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Co-expression of Extensively drug resistant (XDR) clinical isolates of *Pseudomonas aeruginosa* harboring FOX and MOX ampicillinase Gene

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Abstract

This study determines the Co-expression of clinical isolates of XDR *Pseudomonas aeruginosa* harboring *FOX* and *MOX* ampicillinase Gene. A total of five (500) hundred "Clean catch" midstream urine and wound samples collected from patients at a tertiary healthcare institution in Ebonyi State Nigeria were analyzed using standard microbiological techniques. Determination of XDR *Pseudomonas aeruginosa* isolates was by Kirby-Bauer disc diffusion method. Molecular characterization of *FOX* and *MOX* ampicillinase resistant genes were done by PCR using specific primers. In total, the *Pseudomonas aeruginosa* accounted for 22.6 %. XDR *Pseudomonas aeruginosa* accounted for 17. 8 % and 25.3 % in Wound and urine samples respectively. All the XDR *Pseudomonas aeruginosa* harbored *FOX* and *MOX* ampicillinase resistant Gene. The high frequency of XDR *Pseudomonas aeruginosa* in our study is very worrisome and could have significant public health impact such as treatment failures, and possibly death, if not properly managed. The solutions to this crisis are to allocate more resources to basic and clinical research and to infection control and antimicrobial stewardship, to develop new antimicrobials, and to optimize the use of those that are currently available.

Keywords: XDR, *Pseudomonas aeruginosa, FOX*, *MOX* ampicillinase

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium that does not usually cause infections in healthy people, but it can cause infections in organ system of immunocompromised individuals [1]. (Ogba *et al.,* 2022a). This pathogen is a frequent cause of nosocomial infections such as acute and chronic lung infections, urinary tract infections, ocular infections and bacteraemia, with high mortality and morbidity rates [1, 2]. (Ogba *et al.,* 2022a; Ogba *et al.,* 2022b). *P. aeruginosa* is a major cause of hospital acquired infections, accounting for 10-30% of nosocomial infections in patients [1, 3]. (Ogba *et al.,* 2022a; Gill *et al.,* 2021).

Antimicrobial resistance has been recognized as a global public health problem amongst these strain. The rates of infection increase in the presence of MDR strains, due to elaborate expression and emergence of XDR phenotype despite adequate treatment [4, 5, 6]. (Tenover *et al.,* 2022; Lyu*et al.,* 2023; Hafiz *et al.,* 2023). This is a common situation in intensive care units (ICU), where ventilator-associated pneumonia (VAP) is the most common infection. In-hospital mortality, which is frequently linked to widespread high-risk clones, of XDR is higher in patients infected with previous cases of MDR strains than in patients infected with non-MDR strains and infection caused by XDR *P. aeruginosa* is therefore an important determinant of hospital mortality [7]. (WHO, 2019). Extensively drug resistant (XDR) phenotype is defined as *P. aeruginosa*, which is resistant to more than one antimicrobial agent in all the antimicrobial categories, except in two or less.

Previous years have witnessed an increasing prevalence of XDR *P. aeruginosa* strains, with rates of between 6.4 %-35.8 % in some geographical areas [8, 9, 10, 11]. (Pérez *et al.,* 2019; Saleem *et al.,* 2019; Mirzaei *et al.,* 2020; Liu and Qin, 2022). Most countries in Europe report rates of resistance of more than 10% for all antimicrobial groups under surveillance [8, 12]. (ECDC, 2017; Pérez *et al.,* 2019). There are several major reasons why the emergence and dissemination of extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains have recently become issues of public health concern. First, *P. aeruginosa* causes severe infections, particularly in health care settings and in immunocompromised patients. Second, it has an outstanding capacity for being selected and for spreading antimicrobial resistance *in vivo* [13]. (Poole, 2001). Third, the successful worldwide spread of the so called "high-risk" clones of *P. aeruginosa* poses a threat to global public health that needs to be studied and managed with urgency and determination [14]. (Oliver *et al.,* 2015). The so called "high-risk" clones of *P. aeruginosa* possess elaborate inactivating enzymes, such as ampicillinase (AmpC) encode by bla*FOX* and bla*MOX*. AmpC production is constitutive, leading to resistance to expanded-spectrum cephalosporins, as is evident by *in vitro* susceptibility testing[15, 16, 17, 18, 19]. (Akpu *et al.,* 2023a; Akpu *et al.,* 2023b; Joji *et al.,* 2021; Golsha *et al.,* 2021; Ejikeugwu *et al.,* 2018). They have rapidly spread over different parts of the world but the literature of some AmpC β-lactamase genotype such as the bla*FOX* and bla*MOX* circulating in this area are scares. The co-existence of XDR and AmpC β-lactamase resistant determinant has severely hampered patients care due to increase failure of most therapeutic agent. The remedies to this issue are to devote greater resources to scientific and clinical research, hence our research is geared to determine the presence XDR *Pseudomonas aeruginosa* isolates co-habouring bla*FOX* and bla*MOX* from urine and wound samples of patient visiting a tertiary hospital in Abakaliki Ebonyi State Nigeria

