MULTIDRUG-RESISTANCE PATTERNS AND DETECTION OF PstS GENE IN CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA FROM NSUKKA, SOUTHEAST NIGERIA

MARTINA C. AGBO1*, IFEOMA M EZEONU2, ANTHONY C IKEYE3, CELESTINA C UGWU3

1Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Nigeria. 2Department of Microbiology, University of Nigeria, Nsukka, Nigeria. 3Department of Applied Microbiology and Brewing, Enugu State University of Science and Technology, Enugu, Nigeria. Email: martina.agbo@unn.edu.ng

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ABSTRACT

Objective: This study was aimed to determine the antibiotic resistance patterns of clinical Pseudomonas aeruginosa isolates and to detect the presence of PstS gene.

Methods: One hundred and ninety-two clinical isolates of P. aeruginosa were characterized using polymerase chain reaction (PCR) and 16S rDNA sequencing. Antibiotic resistance patterns were determined using the disk diffusion method, while the minimum inhibitory concentrations (MICs) of selected antibiotics against resistant isolates were determined by macro broth dilution and E-test strip methods. The resistant isolates were screened for the presence of PstS gene using PCR.

Results: Of 192 clinical isolates of P. aeruginosa, 136 (70.83%) were resistant to at least two antibiotics. Of these, 135 (99%) could be classified as multidrug-resistant P. aeruginosa (MDR-PA). 63 (46%) were extensively drug-resistant (XDR-PA), while 38 (28%) were pandrug-resistant (PDR-PA). The isolates exhibited high level of resistance to cefotaxime and ticarcillin, and low levels of resistance to meropenem and imipenem. The MIC values for meropenem against the resistant isolates were generally <32 mg/L, while the values for other antibiotics ranged from 32 to >128 mg/L. Multiple antibiotic resistance indexes of the MDR-PA ranged from 0.27 to 0.91 and the most prevalent pattern of resistance was Piperacillin – Ticarcillin – Piperacillin/Tazobactam – Cefotaxime – Cefazidime – Gentamicin – Tobramycin – Ciprofloxacin. About 50% of the resistant isolates possessed the PstS gene.

Conclusions: The results confirmed the presence of XDR, PDR, and PstS gene in P. aeruginosa strains. There is an urgent need for healthcare practitioners to address the problem of multidrug resistance, by implementing a more rational and appropriate use of antibiotics.

Keywords: Pseudomonas aeruginosa, Antimicrobial resistance, Multidrug-resistant Pseudomonas aeruginosa, Extensively drug-resistant, PstS gene.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative aerobic bacillus belonging to the Pseudomonadaceae family. It is highly ubiquitous in nature and may be found in most moist environments in the hospital, including sinks, cleaning buckets, drains, humidifiers, and toilet water [1]. P. aeruginosa is becoming an increasingly important cause of health-care-associated infections [2-5]. It has been reported to be the sixth most common nosocomial pathogen overall and second most common pathogen in ventilator-associated pneumonia in US hospitals [4]. It has also been ranked second among Gram-negative pathogens reported to the United States National Nosocomial Infection Surveillance System [3]. P. aeruginosa constitutes a common pathogen in hospitals, particularly in intensive care units, due to its ubiquitous nature, ability to survive in moist environments, innate resistance to many antibiotics and antiseptics, and ability to acquire resistance to many drug classes [3,6,7]. It is a leading cause of septicemia, pneumonia, meningitis, wound, urinary tract, surgical wound, burn, and ear infections [8].

