., 2002).

The presence of *Escherichia coli* in water bodies is a regular feature and is usually related to fecal coliform contamination due to their relation with gastrointestinal tract of humans and animals. This study demonstrates the presence of this specie in all the three collection sites, with a little difference in terms of quantity founded among both rivers and the river junction

analyzed. The presence of *E. coli* isolates indicating fecal contamination is a worldwide problem once this strains can be found in several aquatic environments such as ethiopian rivers with interference with hospital effluents (Tesfaye et al., 2019), philippine river reciving treated sewage effluent (Suzuki et al., 2019), swedish aquatic environments with influence of wastewaters (Khan, Soderquist, Jass, 2019), brazilian wastewater and river water correlated to rural area (Lanna et al., 2019) and others.

In this study, the presence of MDR isolates was found in most of analyzed strains (54.16% n=26). The MDR E. coli is commonly found and isolated from several water bodies sources like rivers, lakes, wastewater treatment plant, wastewater generated from hospital without correct treatment and aquatic environment that receives treated sewage effluent (Hassen et al., 2018; Tasfaye et al., 2019; Suzuki et al., 2019).

Brander and co-workers (2017) found 60.6% of multidrug resistance strains in fecal samples from children with enteric infections corroborating to our results as an ambient contamination consequence, increasing 76% the propensity to find multidrug resistant isolates in patients with lack of basic sanitation.

In this study, MDR isolates demonstrated co-resistance to all three antimicrobial groups, with at least two of them together in each isolate. The most part of isolates has aminoglycoside resistance (81.2%) differing from others studies such as *E.coli* from mexican irrigation water samples (Canizalez-Roman et al., 2019), *Enterobacteriaceae* isolates from portuguese wastewater and river water (Amador et al., 2015) and nigerian rivers reservoirs of *E. coli* as water fecal contamination (Titilawo, 2015), who did not shown an expressive quantity of aminoglycoside *E. coli* resistance.

Following aminoglycoside resistance isolates with resistance to three and four generation cephalosporins were one of the most found in this study (75%). The presence of isolates resistant to this antimicrobial group in rivers can be an important characteristic, especially when associated with resistance to other antimicrobial groups including all generation cephalosporins, ciprofloxacin (a quinolone antimicrobial), tetracycline, amoxicillinwith clavulanic acid due to the relationship with untreated

hospital sewage (Tasfaye et al., 2019). However, the same study demonstrates 18.7% of carbapenems resistance strain with carbapenemases genes, while in this study, strains demonstrated carbapenems resistance but no gene related was found.

Our results showed 77% of isolates with phenotypic fluoroquinolone resistance (parameters defined ciprofloxacin and/or norfloxacin resistance), a big part of the strains content. *E. coli* resistance to ciprofloxacin and norfloxacin have been found worldwide and this resistance profile of isolates might be related to community water sources, such as wastewater treatment plants, rivers and water samples with untreated hospital sewage (HASSEN et al., 2018; Titilawo et al., 2015).

It is important to emphasize that neither isolates presented antimicrobial resistance to all antimicrobial tested. Tesfaye and co-workers (2019) analyzing water samples from Ethiopian rivers five ciprofloxacin resistant isolates, indicating that not only hospital wastewater contributes to antimicrobial resistance but also other good practices.

Genes coding resistance for our chosen antimicrobials groups was identify in 77% of *E. coli* isolates. ESBL resistance genes aspects are mainly related to water sources as environmental or wastewater and is possible to notice the presence of several resistance genes such as: $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM}not only at hospital but also in rivers and aquatic environment (contaminated or not) (Hassen et al., 2018; Khan, Soderquist, Jass, 2019).

A total of 48.6% *E. coli* isolates presented fluoroquinolone specific resistance genes (*qnrA* and *qnrB*) corroborating to Khan, Soderquist and Jass (2019) that identify these two genes from sweden water samples including hospital source, wastewater treatment plant and river water. Despite both *qnrA* and *qnrB* gene are associated and first found in aquatic environment, few Brazilian studies detect these genes except in water samples (Varella et al., 2015; Conte et al., 2017).

Aminoglycoside modifying enzymes genes *aacC2* and *aacC3* were found in 32.4% of *E. coli* isolates. The *aacC2* gene is frequently found in available literature related to *E. coli* specie, even more when associated to water samples as river waters and other sources (Khan, Soderquist, Jass, 2019; Titilawo, Obi, Okoh, 2015; Hara et al., 2018).

Despite the *aacC3* gene is not usually found in *E. coli* isolates due to their close link to others *Enterobacteriaceae* species (LIN et al., 2017), our study demonstrate the presence of eight (66.6%) *E. coli* isolates presenting this gene while six (34.4%) presented *aacC2* gene.