METHODS

Sample collection

The duration of the research was between November, 2021 and August, 2022. The samples were collected aseptically with the help of a professional health personnel. A total of five (500) hundred "Clean catch" midstream urine and wound samples were collected randomly in a sterile container (or sterile swab stick in the case of wound samples) from different wards which includes surgical, medical, orthopedic, surgical outpatient, intensive care unit, gyneacology, and pediatric wards and grouped according to age and sex of patients at Alex Ekwueme Federal University Teaching Hospital, Abakaliki (AE-FEUTHA). All clinical samples were suspended in a sterile Brain-Heart Infusion broth (Thermo Fisher Scientific™, U.S.A) and incubated aerobically at 37 °C for 24 hrs. After overnight incubation, turbid broth cultured samples were streaked on solidified Cetrimide Selective agar (Thermo Fisher Scientific™, U.S.A) plate. The plates were incubated for 24 hours at 37 °C. Bacterial colonies characteristics with pyocyanin greenish colonies, grape-like odor, oxidase positive, were inferred as *Pseudomonas aeruginosa*. The bacteria were further confirmed using VITEK® 2 Compact system (BioMerieux, France) and store for further studies [1, 2]. (Ogba *et al.,* 2022a; Ogba *et al.,* 2022b).

Determination of XDR resistant

The pattern of XDR *Pseudomonas aeruginosa* isolated was determined using Kirby-Bauer susceptibility test method in compliant with the Clinical and Laboratory Standard Institute [20]. (CLSI, 2019). An overnight culture of the test bacteria grown in nutrient broth was adjusted to 0.5 Macfarland turbidity standards. The inoculum was aseptically inoculated on the surface of Mueller-Hinton agar plate(s) using sterile swab sticks. Commercially available single antibiotics disc was aseptically impregnated on the surface of the inoculated Mueller-Hinton agar. The following antibiotics disc were used: Piperacillin/tazobactam (30 µg), ceftriaxone (30 µg), Aztreonam (30 µg), cefotaxime (30 µg), tetracycline (30 µg), cefoxitin (30 µg), cefepime (10 µg), amoxycillin clavulanic acid (20/10 µg), trimethoprim sulphamethoxazole (125/23.75 µg), nalidixic acid (30 µg), aztreonam (30 µg), colistin (10 µg) and amikacin (30 µg) (Oxoid, UK). The plates were incubated at 37° C for 24 hrs, and the inhibition zone diameters (IZDs) produced by the antibiotics were measured with a meter rule and recorded as earlier recommended [20, 21, 22]. (CLSI, 2019; Oke *et al.,* 2020; Uzoije *et al.,* 2021) for the assessment of XDR = non-susceptible to ≥1 agent in all but ≤2 categories [23]. (Rodulfo *et al.,* 2019). American Type Culture (ATCC) collection strain (*P. aeruginosa* ATCC 27853) was used for quality control in this study.

Genomic Analysis of the bla*FOX* and bla*MOX* genes

Plasmid DNA was extracted from the test bacterial isolates using the Zymo Plasmid miniprep kit [Zymo Research™, USA] [24]. (Edemekong *et al.,* 2022). The primer sequence that were used for the PCR amplification of AmpC gene are shown as follows; bla*MOX* forward-GCT GCT CAA GG AGCA CAG GAT; reverse-CAC ATT GAC ATA GGT GTG GTG C; bla$_{FOX}$ forward-AACA TOG GGG TAT CAG GGA GAT G; reverse- CAA AGC GCE TAA CCG GAT TGG (Invitrogen™, USA). The purified DNA fragment was quantified using a NanoDrop (Thermo Scientific, USA). PCR experiment was conducted according to the procedures described previously [15, 25]. (Akpu *et al.,* 2023a; Adibe-Nwafor *et al.,* 2023). This was carried out using a PCR master mix (50 µl) comprising 5x GoTaq (10µl), 25 mM MgCl2 (3µl), dNTPs (10 mM) 1µl, forward primers (1µl), reverse primers (1µl), 10 pmol 1 µl, DNA Taq (1000 U) 25µl, Ultrapure Water 8µl. The PCR conditions used are an amplification cycle at 95 °C for 5 minutes; 35 cycles at 96 °C for 30 seconds, 58 °C for 90 seconds, and 72 °C for 60 seconds; and a final extension step at 72 °C for 10 minutes.