Resistance in Pseudomonas may be mediated through several distinct mechanisms, including the production of β-lactamases, efflux pumps, target site, and outer membrane modifications, but antimicrobial agents that have been found to be effective against P. aeruginosa include aminoglycosides (gentamicin, tobramycin, amikacin, and netilmicin); carbapenems (imipenem, meropenem, and doripenem); cephalosporins (cefazidime and cepofine); fluoroquinolones (ciprofloxacin and levofloxacin); penicillins (β-lactamase inhibitors (ticarcillin-clavulanic acid and piperacillin-tazobactam); monobactams (aztreonam); phosphonic acids (fosfomycin); and polymyxins (colistin and polymyxin B) [9,10]. Antibiotic resistant P. aeruginosa can be classified today Multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR). MDR has been defined as a strain that develops resistance to at least one agent in three or more antimicrobial classes [9,11]. XDR is a strain resistant to one agent in all categories except two or less, while PDR is a strain resistant to one agent in all categories [9,11].

Of all the anti-Pseudomonas agents, the carbapenems have been widely used for empirical or directed therapy, whenever MDR P. aeruginosa is suspected. However, recent evidence indicates that resistance to the carbapenems is emerging in P. aeruginosa and improper use of these drugs could accelerate the occurrence [4,12]. This is a global concern, not just for Pseudomonas, but for other bacterial pathogens. However, for the developing world, especially, this concern appears to be more reality than speculation. The concern arises from certain practices observed in the developing world, including unregulated use/sale of antibiotics, over-prescription of antibiotics, release of antibiotics into wastewaters by drug manufacturers, poor sanitation, poor infection control, misuse of antibiotics, and among others [13]. These problems can, however, be solved by better surveillance and monitoring of drug resistance. To this effect, the World Health Organization (WHO) has proposed a new global surveillance network, although there are still questions about how the initiative will be funded [13].

Considering the constantly evolving pattern of antibiotic resistance in P. aeruginosa and high mortality rates associated with Pseudomonas infections, it is of great public health importance to continuously...
track the occurrence and spread of antibiotic resistance in this rapidly emerging superbug. In a WHO global map of antibiotic resistance data from 194 member countries, Nigeria was one of the countries for which no information was available [13].

Studies have reported that expression of the PstS gene enhances the virulence of MDR P. aeruginosa strains [14]. PstS proteins are the cell-bound phosphate binding elements of the ubiquitous bacterial ATP-binding cassette phosphate uptake mechanisms. This study was, therefore, undertaken to determine the prevalence of MDR among clinical isolates of P. aeruginosa in Nsukka, Southeast Nigeria, determine the patterns of resistance and screen the isolates for the presence of PstS gene, which has been reported to confer increased virulence on MDR P. aeruginosa (MDRPA).

**METHODS**

The bacterial strains
A total of 192 strains of P. aeruginosa were collected from Microbiology Laboratories from various hospitals in Nsukka, from March 2016 to February 2017. Ethical approval and informed consent were not required by our Institution Ethics Committee because all bacterial isolates were collected, processed, and stored as part of routine diagnosis by the hospitals. No patient information was associated with the data. The isolates obtained from the various laboratories were subcultured onto Pseudomonas ceftiramide agar (Oxoid, UK) which was supplemented with 10 ml/l of glycerol and characterized using standard microbiological techniques such as grade like odor, pigment production, positive oxidase test, growth at 42°C, and molecular standard methods (Table 1). The ten antibiotics represented six classes of antibiotic (Table 1). The isolates were considered susceptible or resistant according to the zone of inhibition recommended by the Clinical and Laboratory Standard Institute [15]. P. aeruginosa ATCC 27853 was used as the control strain. Isolates were considered MDR (MDRPA) if they showed resistance to three or more classes of the tested antibiotics.