In our study none of the isolates presented *bla*KPC resistance gene, and it is confirmed by Khan, Soderquist and Jass (2019) that did not find the same gene to *E. coli* isolates from household and hospital wastewater from Sweden indicating that despite phenotypical resistance, not all isolated presentes genotipical related gene.

The pathotypes of *E. coli* that are associated with intestinal disease are known as diarrheagenic *E. coli* (DEC). These strains have been isolated from several ecological niches ranging from mammalians intestine to different food products and aquatic environments including river waters. Different DEC pathotypes have been associated with sporadic cases of diarrheal diseases in humans causing waterborne gastroenteritis outbreaks (Aiuja et al., 2018; Canizalez-Roman et al., 2019).

This study demonstrates that 27% (n=13) of *E. coli* isolates in rivers waters presented virulence pathotypes coding for EAEC, EIEC, ETEC, EPEC and EHEC. These pathotypes are important waterborne pathogens and these isolates are commonly transmitted through contaminated water and have been isolated from gastroenteritis patients (Ram et al., 2009; Yang et al., 2007; Canizalez-Roman et al., 2019).

The most frequently isolated pathotypes was EAEC (n=3), EIEC (n=3) and ETEC (n=3), followed by EHEC (n=2) and EPEC (n=2). All pathotypes were present in all river water sample collection. All *E. coli* isolates with virulence pathotypes also demonstrated a MDR profile and therefore, represent a threat to those in risk of infection. Contamination of surface waters by pathogenic *E. coli* is a big concern. Variation in epidemiology of diarrheal disease and associated pathotypes has been linked to geographical, temporal and climatic conditions complicating public health prevention and control initiatives. Besides studies describing outbreaks of waterborne bacterial infections, the prevalence and categorization of DEC isolates in water samples has been poorly studied. Understand the prevalence of DEC pathotypes in environmental sources would help link observed

infections/outbreaks information helping public health interventions (Aijuka et al., 2018; Carlton et al., 2016; Baker et al., 2016).

Among other factors, biofilm are known to play an important role in virulence pathotypes, especially because persistence of DEC in the environment may be related to biofilm development on plastic surfaces such as water feeding systems. Biofilms are a distinct bacterial lifestyle since biofilm formation also promotes resistance to antibiotics as well as exchange of DNA (Beloin et al., 2008; Nielsen et al., 2018).

Although biofilm analyses have been done independently, *E. coli* strains who presented virulence pathotypes not only were considered as MDR, but also 76.9% (n= 10) were classified as strongly adherent and the remain three isolates were considered moderately adherent. The ability of DEC to exchange DNA in biofilms is also of concern due to potential acquisition of virulence and antimicrobial resistance plasmids (Johnson et al., 2005; Nielsen et al., 2018).

Biofilm formation occurs in four processes: attachment, development, maturation, and dispersal. Biofilms show increased resistance to environmental stresses and to the host immune system, as well as tolerance to antibiotics. In the gastrointestinal tract, commensal and pathogenic *E. coli* faces a wide range of environmental signals, not only from the host but also from other commensal bacteria (Kang et al., 2018; Rossi et al., 2018).

In this study, we compared five *E. coli* pathotypes (ETEC, EICE, EAEC, EHEC and EPEC) and non-pathogenic *E. coli*. All non-adherent strains and weakly adherent strains were classified as non-pathogenic *E. coli* isolated from Maracanã river (n=2), Joana river (n=3) and the junction of both rivers (n=4). Strongly and moderately adherent strains were pathogenic *E. coli*. Some studies have reported strongly, moderately and weakly adherent pathogenic *E. coli* demonstrating that there are no standard of this specie in biofilm formation (Kang et al., 2018; Raya et al., 2019). Schiebel and co-workers (2017) observed EAEC isolates exhibits the highest capacity for biofilm formation corroborating our study once the EAEC pathotypes was present in 23% of *E. coli* isolates.

In non-pathogenic *E. coli*, cell surface components are well known and play a indispensable role in biofilm formation. In contrast there is poor

information regarding biofilm formation by pathogenic *E. coli* isolate. The results clearly demonstrate the importance of further studies to define the roles of different components of pathogenic *E. coli* biofilms and how they are regulated.

CONCLUSION

Aquatic environments, such rivers, are considered a complex community acting as a reservoir of microorganisms with different genotypes and virulence pathotypes. Environmental selection pressures, such as antimicrobial and other chemical compounds in aquatic environment could lead to increased problems with infections caused by resistant bacteria.

This study demonstrated the presence of MDR *E. coli* strains with virulence pathotypes able to form biofilm who might be related to hospital sewage disposal. Despite our results, is necessary further investigation of biofilm formation in *E. coli* isolates with virulence pathotypes.

The presence of *E. coli* isolates with these characteristics is found worldwide and has been considered a health care problem. Public politics such as monitoring aquatic environments, treat hospital sewage and limit the antimicrobial use must be implemented to avoid community and nosocomial infections caused by MDR isolates.

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