RESULT

Pseudomonas aeruginosa accounted for 113(22.6 %) as presented in Table 1. XDR *Pseudomonas aeruginosa* accounted for 17. 8 % and 25.3 % in Wound and urine samples respectively while 56.9 % were non XDR *Pseudomonas aeruginosa* (Figure 1). All the XDR *Pseudomonas aeruginosa* harbored *FOX* and *MOX* ampicillinase resistant Gene. Distribution of *bla*$_{fox}$ and bla$_{mox}$ in XDR-*P. aeruginosa* accounted 100 % (Figure 2).

Table 1: Distribution of *P. aeruginosa* in different Clinical Samples collected from Patients Attending AE-FUTH

Patients Ward	Clinical sample	No. sampled	*P. aeruginosa* (%)
Surgical	Wound	10	3(30.0)
	Urine	20	7(35.0)
Medical	Wound	50	10(20.0)
	Urine	100	19(19.0)
Orthopedic	Wound	100	20(20.0)
Surgical Outpatient	Wound	150	30(20.0)
Intensive Care Unit	Urine	20	10(50.0)
Gynecology	Urine	22	5(22.7)
	Wound	8	3(30.0)
Pediatrics	Wound	15	4(26.7)
	Urine	5	2(40.0)
Total		**500**	**113(22.6)**

Table 2: Antibiotics Categories and agent selected for XDR *P. aeruginosa* Identification

Categories	Antibiotics (µg)	Non-susceptible or Resistant strain (%)
Aminoglycoside	Amikacin (30)	0.0
β- lactam/inhibitor	Ticarcillin-clavulanic acid (85)	100
	Piperacillin-tazobactam (30)	58.8
	Amoxicillin/clavulanic acid (20/10)	100
Monobactam	Azetronam (30)	76.5
Cephalosporin's	Cefotaxime (30)	82.4
	Ceftriaxone (30)	100
	Cefepime (30)	100
Fluoroquinolones	Nalidixic acid (30)	70.6
Polymyxin	Colistin (10)	100
Sulfonamide	Trimethoprim-Sulfamethoxazole (25)	100
Tetracycline	Tetracycline (30)	100
	Doxycycline (30)	100

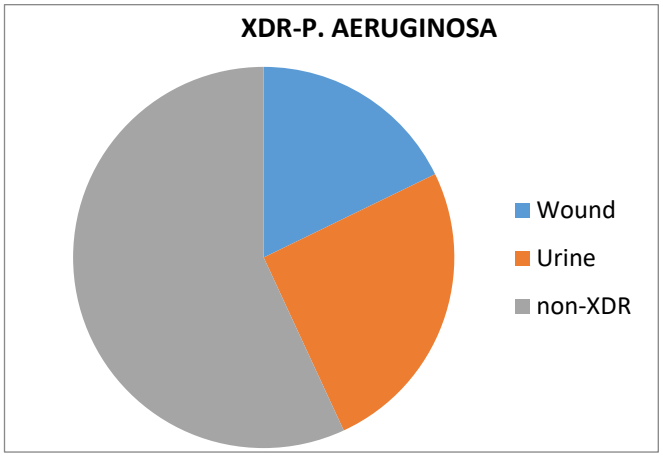

Figure 1: Pie chart showing the distribution of XDR-*P. aeruginosa*

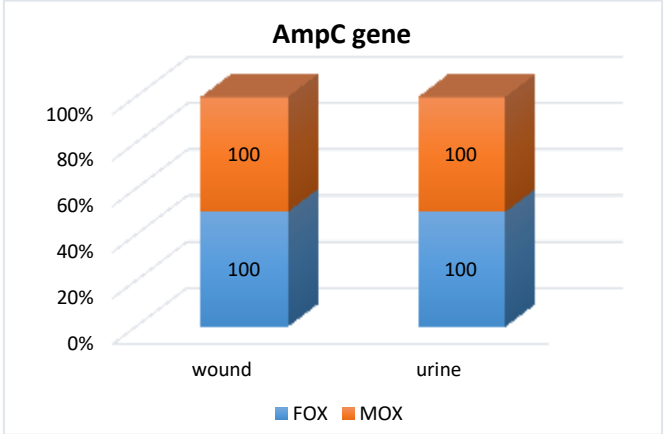

Figure 2: 3D combo chart showing the distribution of bla_{FOX} and bla_{MOX} in XDR-*P. aeruginosa*