**Table 1: Zone diameter interpretive standards and equivalent MIC breakpoints for Pseudomonas aeruginosa** (Clinical and Laboratory Standard Institute, 2014)

| Antimicrobial class | Antimicrobial agent and disk concentration | Zone diameter (mm) | Equivalent MIC break point (ug/ml) |
|---------------------|-------------------------------------------|--------------------|-----------------------------------|
| Penicillins         | Ticaricillin 75 µg                         | ≤15                | ≥128                             | ≤64 |
|                     | Piperacillin 100 µg                        | 15–23              | ≥24                              | ≥128/4 |
| β-lactam/β-lactamase inhibitor | Pipercillin–tazobactam 100/10 µg | ≤14               | ≥21                              | ≥64/4 |
| Cephalosporins      | Cefazidim 30 µg                           | ≤14                | ≥21                              | ≥64/4 |
|                     | Cefotaxime 30 µg                          | 15–17              | ≥18                              | ≥8   |
| Carbapenems         | Imipenem 10 µg                            | ≤15                | ≥16                              | ≤8   |
|                     | Meropenem 10 µg                           | 16–18              | ≥16                              | ≤4   |
| Aminoglycosides     | Gentamicin 10 µg                          | ≤12                | ≥16                              | ≤4   |
| Fluoroquinolones    | Tobramycin 10 µg                          | ≤12                | ≥15                              | ≤8   |
|                     | Ciprofloxacin 5 µg                        | 16–20              | ≥4                               | ≤1   |

| R: Resistant, 1: Intermediate, S: Sensitive, MIC: Minimal inhibiting concentration |

**Table 2: Primers used for Identification of Pseudomonas aeruginosa and detection of PstS gene**

| Target gene | Function | Sequence (5′−3′) | Ampliprin size (pb) | Accession No. |
|-------------|----------|-----------------|---------------------|---------------|
| 16s rRNA    | Consensus region | AGACTTTGATCCTGCTGTCGAC AGCGCTACTGTATGAGACTTT GGCCTTCAGACAGAAGTACAG ATGATCCGCTTCCAGCAC | 1499 | HM045838 |
| PstS        | Enhances virulence of MDR | GGCCTTCAGACAGAAGTACAG ATGATCCGCTTCCAGCAC | 606 | EF601159 |

**Determination of minimum inhibitory concentrations (MIC)**

The MIC for the MDR P. aeruginosa was determined using macro broth dilution method and E-test method according to the CLSI standard [15]. P. aeruginosa isolates that were resistant to meropenem; ceftazidime; ciprofloxacin; and gentamicin were used for MIC study. Macro broth dilution was used to determine the MIC for ciprofloxacin and gentamicin while E-strip test was used for meropenem and ceftazidime.

**Genomic DNA extraction**

The genomic DNA was extracted from 20 MDR P. aeruginosa strain using Zymo research fungal/bacterial DNA Miniprep Kit (Zymo Research, USA) according to the manufacturer’s protocol.

**DNA amplification**

The extracted DNA was amplified with 16s rRNA primer targeting P. aeruginosa consensus region and another primer targeting PstS gene that enhances the virulence of MDR Pseudomonas strains (Inqaba Biotechnical Company, South Africa), Table 2. The polymerase chain reaction (PCR) was carried out using the New England Biolabs one Taq mix master with standard buffer. PCR reaction mixture was prepared in a 25 µl reaction volume containing 12.5 µl of 1X Master mix with standard buffer, 20 nM Tris-HCl, 1.8 mM MgCl₂, 22 mM NH₄Cl, 22 mM KCl, 0.2 mM DNTPs, 5% glycerol, 0.06% GEPEL CA-630, 0.05% Tween 20, 25 units/ml Taq DNA polymerase (Biolab, England). 0.5 µl (10 µM) each of the forward and reverse primers (Inqaba Biotechnical, South Africa), 3 µl of the extracted DNA, and 6.5 µl of sterile nuclease-free water (Norgen Biotek, Canada) to make up to 25 µl of reaction volume. This was vortexed at low speed and placed in a thermal cycler machine (BIBBY – Scientific Ltd., UK). The parameters for amplification were as follows initial denaturation of 94°C for 5 min, followed by 35 amplification cycles of denaturation at 94°C, 30 s, annealing at 55°C, 30 s, and extension at 72°C, 1 min.

**Table 2. Primers used for Identification of Pseudomonas aeruginosa and detection of PstS gene**

| Target gene | