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Phenotypic and genotypic detection of antibiotic resistance among *Pseudomonas aeruginosa* isolates from orthopedic wounds from Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (Coouth), Awka Anambra Nigeria

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Abstract

Pseudomonas aeruginosa is well known orthopedic wounds-associated bacterial pathogen, responsible for prolonged wound treatment, due to its resistance, mediated by different types of extended-spectrum beta-lactamases (ESBL). The present study was undertaken to find the prevalence of extended-spectrum β lactamase (ESBL), Metallo-β lactamase (MBL), and AmpC β lactamase in *P. aeruginosa* isolated from orthopedic wound samples. Wound swabs were processed and *P. aeruginosa* was identified by standard protocols. The Kirby-Bauer disc diffusion method was used in ascertaining the antibiotic sensitivity of the isolates. Special screening and confirmatory tests were performed for the phenotypic and genotypic detection of resistance. The resistance rate to gentamicin, ofloxacin, ciprofloxacin and ceftazidime were 2.6%, 2.6%, 2.6, and 5.1% respectively. *P. aeruginosa* expressed very high resistance to amoxicillin (97.4%), nitrofurantoin (100%), and two of the 3rd generation cephalosporins: cefuroxime (100%), and cefixime (100%). The aminoglycoside and the quinolones were the most effective drugs against the P. aeruginosa isolates with susceptibilities of 97.4% and 94.9% respectively. Out of the 38 isolates of *P. aeruginosa*, 11 (28.9%) and 14 (36.8%) were ESBL and MBL positive, respectively. The blaTEM genes were the only prevalent genes detected in all 38 (100%) isolates and mediated the phenotypic and genotypic resistance observed. MBL production was observed in 14 (36.8%) while AmpC and co-expression of genes were not observed. The blaTEM genotypic resistance was observed to be common among the isolates. This calls for great concern and continuous monitoring of antimicrobial resistance with the implementation of stringent mediation practices toward infection prevention and control.

Keywords: *Pseudomonas aeruginosa*; Pathogen; Resistance genes; Extended spectrum β lactamase; Sensitivity; resistance

1. Introduction

The confirmation by the clinical microbiology laboratory of the susceptibility of bacterial isolates to chosen antimicrobial agents via the performance of antimicrobial susceptibility testing is of great importance. Antimicrobial resistance expressed by certain bacterial isolates is believed to be mediated by a family of enzymes capable of degrading certain parts of the antimicrobial molecule thus rendering it inactive [1, 2]. The Gram-Negative cell wall structure which comprises the outer membrane, the periplasmic space, and the cytoplasmic membrane is a target for various antimicrobial agents due to their important roles in the survival of the bacterium. Among these, the penicillin-Binding-Proteins (PBPs) which are periplasmic proteins are thought to have the same origin as Beta-lactamases and are responsible for the synthesis of peptidoglycan. Thus, certain Gram-negative bacteria isolates evade (or resist) the actions of beta-lactams antibiotics through the production of beta-lactamase enzymes which degrade beta-lactam

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molecules and have evolved thus express resistance to a wide range of beta-lactam antibiotics [3, 2]. Regrettably, the expression of co-resistance by ESBL-positive bacteria isolates to many other different antibiotics has resulted in the limitations of available therapeutic options [4].

Pseudomonas aeruginosa is one of the most widely implicated pathogens associated with nosocomial, septicemia, and burn infections, [5, 6], which has shown difficult susceptibility to treatments. ESBL-positive Enterobacteriaceae especially *Pseudomonas aeruginosa* has been established to possess SHV, CTX-M-, PER, VEB, and TEM genes and as a major cause of wound infections and surgical infections [5].

This study was carried out to investigate the multidrug resistance of isolates of *P. aeruginosa* and the phenotypic and molecular detection of ESBL-positive strains in patients with wound infections in Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Awka Anambra Nigeria.

2. Material and methods

A total of 121 (49%) isolates of *Pseudomonas aeruginosa* were isolated from 245 wound swabs obtained from patients having post-operative orthopedic wounds.

All the isolates were analyzed by combining standard macroscopic and microscopic protocols. The isolates were confirmed by conventional biochemical methods.

Ethical approval was granted (COOUTH/CMAC/ETH.C/Vol.1/0050) by the Ethical Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Awka before sample collection.

2.1. Determination of resistance

Testing for antibiotic sensitivity of the isolates was done using the Kirby-Bauer method [7]. Commercially available single antibiotic discs of Ceftazidime (CAZ 30 µg), Cefuroxime (30 µg), Gentamicin (10 µg), Cefixime (5 µg), Ofloxacin (5 µg), Amoxicillin clavulanic (30 µg), nitrofurantoin (300 µg), Ciprofloxacin (5 µg) (Abtek, UK) were placed on the inoculated Mueller-Hinton agar plates. The plates were incubated at 37°C for 24 h in an inverted position after which the zones of inhibition around each disc were measured to the nearest millimeter (mm) with a meter rule, recorded, and interpreted according to the CLSI [8] guidelines.

2.2. Preliminary ESBL Screening

For the preliminary screening of ESBL-producing strains, isolates that expressed multidrug resistance including resistance to third-generation cephalosporins (ceftazidime and/or cefuroxime) were further screened for ESBL production by checking their susceptibility against ceftazidime, cefotaxime, and cefoxitin and in combination with amoxicillin-clavulanic acid on Mueller-Hinton agar. In each plate, four discs were placed at inter-disc distances of 25 or 30 nm away from an amoxicillin/clavulanic acid disc (20/10 µg). Plates were incubated at 37°C for 18 to 24 h. Strains were considered ESBL-positive when the difference of zone diameters between the beta-lactam disc and disc containing antibiotic associated with clavulanic acid was > 5 mm. Suspected ESBL producers were further subjected to Double Disc Synergy Test (DDST) for phenotypic confirmation [9].

2.3. Phenotypic ESBL confirmation

Probable ESBL producers were confirmed using the DDST. Here, synergy was determined between a disc of lactamases inhibitor (Amoxicillin 20 µg and Clavulanic acid 10 µg) and a 30 mg disc of each of third-generation cephalosporins (Ceftazidime30 µg and Cefotaxime 30 µg) test antibiotic placed at a distance of 15 mm apart on lawn culture of the resistant isolates on Muller Hinton Agar. The test organism was confirmed to produce ESBL if the zone size around the antibiotic disc increased from 5mm above in the presence of a beta-lactamase inhibitor disc (Amoxicillin 20 µg and Clavulanic acid 10 µg). This increase occurs because the clavulanic acid inactivates the ESBL produced by the test organism resulting in the formation of an extended inhibitory zone.

2.4. Metalo Beta-Lactamase (MBL) Screening and Confirmation

Isolates resistant to meropenem (MRP) and ceftazidime were further confirmed for MBL production by combined disc test. Meropenem+ EDTA disc and meropenem disc alone were each placed on a lawn of Mueller-Hinton agar plates inoculated with the test organism and incubated at 37°C for 24 h in an inverted position. An organism is considered to be MBL-positive if there is an increase of ≥7 mm in the zone of inhibition around the meropenem+ EDTA disc as compared to the meropenem disc alone [10].

2.5. Molecular Characterization of ESBL-genes

2.5.1. Bacterial Cell Preparation

All the ESBL-positive *P. aeruginosa* isolates were prepared for DNA isolation. An overnight culture was used. The overnight culture was transferred into a 1.5 ml capped Eppendorff collect tube and bacterial cells were harvested by centrifuge for 2 min at 13100 rpm.