DISCUSSION

Among the samples from patients in AE-FUTHA, the overall prevalence of *P. aeruginosa* was 22.6 %. Although there seem to be geographical differences in the proportions between the species earlier identified in other study, this observation is not parallel with reports in China which were 10.7 % and in Zaria were 10.5 % and in North-eastern Nigeria were 2.1 % was reported [11, 26, 27]. (Liu and Qin, 2022; Olayinka *et al*., 2004; Okon *et al*., 2010), but strongly agrees with previous report of higher prevalence rates of 32.1 % and 20.3 % published by [28]. Rajat *et al*. (2012) and [29]. Javiya *et al*. (2008) in Ahmadabad and Gujarat, India, 31.7 % in Ethopia [30]. (Sewunet *et al.,* 2022) and other studies that reported their presence in clinical samples in Germany and U. S. A [31, 32]. (Schäfer *et al*., 2019; Zilberberg and Shorr, 2013). It is worth noting that the proportion of *P. aeruginosa* isolates varied with medical conditions and samples, and that comparing epidemiological data of *P. aeruginosa* as in this study may be difficult because other variables influence the outcome of results, such as clinical specimens received for testing, studied population, type of hospitals, and geographical locations.

However, there was more frequent isolation of this bacteria from Intensive Care Unit patients 50.0% and Pediatric 40.0 %. The high prevalent of *P. aeruginosa* at ICU reported in this study was consistent with previous reports in Saudi Arabia [5]. (Lyu *et al.,* 2023) and indicates that the ICU are at high-risk of infection due to increase presence of patients with prolong hospitalization while its prevalence among pediatric patient may be due to poor hygiene conditions.

AmpC β-lactamases producing *P. aeruginosa* in this study express XDR phenotype recording 43.1 % and it reiterate with reports from other studies; according to [8]. Perez *et al*. (2019) after collecting fifty-nine *P. aeruginosa* from twelve different hospitals in Spain, Italy and Greece indicated that the prevalence of the XDR was 88.9% and 19(35.8 %) respectively [8]. (Perez *et al*., 2019). Another published study conducted by Saleem *et al.* (2019) in Pakistan reported that 18.1 % of XDR *P. aeruginosa* isolates were 30.2 %, 17.4 % and 37.2 % resistant to imipenem, ciprofloxacin, and amikacin [9]. (Saleem and Bokhari, 2019). In Iran 15.53 % of the *P. aeruginosa* were XDR [10]. (Mirzaei *et al.,* 2020). Improper antibiotic prescriptions in our hospital could be a possible reason for this resistant features. All *P. aeruginosa* isolates highly susceptible to Amikacin. This aminoglycoside are alternatives that can be used as first line chemotherapy for this XDR *P. aeruginosa.* However, this will depend on reported resistance proportions, which this study have provided at the moment.

The amplified AmpC β-lactamase genes (bla_{FOX} and bla_{MOX}) was harboured in all the XDR *P. aeruginosa* tested. Although bla_{MOX} genes are not commonly reported in *P. aeruginosa* elsewhere but bla_{FOX} genes were detected by PCR in 3 (12.5 %) *P. aeruginosa* isolates by earlier study in south eastern Nigeria [19]. (Ejikeugwu*et al.,* 2021). A striking finding is the predominance of FOX/MOX plasmids newly observed in Mozambique [33]. (Estaleva *et al.,* 2021). Also, [34]. Wassef *et al*. (2014) reported MOX and FOX families as the most prevalent AmpC subtypes in Egypt [34]. (Wassef *et al.,* 2014). The plasmid-derived AmpC β-lactamase FOX and MOX among enteric clinical strains of *Klebsiella pneumoniae* and *E. coli* are most common reported [35, 36, 37, 38, 39]. (Alvarez *et al.,* 2004; Manoharan *et al.,* 2012; Chérif *et al.,* 2015; Govindaswamy *et al.,* 2019; Rizia *et al.,* 2020). However, the frequency distribution of plasmid-borne *AmpC* family genes with respect to type of producer species and source of clinical isolates has been predominantly found in *Escherichia coli* accounting for the greatest number of plasmid-borne AmpC-producers [39]. (Rizia *et al.,* 2020). This clearly based on their genetic similarities to species specific AmpC β-lactamase and plasmid variants groups. To the best of our knowledge, this is the first study from Nigeria that reveals the prevalence of XDR *P. aeruginosa* infection in clinical sample from disease patients. The infections due to the co-existence of XDR *P. aeruginosa* strain with FOX and MOX producers were more frequently healthcare related and patients with such infections had more comorbidities. No other differences were found in terms of risk factor distribution between XDR harboring FOX and MOX.

CONCLUSION

XDR *P. aeruginosa* with the co-expression of FOX and MOX gene are the mainstay of antimicrobial failure and prolong hospitalization amongst the patient. This shows the need for hasty identification, implementation of strict antimicrobial stewardship policies and strong microbiological surveillance procedures in the hospitals to limit the spread of XDR or elaborate their dissemination to pan-drug resistant clone.

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Conflict of Interest: None

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