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Detection of antibiotic resistance genes among multiple drug resistant *Pseudomonas aeruginosa* strains isolated from clinical sources in selected health institutions in Kwara State.

***Adekunle O.C.[1], Mustapha A.[2], Odewale G.[3], Ojedele R.O.[4]**

Abstract

Introduction *Pseudomonas aeruginosa* (*P. aeruginosa*) is a frequent nosocomial pathogen that causes severe diseases in many clinical and community settings. The objectives were to investigate the occurrence of multiple antibiotic resistant P. aeruginosa strains among clinical samples and to detect the presence of antibiotic resistance genes in the DNA molecules of the strains.

Methods: Clinical specimens were collected aseptically from various human anatomical sites in five selected health institutions within Kwara State, Nigeria. Multiple drug resistance patterns of isolated micro-organisms to different antibiotics were determined using the Bauer Kirby disc diffusion technique. The DNA samples of the multiple resistant *P. aeruginosa* strains were extracted and subjected to Polymerase Chain Reaction (PCR) for resistance gene determination.

Results: A total of 145 isolates were identified as *P. aeruginosa* from the clinical samples. Absolute resistance to ceftazidime, gentamicin and ceftriaxone was observed while low resistance to ciprofloxacin, piperacillin and imipenem was documented. The prevalence of bla_{VIM}, ,bla_{CTX-M} and bla_{TEM} were 34.4 %, 46.7 % and 16.7 % respectively.

Conclusion: This study has shown that there is a high occurrence of metallo β-lactamase- producing and antibiotic-resistant strains of *P. aeruginosa* in clinical specimens from the studied area.

Keywords: Metallo β-lactamase enzyme, *P. aeruginosa,* clinical samples, antibiotic-resistance genes.

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Détection de gènes de résistance aux antibiotiques parmi plusieurs souches de Pseudomonas aeruginosa résistantes aux médicaments isolés à partir de sources cliniques dans des établissements de santé sélectionnés dans l'état de Kwara

***Adekunle O.C.[1], Mustapha A.[2], Odewale G.[3], Ojedele R.O.[4]**

Objectifs de l'étude: Pseudomonas aeruginosa (P. aeruginosa) est un pathogène nosocomial fréquent qui provoque des maladies graves dans de nombreux contextes cliniques et communautaires. Les objectifs étaient d'étudier la présence de multiples souches de P. aeruginosa résistantes aux antibiotiques parmi des échantillons cliniques et de détecter la présence de gènes de résistance aux antibiotiques dans les molécules d'ADN des souches.

Méthode de l'étude : Des échantillons cliniques ont été prélevés de manière aseptique sur divers sites anatomiques humains dans cinq établissements de santé sélectionnés dans l'état de Kwara, au Nigéria. De multiples profils de résistance aux médicaments de micro-organismes isolés à différents antibiotiques ont été déterminés à l'aide de la technique de diffusion sur disque de Bauer Kirby. Les échantillons d'ADN des multiples souches résistantes de P. aeruginosa ont été extraits et soumis à une réaction en chaîne par polymérase (PCR) pour la détermination des gènes de résistance.

Résultat de l'étude : Un total de 145 isolats a été identifié comme P. aeruginosa à partir des échantillons cliniques. Une résistance absolue à la ceftazidime, à la gentamicine et à la ceftriaxone a été observée tandis qu'une faible résistance à la ciprofloxacine, à la pipéracilline et à l'imipénem a été documentée.

Conclusion : Cette étude a montré qu'il existe une forte occurrence de souches productrices de métallo β-lactamases et résistantes aux antibiotiques de P. aeruginosa dans les échantillons cliniques de la zone étudiée.

Mots-clés : Enzyme métallo β-lactamase, P. aeruginosa, échantillons cliniques, gènes de résistance aux antibiotiques.

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INTRODUCTION

Pseudomonas aeruginosa is a bacterium characterized by its high genetic plasticity and potential for adapting to various environments. The species are frequently isolated from soil and water or colonize several anatomical sites of plants, insects, animals, and humans (1, 2). The bacterium may be involved in food infection and has many virulence factors (3, 4).

P. aeruginosa is widespread in natural environments and considered as an opportunistic secondary pathogen for humans that is capable of causing major nosocomial infections and a broad spectrum of infections including urinary tract infection, burns, respiratory tract infection, meningitis, chronic otitis media and otitis externa, pseudomonal endocarditis and septicemia (5, 6). The extensive use of antimicrobial agents and the evolution of antimicrobial resistance strategies by bacteria have resulted in the emergence of nosocomial bacterial pathogens (superbugs) with acquired resistance to almost all available antimicrobial agents (7). These superbugs have severely threatened therapeutic choices in the last few decades (8, 9). *P. aeruginosa* is considered multidrug resistant (MDR) if the isolate is resistant to more than two of the following drugs: piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem, meropenem and colistin (10, 11). These agents are representatives of the primary antibiotic classes used in the treatment of *P. aeruginosa* infections.