2.5.2. DNA Extraction

DNA extraction was carried out using the HiPurATM Bacterial Genomic DNA purification Kit (HIMEDIA, category no MB505-50PR) by following the manufacturer's instructions. Briefly, the harvested bacterial cells were re-suspended in 180 ml of lysis solution (AL) followed by the addition of 20 mL of Proteinase K (20 µg/ml), and were incubated at 55 °C for 30 mins. Thereafter, 200 mL of lysis solution (C1), was added, vortexed for 15 secs, and incubated at 55 °C for 10 min. Following incubation, 200 mL ethanol (95-100%) was added, vortexed for a few seconds, transferred to a miniprep spin column, and centrifuged at 10,000 rpm for 1min. afterward, the flow-through liquid was discarded and placed in the same collection tube. Thereafter, 500 ml of prewash solution was added and centrifuged at 10,000 rpm for 1 min. The flow-through liquid was discarded and placed in the same collection tube. A 500 mL of wash solution was added and centrifuged at 13,000-16,000, for 3 mins. The flow was again discarded and further spun for 1 min. It was then transferred to a fresh uncapped collection tube where 200 ml of elution buffer was added into the column and incubated at room temperature for 5 min followed by centrifuging at

10,000 rpm for 1 min. Finally, the eluent was transferred to a fresh capped collect tube for storage at -20°C and used for various PCR reactions.

2.5.3. Polymerase Chain Reaction (PCR) Amplification of Genomic DNA for ESBL detection

The extracted DNA of each positive ESBL producer was subjected to multiplex PCR using specific primers for different families of ESBLs (blaCTX-F, blaCTX-R, blaSHV, blaTEM-F, and blaTEM-R). The PCR amplification was performed using 2 µl of sample DNA in a total volume of 25 µl reaction mixture with amplification conditions.

The PCR products were separated on 1.5% agarose gel electrophoresis and visualized with 0.5 µg/ml ethidium bromide under an ultraviolet transilluminator (Biozen lab, UK). The gel was poured into a gel tray and was filled with Tris base, acetic acid EDTA (TAE) buffer until the gel is covered. The DNA samples were carefully loaded into additional wells of the gel. The Agarose gel Electrophoretic machine was run at 80 V – 150 V for 1hr, afterwards, the gel was carefully removed from the gel box and viewed under UV light to visualize the DNA fragments. The DNA ladder in the first lane was used as a guide to interpreting the bands.

Low molecular weight and a 50bp ladder were used as the molecular weight standards.

3. Results

3.1. Identification of *Pseudomonas aeruginosa*

Following the analysis of the 245 clinical (wound swabs) samples obtained from patients in orthopedic wards, 39 non-duplicate isolates were confirmed to be *Pseudomonas aeruginosa* based on their cultural features and biochemical reactions and production of pyocyanin.

3.2. Antibiotic susceptibility patterns

We observed varying patterns of susceptibility of the *P. aeruginosa* isolates to the tested antibiotics. *P. aeruginosa* isolates expressed resistance to gentamicin (2.6%), ofloxacin (2.6%), ciprofloxacin (2.6%), and ceftazidime (5.1 %). Other percentages of resistance were observed to be very high and included amoxicillin-clavulanic acid (97.4%), cefuroxime (100%), cefixime (100%) and nitrofurantoin (100%). Also, the *P. aeruginosa* isolates expressed 100% resistance to two of the 3rd generation cephalosporins (3GCs) which included cefuroxime and cefixime while the aminoglycoside and the quinolones were the most effective drugs against the *P. aeruginosa* isolates with susceptibility of 97.4% for each of the antibiotic in these classes used, followed by ceftazidime (5.1 %) (Figure 1).

Among the 3GCs tested, ceftazidime was the only active (94. 9 %) drug against the *P. aeruginosa* isolates. this implied that 66.7 % of the 3GCs used may have been hydrolyzed by a beta-lactamase thus indicating the presence of broad-

spectrum beta-lactamases producing *P. aeruginosa*. The study also revealed that all thirty-eight isolates (100%) were multidrug-resistant.

