In-vitro Biofilm Formation and Antimicrobial Resistance of Metallo Beta-lactamase Producing *Pseudomonas aeruginosa* of Clinical Origin

Nwankwo F. M.* Iroha I. R. Awoke O. A. Ugbo E. N. and Nwankwo L. N.
Department of Applied Microbiology, Faculty of Science, Ebonyi State University, Abakaliki, Ebonyi State, Nigeria.
* Corresponding author: mbamfidelis@gmail.com

Abstract: *Pseudomonas aeruginosa* is an important cause of morbidity and mortality in hospitalized patients and patients with underlying medical conditions. The prevalence of biofilm formation and muti-drug resistant strains of *P. aeruginosa* isolates has been on the increase. This study was aimed at *in-vitro* biofilm formation in metallo beta-lactamase producing *Pseudomonas aeruginosa* of clinical origin. A total of 590 different clinical samples were used for this study, during which the samples were collected from different units of Alex-Ekwueme Federal Teaching Hospital and Mile 4 Hospital all in Abakaliki. Standard microbiological methods were used to identify the isolates. The isolated *P. aeruginosa* were further subjected to imipenem-ethylene diamine tetractic acid combine disc test (CDT) to ascertain the metallo beta-lactamase production, biofilm assay using tube method to determine the ability of isolates to form biofilm. The isolates were also subjected to antibiotics susceptibility studies against different classes of antibiotics through disc diffusion method. Out of the 590 samples collected and screened, fifty nine (59) isolates were identified and characterized as *P. aeruginosa.* Thirty four (34) were metallo beta-lactamase (MBL) producer, and 21 were biofilm producers. The antibiogram of the biofilm producing *P. aeruginosa* revealed high resistance rate to cefoxitin (95.2%), nalixidic acid (85.7%), cefepime (80.9%), piperaciilin (80.9%), ofloxacin (76.2%), colistin (76.2%), amikacin (76.2%), tetracycline (71.4%), amoxicillin (71.4%), and ceftriaxone (66.7%). Strict implementation and adherence to antibiotics stewardship in the hospital setting is highly recommended to control and manage the rise antibiotic resistance.
Key word: *In-vitro*, biofilm, metallo beta-lactamase, isolates

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen in nosocomial infections and responsible for high mortality rates in burn centers, cystic fibrosis, pneumonia (Altoparlak, 2004; Lipovy, 2010). Infections caused by *P. aeruginosa* are difficult to treat, as the majority of isolates exhibit innate resistance to several antibiotics, due to poor outer-membrane permeability, constitutive expression of various efflux pumps and production of antibiotic inactivating enzymes (Lambert, 2002). Among these, the important roles of various β-lactamases such as AmpC, extended-spectrum β-lactamases (ESBL) and carbapenemases have been reported (Mesaros, 2007; Kumar, 2012). AmpC β-lactamase is responsible for resistance to cephalosporins and ESBLs confer resistance to all β-lactams except for the carbapenem family. Carbapenemases, particularly metallo beta-lactamases (MBL), hydrolyze all β-lactam antibiotics with the exception of monobactams. Co-existence of multiple β-lactamases in clinical isolates of *P. aeruginosa* is common, causing resistance to almost all β-lactam antibiotics (Upadhyay, 2010).

Another important factor contributing to the pathogenesis of *P. aeruginosa* in causing fatal infections is its potential to form biofilms on biotic and abiotic surfaces (Karatuna, 2010). The bacterial populations in biofilms are usually more resistant to antibiotics and host-mediated clearance strategies compared to their planktonic counterparts, giving rise to chronic infections that are notoriously difficult to eradicate (Costerton, 1995; Mah, 2003). Bacteria growing in biofilms produce one or more extracellular polymeric matrices which hold the cells of the biofilm community together. Polysaccharides are important components of the biofilm matrix, as they contribute to the overall biofilm architecture and to the resistance of biofilm-grown bacteria to certain antibacterial agents.