P. aeruginosa uses distinctive mechanisms to become resistant to a wide range of antimicrobials agents (12, 13).These include the up-regulation of efflux systems, decreased outer membrane permeability and β- lactamase production. However, acquired extended spectrum β-lactamase (ESBL) and metallo β-lactamase (MBL) mediated resistance is important in emerging resistance mechanisms of *P. aeruginosa* (14, 15). *P. aeruginosa* has been reported to have constitutive expression of multidrug efflux pumps and AmpC β-lactamase associated with loss in permeability of the outer membrane (16).Therefore, this study is aimed at investigating the occurrence of metallo β-Lactamase enzyme and detecting antibiotic resistance genes among multiple antibiotic resistant *P. aeruginosa* isolated from clinical samples.

MATERIALS AND METHODS

Description of Study Location

The selected hospitals and healthcare located in Kwara State, Nigeria facilities provide quality health care services to the residents of the State and neighbouring States like Oyo, Kogi, Niger, Osun, and Ekiti. Ilorin is the capital of Kwara State in the central Nigeria, West Africa. Ilorin coordinates on the globe at 8°30'N 4°33'E.

Sample Collection

One hundred and forty five strains of *P. aeruginosa* were isolated from two hundred and thirty five clinical samples which included urine, wound, sputum, blood and indwelling medical devices. Well labelled screw-top containers were used to collect mid-stream urine. Swab samples were taken by expert technicians from hospitalized patients admitted to burn ward. The swabs were collected in sterile normal saline (0.85(w/v)) with all aseptic precautions. Sputum was produced by coughing from deep in the chest and coughed into plastic collection bottle.

Ethical considerations: Ethical approval was obtained from the Ethical Review Committee (ERC) of Kwara State Ministry of Health, Ilorin.

Isolation of *P. aeruginosa* and Bacterial examination

The clinical samples were collected into a transport medium (Nutrient broth) before transporting to the laboratory. The collected samples were inoculated into nutrient broth for culture 42^{o}C for 24 hours. The overnight broth culture was further sub-cultured on MacConkey agar and Blood Agar. Incubation of the cultured plates was done under aerobic conditions. The bacterial growth was observed for colonies morphologically resembling *P. aeruginosa. P. aeruginosa* formed smooth round colonies with a fluorescent greenish colour. Colonies morphologically resembling *P. aeruginosa* were subjected to further characterization.

Confirmation of *Pseudomonas aeruginosa* strains

The test for the identification of *P. aeruginosa* in clinical samples was carried out using cetrimide agar for confirmation. Morphological Characterization such as Gram Staining and Biochemical Characterization such as indole, citrate, and oxidase tests were carried out. Results obtained were compared with specifications in 21st edition of Bergey's Manual of Systematic Bacteriology.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed using modified Kirby-Bauer disk diffusion method following the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2015).

Metallo –β lactamase detection

The inoculum of *Pseudomonas aeruginosa* was prepared (CLSI, 2015). Each isolate of *P. aeruginosa* was inoculated on a separate Mueller Hinton plate. MBL E-test strips containing concentration gradients (1-64 µg/ml) of imipenem (IP) on one end of the strip and imipenem overlaid with a constant concentration of EDTA (IPI) on the other end of the strip were placed onto the plate and incubated at 42°C aerobically for 18-24 hours as shown in plates 1 and 2. Negative control was set along with the test using *P. aeruginosa* ATCC 27853. A reduction in the imipenem minimum inhibitory concentration (MIC) in the presence of EDTA of greater than or equal to eight-fold (IP/IPI $\geq$8) indicated MBL positivity.

Detection of multiple drug resistant *Pseudomonas aeruginosa*

Multiple drug resistance in *P. aeruginosa* isolates was defined as resistance to three or more Pseudomonal anti-microbial classes (18).

DNA Extraction and Amplification in Thermal Cycler

Five (5) colonies of the isolate was scooped into a sterile microtube containing 200 µl of 1 X TBE. It was boiled for 15 minutes after which the preparation was allowed to cool rapidly on ice at -20 °C for 30 minutes. It was incubated for 20 minutes at 66 °C. The lysate was centrifuged (5 min at 13,000 rpm), and 3 µl of the supernatant was used as the DNA sample for the PCR reaction.

For PCR amplification, a total volume of 20 µl reaction mix containing 2 µl of 10X buffer, 1µl $MgCl_2$, 0.8 µl dNTPs, 0.5µl of forward primer, 0.5µl of reverse primer, 0.2 µl Taq polymerase, 10 µl of nuclease free water and 5 µl of DNA lysate was used.