3.3. Phenotypic expression of ESBL and molecular detection of ESBL and MBL encoding genes

All the isolates that were resistant to two of the three 3GCs also expressed ESBL and MBL. Out of the 39 isolates of *P. aeruginosa*, ESBL production was observed in 11 (28.2%) while MBL production was observed in 14 (35.8%) as shown in Table 1. Also, of the 39 *P. aeruginosa* isolates, 28 were tested by multiplex PCR for the detection of ESBL and MBL. These data revealed the ESBL-phenotypic and genotypic resistance observed in this study are mediated by bla_{TEM} (100%) only. However, none of the isolates exhibited co-expression of other genes including bla_{SHV} and bla_{CTX}. Also, MBL-genotypic resistance bla_{VIM} was not detected in any of the strains tested. The genotypic resistance observed to be common among the *P. aeruginosa* isolates was $bla_{TEM\text{-}F}$.

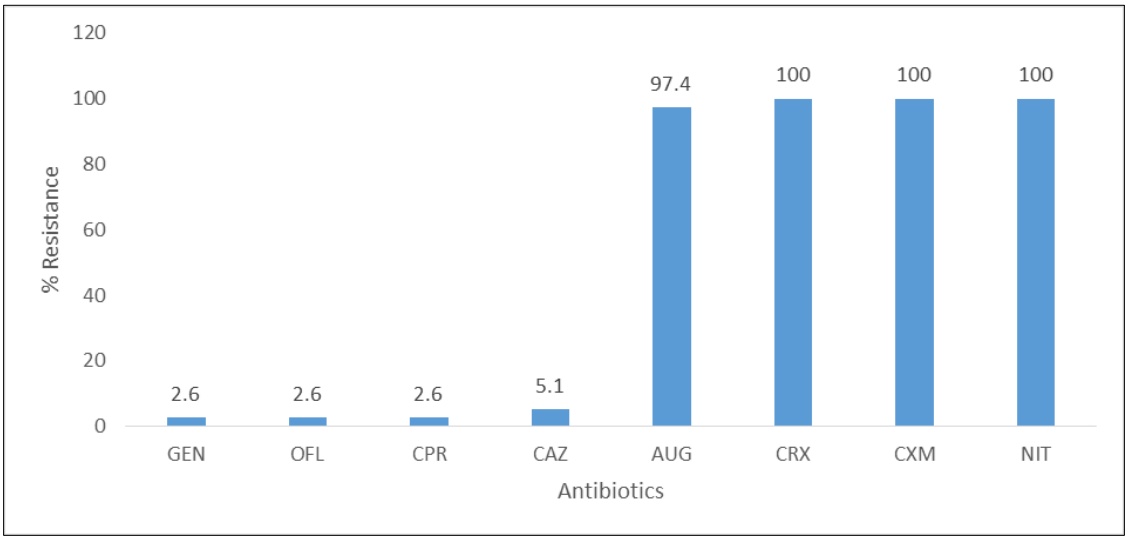

Figure 1 Antibiotic susceptibility patterns of *P. aeruginosa* (N=39)

Table 1 Prevalence of ESBL and MBL-positive *P. aeruginosa* isolates

No. of *P. aeruginosa* isolates	No. of ESBL-positive *P. aeruginosa* isolates	No. of MBL-positive *P. aeruginosa* isolates
39	11	14

4. Discussion

The outcome of this study has detected the presence of *P. aeruginosa* as one of the pathogenic Enterobacteriaceae implicated in the wound infection of the patients sampled. *P. aeruginosa* was detected in 39 (15.9%) wound swab samples while 206 (84.1%) had other Enterobacteriaceae. In the present study, the incident rate of *P aeruginosa* isolated from wounds was lower than in 2005 (51.5%) and 2002 (58.95%) reported previously in Chandigarh, India [11] and much higher than 18.6% reported in the western part of Nigeria [12]. However, a similar incident rate of 34.92% was reported by Oli *et al*., [13]. Also, the presence *of P. aeruginosa* in the wound samples analyzed in this study is in accordance with other studies [13, 11, 12, 14, 15]. The changing trend in the detection and prevalence of *P. aeruginosa* may be attributed to important factors including disinfection policies, hospital infection control measures, and assessment and control of bioaerosols.