At least three exopolysaccharides have been shown to be involved in biofilm formation by *P. aeruginosa*, including alginate, *psl*,

and *pel* (Ghafoor, 2011). Among these, *psl* is a mannose-rich polymer with an essential role in the initial steps of biofilm formation by non-mucoid *P. aeruginosa* as well as in its maintenance. *psl* forms a helical structure around *P. aeruginosa* cells which increases the cell-to-surface and cell-to-cell interactions necessary for biofilm formation (Jackson, 2004; Ma, 2006). Synthesis of *psl* is mediated by the *psl* gene cluster (pslA-pslO) and pslA has been reported to be the first and most important gene necessary for *psl* synthesis (Matsukawa, 2004; Overhage, 2005). To the best of our knowledge this research study is the first documented epidemiological analysis of *in-vitro* biofilm formation and antibiotics resistance pattern of MBL producing clinical isolates of *P. aeruginosa* in a tertiary hospitals in Abakaliki, Ebonyi state. As such our present study, therefore, would be useful to understand the dynamics of biofilms forming MBL *P. aeruginosa* strains in our study center.

MATERIALS AND METHODS

Study area: This study was carried out Alex Ekwueme Federal University Teaching Hospital Abakaliki and Mile 4 Missionary Hospitals all in the capital city of Ebonyi State, Nigeria. Ebonyi State is located in the South Eastern part of Nigeria. The state shares boundary with Benue, Cross River, Abia and Enugu States. It is between longitude 7°30' and latitude 60°45' E and about 3,242,500 in population mainly farmers and traders.

Sample collection: The samples used were collected from Alex-Ekwueme Federal Teaching Hospital and Mile 4 Hospital as follows: wound (185) using sterile swab stick to rotate on the wound surface in a zigzag motion, urine (230) through the use of well labeled sterile asptic container to collect early morning urine, ear swab (90) by the use of sterile cotton swab deeped into the ear canal while sputum (50) the patients was asked take a deep breaths, then force out deep cough and expectorate into a sterile screw-top container and ear (35) were from Mile 4 hospital using previous method. The collected specimens were transported to the Department of Applied Microbiology Laboratory unit, Faculty of Science, Ebonyi State University, Abakaliki within two hours of collection for bacteriological analysis. Then specimens were cultured in nutrient broth and to nutrient agar for isolation and identification of *P. aeruginosa*.

Isolation, identification and Characterization of the isolates: The various clinical samples were collected from patients who visited the hospitals and inoculated in a sterile nutrient broth. This was incubated for 24 hrs at 30°C and observed for turbidity. Test tubes with turbidity was aseptically streaked on agar plate and incubated at 37°C for 24 hrs. The colonies they were subcultured to obtain pure cultures which were observed for colony morphology. Gram staining and biochemical tests such as catalase test, oxidase test, citrate utilization, indole test and sugar fermentation test were carried out on the pure cultures. Culturally morphologically and biochemically identified *P. aeruginosa* was done and further characterized by PCR using 16s rRNA specific primers (Lamont, 2003).

Antibiotic susceptibility studies of the bacterial isolates: Antibiotic sensitivity of the biofilm producing metallo beta-lactamase *P. aeruginosa* isolates was determined using Kirby – Bauer disc – diffusion method as described by CLSI (2022). Briefly, a sterile swabs stick was used to inoculate the test organism onto Mueller-Hinton agar. Sterile forceps was used to carefully distribute the following antibiotic disc, meropenem (MEM10µg), ceftriaxone (30µg), ofloxacin (5µg), tetracycline (30µg), piperacillin/TAZ (40µg), amikacin (30µg), colistin (10µg), cefoxitin (30µg), amoxicillin/clavulanic (30µg), nalidixic acid (30µg), imipenem (10µg), cefepime (10µg) (Oxoid, UK) evenly on the inoculated plates at a distance of 30 mm. The plates were placed on the bench for 30 minutes to allow pre-diffusion of the antibiotics, inverted and incubated

aerobically at 35ºC for 18-24 hours. The zones of inhibition was measured using a meter rule recorded as milliters (mm) and compared with CLSI guidelines on antimicrobial susceptibility studies (Chigbu, 2003).

Determination of matallo beta-lactamse production of the isolates: Using imipenem-ethylene diamine tetracetic acid combine disc test (CDT)**.** A lawn culture of the test isolate was prepared and allowed to dry for five minutes. Two imipenem (10 µg) discs, one with 0.5 M EDTA and other a plain imipenem disc, was placed on the surface of agar plates approximately 30 mm apart. The plates was then incubated overnight at 37ºC for 16-18hrs and then observed for an increase in zone diameter of >7 mm around the imipenem-EDTA disc in comparison to imipenem disk alone which indicates the production of MBL (Chigbu, 2003).