The polymerase chain reaction was set up in a PCR vial after adding the master mix, the forward and reverse primers and the extracted DNA. Amplification was subjected to initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 60 °C, 54° C, 47°C 50°C for 1 minute, for CTX-M, VIM, IMP and *oprD* respectively, extension at 72 ^{0}C for 1 minute and final extension procedure was carried out at 72 C° for 10 minutes. Primers used are shown in Table **1**.

Agarose gel electrophoresis

The PCR products were analyzed after electrophoresis in 1.5 % agarose gel stained with ethidium bromide to detect specific amplified products by comparing with standard molecular weight marker.

Data analysis

Data were entered into the computer and statistical analysis was performed using Chi-square. P values $\leq$0.05 were assumed statistically significant.

RESULTS

Out of the 145 *P. aeruginosa* isolates examined, 30 (21 %) were MBL-producing while 115 (79 %) were negative for MBL production.

During the study period, MBL positive isolates were found to be resistant to ceftriaxone and ceftazidime (100 %) while the susceptibility patterns of the MBL negative isolates to these antibiotics were 55 (37.9 %) and 90 (62.1 %) respectively as presented in Table 2.

Plate 1 displays the photograph of MBL positive *P. aeruginosa* showing a phantom zone which is characteristic of MBL producers while plate **2** displays the photograph of MBL positive *P. aeruginosa* showing IP/IPI $\geq$8

Electrophoresis gel picture of the amplified resistance genes showed *oprD* gene at band 585 bp,*ctx-M* gene at band 1300 bp, *VIM* gene at band 390 bpand TEM gene at band 517 bp (Figures 1 – 4).

DISCUSSION

Pseudomonas aeruginosa is commonly implicated as a cause of health care acquired infections with high mortality rates (19). High rate of microbial resistance to the cephalosporins among the *P. aeruginosa* isolates was observed in this study. The high incidence of resistance to the cephalosporins and quinolones may be attributed to cross-resistance which may be as a result of indiscriminate use of ciprofloxacin in the studied area. Resistance of the isolates to beta-lactam antibiotics showed an increased beta-lactamase enzyme production among the isolates. All the MBL producing isolates were resistant to ceftriaxone (100 %), ceftazidime and ciprofloxacin (100 %) respectively as was also observed by Oladipo *et al.* (20) who reported high

prevalence of cephalosporin resistant *P. aeruginosa* isolates among in- and out-patients.

In this study the prevalence of MBL producing *P.aeruginosa* was found to be 21 %. This result was lower than the 36.07 % reported by Sasirekha *et al.,* (21) and higher than the prevalence reported in Lagos (22), and in Belgium (23): 4.1 % and 4.4 % respectively. It is also higher than the prevalence of 10.0% and 14.0% obtained in Enugu, South east Nigeria (24) and India (25) respectively.

The highest rates of susceptibility of *P. aeruginosa* isolates were recorded for colistin (77.2 %) followed by imipenem (65.5 %). This result for colistin corroborates studies from Nwankwo and Shaibu and Aibinu *et al.* (22) who reported 78.9 % and 95.6% susceptibility rates respectively for *P. aeruginosa* isolates. Colistin is nephrotoxic, which is why it is always used as a last resort drug for resistant strains. It is therefore not abused; this explains the high susceptibility of the isolates to the drug.

Gentamicin on the other hand had less activity and showed same microbial resistance profile as the β-lactam drugs. The reason for this high resistance to gentamicin could be as a result of indiscriminate use of the drug in this area. The drug, though a prescription only medicine is purchased as over-the-counter in the open markets littered in the community and is commonly used by unqualified personnel in the treatment of "infections". Several studies have shown that gentamicin is effective against *Pseudomonas* species but if misused, the organisms may develop resistance to them.These research findings are similar to that of Sasirekha*et al.* (21) where they recorded 82.1 % susceptibility of the isolates to gentamicin.In previous studies by Oduyebo *et al.* (26) in Kwara state, *P. aeruginosa* strains were found to be highly susceptible to the ciprofloxacin and well tolerated. This result implies that quinolones alone, cannot be depended upon as an antipseudomonal antimicrobial in this area. They will have to be used in combination or replaced with another antimicrobial preferably the broad-spectrum beta-lactams/penicillins. In the present study, isolates positive for *oprD* gene were 42 (46.7 %), VIM gene 31 (34.4%), *ctx-M* gene 15 (16.7 %), TEM gene 34 (37.7 %) respectively. The prevalence of *oprD* gene in this study is lower than 90 % reported by Eucharia *et al.* (27). The prevalence of VIM gene observed from this study is lower than 52.3 % reported by Shaikh *et al.* (28) and higher than 2.5 % obtained from Zubair and Iregbu, (29). Also, from this study the result of *ctx-M* gene is slightly lower than 55.7 % reported by Shaikh *et al.* (28) while the TEM gene is higher in this study as compared 31.4 % reported by Shaikh *et al.* (28). The resistance to these drugs is a major problem for chemotherapy and pro-active measures must be put in place at various levels to forestall this trend.