The antibiotic sensitivity revealed varying susceptibility patterns by the *P. aeruginosa* isolates to antibiotics used. A high level of resistance by the isolates to amoxicillin-clavulanic (97.4%), cefuroxime (100%), cefixime (100%), and nitrofurantoin (100%) was recorded. This is in accordance with Oli et al., [13] who observed resistance to cefuroxime (94.12%) and ceftriaxone (61.76%), and also Zafer *et al*., [16] who reported a resistance rate of 87.7%, 80.3%, and 60.6% for the antibiotics cefuroxime, cefoperazone, and ceftazidime respectively. While gentamicin, ofloxacin, and ciprofloxacin showed potency with a similar bacteriostatic activity of 97.4% followed by ceftazidime at 94.9%. Our findings are not in agreement with the data from surveillance on clinical isolates of *P. aeruginosa* from Chukwuemeka

Odumegwu Ojukwu University Teaching Hospital Amaku-Awka, as reported by Oli *et al.,* [13]. The study reported resistance of 32.35% and 47.06%, respectively to the quinolones (sparfloxacin and ciprofloxacin). Also, resistance to gentamicin 23.53% was observed. In this study, we recorded 2.1% of resistance to the quinolones and gentamicin which was far lower than the range of 32.35% - 47.06% reported by Oli *et al*., [13]. Due to their mechanisms of action, which results in the loss of Penicillin-Binding Protein (PBP) enzymatic activity, Beta-lactams are the most used and also the most important group of antibiotics [17]. Members of the antibiotic class β-lactam, are essential to the practice of medicine in the 21st century, where it is included in almost two-thirds of recent hospital prescriptions [18]. In this study, the observed variation in the resistance to the antibiotics tested may be due to increasing selective pressure resulting from extensive use of these antibiotics. Thus, the importance of continuous surveillance on the prescription of these antibiotics and antimicrobial resistance should be given a World health emergency status with strict monitoring of the adherence to appropriate antibiotic treatment within the study area.

The data presented in this study on the observed change in the pattern of antibiotic resistance in the orthopedic unit should be studied and applied both for clinical settings and epidemiological purposes. In this study, high percentage resistance was observed for amoxicillin-clavulanic while all the isolates were resistant to two 3GCs cefuroxime and cefixime. Similarly, Zafer *et al*., [16] reported a high resistance rate of 87.7%, and 80.3% by *P. aeruginosa* to cefuroxime, and cefoperazone respectively. Whereas, Oli *et al*., [13] and Hosu *et al*., [19] detected an average resistance of 51.3% to the cephalosporins (ceftazidime and cefepime). This further confirms the increasing resistance trend for *P. aeruginosa* and other members of the Enterobacteriaceae family. In Nigeria, the prevalence of ESBL-producing Gram-negative bacteria from southeast Nigeria ranged from 8.1% to 74.3%. Antibiotic resistance is a global health challenge that is receiving tremendous attention that may have been in part triggered by the dwindling development of newer medicines to tackle this menace. Also, several studies have made available useful data on the incident and antibiotic status of *P. aeruginosa* and have identified a link between antibiotic prescription and the emergence and widespread of antibiotic resistance [5, 20, 21, 22, 23, 24].

The production of specialized enzymes such as the extended-spectrum beta-lactamases and metallo beta-lactamases by some bacteria that confer resistance to certain antibiotics has further hampered antimicrobial treatment [19]. Among these bacteria is *P. aeruginosa*, which is constantly implicated as a major pathogen of healthcare-associated infections. Within the hospital setting, treatment of infections caused by *P. aeruginosa* has become a challenge and this is due to the constantly changing antibiotic status of this bacteria to commonly recommended antibiotics [25, 26]. In this study, the genotypic detection of resistance confirmed *bla*TEM in 11 (28.9%) isolates as the only genotype that encodes ESBL. This is in accordance with an earlier study carried out by Tanko *et al.,* [27] who recorded a prevalence of 8.1-74.3%. However, *bla*SHV and *bla*CTX-M genes were not detected in any of the isolates identified in this study. Similarly, Miranda *et al*., [28] observed *bla*CTX-M as the least detected ESBL genotype. Furthermore, data from previous studies on the molecular detection of resistance genes showed *bla*TEM as the most prevalent resistance gene as reported by Hosu *et al*., [19]; Kaur and Singh [29]; Erhlers *et al*., [30]; Chen *et al*., [5]; and Miranda *et al*., [28].

In contrast, Laudy *et al*., [31] who investigated the occurrence of ESBLs in *P. aeruginosa* isolated from patients from hospitals in Warsaw, showed the presence of only the *bla*TEM-1 gene encoding TEM-1 β-lactamase in three isolates. Also, another study by Tanko *et al*., [27], observed *bla*TEM as the most prevalent gene detected in their study. Whereas the genes encoding enzymes of the families VEB, GES, PER, OXA, TEM, SHV, BEL, and CTX-M were not detected. As reported in several studies, the incidence rate of ESBL was observed to vary from one geographical location to another, or from one health setting to another, and even for a given place over time [18, 27, 32, 33, 34]. Thus, the increase and spread of antibiotic resistance is time-dependent and should be monitored with the sole aim of containment in cases of emerging resistant strains.

Carbapenem resistance in *Pseudomonas aeruginosa* is most commonly due to the production of metallo beta-lactamases (MBLs). Of the 39 clinical strains of MEM-nonsusceptible *P. aeruginosa* from the orthopedic unit, 14 (35.8%) were MBL positive, detected phenotypically while none of the strains tested expressed the *bla*VIM genes thus were not PCR positive for MBL genes. Similarly, Hosu *et al*., [19] reported the phenotypic resistance displayed by 82 strains of *P. aeruginosa* to imipenem antibiotic was not validated by the genotypic MBL result. Thus, the observed phenotypic resistance by *P. aeruginosa* to the antibiotics of the carbapenem used may be linked to other mechanisms such as the efflux pump by which these isolates neutralize the effects of antibiotics in this class such as meropenem. This is in accordance with Sumita and Fukasawa, [35], who observed meropenem-low-susceptibility in *Pseudomonas aeruginosa* strains. Also, Xu *et al.,* [36] observed a strong correlation between increased usage of anti-pseudomonal carbapenems ($P<0.001$) with the prevalence of imipenem and meropenem-resistant *P. aeruginosa*. In contrast, Begum *et al.,* [15] observed the susceptibility of *P. aeruginosa* isolates to imipenem.

In this study, AmpC was not detected in any of the 38 clinical isolates tested. Moreover, the genes that encode this resistance were not confirmed due to the absence of genotypic resistance genes. This may be due to Mutations in the genes encoding AmpC that also mediate resistance to cefepime [37]. The detection of combinations of ESBL genes present in the same strain has been reported by several researchers [19, 16, 38]. This contradicts our findings, where combinations of ESBL genes in the *P. aeruginosa* isolates reported in this work were not detected. This study revealed *bla*TEM as the most prevalent which is corroborated by other studies.

5. Conclusion

P. aeruginosa was detected in 39 wound swab samples. The detection of a high rate of multidrug-resistant *Pseudomonas aeruginosa* that expressed ESBL- genetic resistance genes in this study is an indication of negligence in the policy on infection control within the study area that must be reviewed and monitored aggressively in order to mitigate the possibility of mutation and spread of these resistance genes within the hospital and in the community. The expression of the *bla*TEM resistance genes by the majority of the isolates calls for stringent measures for early detection, characterization, and prevention of further mutation of the genes that confer the broad spectrum resistance currently being faced and has re-emphasized the importance of susceptibility testing as a crucial aspect in the diagnosis and treatment of serious bacterial infections. Therefore, screening and surveillance for beta-lactamase-*Pseudomonas*-producing isolates implicated in wound infection including other *Enterobacteriaceae* should be made a routine exercise and monitored strictly. This calls for enacting and monitoring the implementation of policies on antibiotic usage in healthcare settings.

Although resistance patterns have been reported to be geographically distinct, there is a need for efficient containment strategies to reduce the spread of emerging resistance.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

Statement of ethical approval

Ethical approval was granted (COOUTH/CMAC/ETH.C/Vol.1/0050) by the Ethical Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Awka before sample collection.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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