Biofilm assay of the bacterial isolates: A loopful of the test organisms was inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 hrs. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong (Chigbu, 2003).

Ethical consideration: Ethical clearance for this study was obtained from the institutional ethical committee and research committee of Ministry of Health Abakaliki Ebonyi State, Health Committee Assigned Number: HC/032

RESULTS

Table 1: Prevalence of *Pseudomonas aeruginosa* from different clinical samples

Location	specimen	No. sample	No. Isolates (%)
AE-FETHA 1	Wound	80	9(11.2%)
AE-FETHA 1	Urine	130	6(4.6%)
AE-FETHA 1	Ear swab	70	9(12.8%)
AE-FETHA 2	Wound	105	18(17.1%)
AE-FETHA 2	Urine	100	4(4%)
AE-FETHA 2	Ear swab	20	4(20%)
Mile 4	Sputum	50	5(10%)
Mile 4	Ear swab	35	4(11.4%)
Total		590	59(10%)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

Table 2: Age distribution of *Pseudomonas aeruginosa* among patients from AE-FETHA and Mile 4 hospitals

Variable	Total samples	Total isolates	AE-FETHA (%)	Mile 4 (%)
Age				
0-20	98	7	5(71.4)	2(28.6)
21–40	175	21	16(76.2)	5(23.8)
41–60	180	11	4(36.4)	7(63.6)
61+	137	20	13(65)	7(35)
Total	590	59	38(64.4)	21(35.6)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

Table 3: Frequency of isolation of MBL producing *Pseudomonas aeruginosa* from different clinical samples

Location	Specimen	Isolates	MBL (%)
AE-FETHA 1	Wound	9	3(33.3%)
AE-FETHA 1	Urine	6	2(33.3%)
AE-FETHA 1	Ear swab	9	5(55.5%)
AE-FETHA 2	Wound	18	13(72.2%)
AE-FETHA 2	Urine	4	3(75%)
AE-FETHA 2	Ear swab	4	3(75%)
Mile 4	Sputum	5	2(40%)
Mile 4	Ear swab	4	3(75%)
Total		59	34(57.6)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

Table 4: Antibiogram of biofilm producers from the MBL producing *Pseudomonas aeruginosa*

Antibiotics	No of isolates	Susceptible (%)	Resistance (%)
Imipenem	21	11(52.4)	10(47.6)
Meropenem	21	13(61.9)	8(38.1)
Ceftriaxone	21	7(33.3)	14(66.7)
Tetracycline	21	6(28.6)	15(71.4)
Ofloxacin	21	5(23.8)	16(76.2)
Piperacillin	21	4(19.1)	17(80.9)
Colistin	21	5(23.8)	16(76.2)
Cefoxitin	21	1(4.8)	20(95.2)
Amoxicillin	21	6(28.6)	15(71.4)
Nalidixic Acid	21	3(14.3)	18(85.7)
Cefepime	21	4(19.1)	17(80.9)
Amikacin	21	5(23.8)	16(76.2)

Table 5: Distribution of MBL biofilm forming *Pseudomonas aeruginosa* from different clinical samples

Location	Specimen	MBL	Biofilm positive (%)
AE-FETHA 1	Wound	3	1(33.3%)
AE-FETHA 1	Urine	2	0(0%)
AE-FETHA 1	Ear swab	5	3(60%)
AE-FETHA 2	Wound	13	9(69.2%)
AE-FETHA 2	Urine	3	1(33.3%)
AE-FETHA 2	Ear swab	3	2(66.6%)
Mile 4	Sputum	2	2(100%)
Mile 4	Ear swab	3	3(100%)
Total		34	21(61.8)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

DISCUSSION

In this study, the total of 59(10%) samples collected from (AE-FETHA 1 and 2; and Mile 4) were colonized with *P. aeruginosa* isolates and the highest frequency distribution of 20% was observed in ear swab samples compared to the frequencies across the isolates. This is similar to the 10.5% reported in Zaria, northern Nigeria by Olayinka *et al.* (2004) while contrary to the 20.3% as reported in a study conducted by Savas *et al.* (2005) in India and 30% in