CONCLUSION

Effective and good antibiotic prescription as well as infection control practices must be put in place to prevent therapeutic failures and further spread of the resistance genes.

Ethic approval and consent to participate: Ethical approval was obtained from the ethical review committee (erc) of kwara state ministry of health, ilorin.

Authors' contributions: AOC, MA, OG and ORO were involved in the conception and design, data collection and collation, data analysis, data interpretation and manuscript proofreading.

Conflict of interest: The authors declare no conflict of interest, financial or otherwise.

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Table 1: Primers used for the Amplification of genes

Primer	Sequence 5^1 -3^1	Base pair (bp)	Annealing Temp. (ºC)	Reference
CTX-M F	CGATGTGCAGTACCAGTAA	585	60	Lim *et al.* (2009)
CTX-M F	CGATGTGCAGTACCAGTAA	585	60	Lim *et al.* (2009)
CTX-M R	CGATGTGCAGTACCAGTAA			
VIM2004A	GTTTGGTCGCATATCGCAAC	390	54	Fazeli *et al.* (2009)
*VIM2004*A	AATGCGCAGCACCAGGATAG			
BLATEM F	TGAGCAAGTTATCTGTATTC	931		Lim *et al*., (2009)
*BLATEM*R	TTAGTTGCTTGGTTTTGATG			
OPRD F	ATGAAAGTGATGAAGTGGAG	1329	50	Fazeli *et al.* (2013)
OPRD R	CAGGATCGACAGCGGATAGT			

Plate 1: Photograph of MBL positive *P. aeruginosa* showing a phantom zone which is characteristic MBL producers.

Plate 2: Photograph of MBL positive *P. aeruginosa* showing IP/IPI=8.

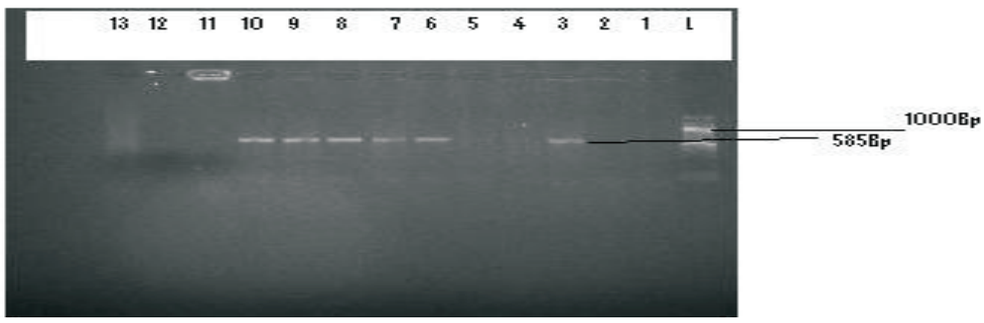

Figure 1: Agarose gel electrophoretogram of *oprD* gene.
Lane1: Negative control; Lanes 6, 7, 8, 9 and 10: *oprD* gene positive isolates;
lane 3: positive control, L: Ladder, 100 bp

Figure 2: Agarose gel electrophoretogram of *ctx-M*–type β-lactamaseafter PCR which bands at 1300 bp .
Lane 1: Positive control; Lanes 2-7 positive for *ctx-M* gene. L is the ladder (100bp).

Figure 3: Agarose gel electrophoretogram of (VIM) after PCR which bands at 390 bp
L: DNA Ladder; Lane2: Positive control; Lane 3: Negative control;
Lane 1-11: Presence of *VIM* gene.

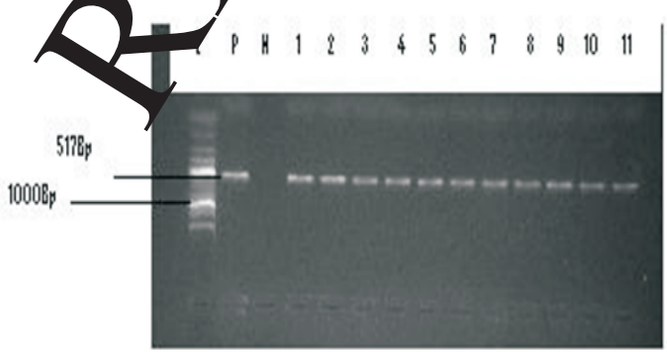

Figure 4: Agarose gel electrophoretogram of TEM after PCR which bands at 517 bp
L: Ladder; Lane P: Positive control lane N: Negative control;
Lanes 1-11: *TEM* gene positive isolates.