Pakistan by Nadeem *et al.* (2009) as this similarity could be attributed to the prevalence of nosocomial infection. This study revealed that *P. aeruginosa* prevalence rate to be 11.2% and 17.1% in wound samples of AE-FETHA 1 and 2 respectively, 4.6% and 4% in urine samples of AE-FETHA 1 and 2, 12.8% and 20% in ear swab of AE-FETHA 1 and 2 respectively, while 10% and 11.4% in sputum and ear swab from mile 4 respectively. This is largely different from the study of Ndip *et al.* (2005), but similar to the 17.2% wound sample as report of 17.85% compared to Ekrem *et al.* (2014). The highest rate of 76.2% and 71.4% prevalence of *P. aeruginosa* was observed among patients within the age range of 0-20 and 21-40 in AE-FETHA 1 and 2 respectively and 63.6% at the age range of 41-60 in Mile 4 hospital was recorded in this study. This is similar to the report of Okon *et al.* (2009) on resistance pattern of *P. aeruginosa* isolated from clinical specimens in a tertiary hospital in northeastern Nigeria.

This study examined the prevalence rate across the age distribution, it showed greatest percentage 76.2 %(21-40), 71.4 %(0-20) in AE-FETHA. This likely indicates that several youthful activities can contribute to the emergence and spread of bacterial pathogens like *Pseudomonas aeruginosa.*

In this study, the total of 34(57.6%) out of the whole isolates were implicated to have produced metallo beta-lactamase (MBL) enzyme. The highest rate of 75% was recorded in urine and ear swab samples collected from AE-FETHA urine and ear swab samples and Mile 4 ear swab samples. This finding is in tandem with reports of studies in and outside Nigeria by Umar *et al.* (2020) and Peshattiwar *et al.* (2011) with similar 75% prevalence rate as reported in this sudy. Biofilm-forming bacteria play a significant role in the development of chronic and recurrent infections that pose challenges for treatment. Managing and effectively treating biofilm-associated infections is of utmost importance in healthcare settings (Da Costa Lima, 2017). The distribution of biofilm forming *P. aeruginosa* from different clinical samples revealed in this study that, 0(0%) in AE-FETHA 2 (ear swab), 100% of MBL producing isolates from sputum and ear swab samples from Mile 4 hospital. This implies that those isolates harbour greater percentage of gene mediating biofilm formation in them which is in contrast to the report of Da Costa Lima *et al.* (2017).

This study also examined the antibiotic susceptibility of *P. aeruginosa* and it was found that cabapenem class of antibiotics has the highest effect on the majority of the isolates. The antibiogram findings showed low level of resistance to members of carbapenem class of antibiotics, specifically imipenem and meropenem of 47.6% and 38.1% respectively. The high susceptibility pattern of these drugs could be associated to less drug abuse by the population being that the cost of these antibiotics prevents patient's self-medication. However, recent studies by Ranjbar *et al.* (2011) and Hamze *et al.* (2012) in Lebanon revealed a high resistance of 97.5% and 33.3% of *P. aeruginosa* to imipenem respectively, thus, demonstrating the evolution of imipenem-resistant strains of *P. aeruginosa*. Nevertheless, imipenem remain a potent anti-pseudomonal antimicrobial agent in Nigeria contrary to other report outside Nigeria where high imipenem resistance is prevalent (Odumosu, 2012). However, the antibiogram of the biofilm formers revealed high resistance rate of cefoxitin 20(95.2), nalixidic acid 18(85.7), cefepime 17(80.9), piperaciilin 17(80.9), ofloxacin 16(76.2), colistin 16(76.2), amikacin 16(76.2), tetracycline 15(71.4), amoxicillin 15(71.4), ceftriaxone 14(66.7), this is in disagreement to an international multicenter study done by Micek *et al.* (2015).

In the present, it is interesting to note that MBL *P. aeruginosa* isolated from ear swab and sputum samples in Mile 4 hospital apparently are all biofilm formers. Meanwhile 0% biofilm formation was seen in urine samples from AE-FETHA 1.

CONCLUSION

This study reported the presence of biofilm formation in metallo beta-lactamase producing *Pseudomonas aeruginosa* isolated from Alex-Ekwueme Federal Teaching Hospital and Mile 4 Hospital. Metallo beta-lactamase producing *Pseudomonas aeruginosa* had percentage prevalence of 57.6 and biofilm producing *Pseudomonas aeruginosa* had a prevalence of 61. 8%. Thus, stringent measure is needed to tilting proper usage of antibiotics in the patient treatment at hospital and strict implementation and adherence to antibiotics stewardship in the hospital setting is highly recommended.

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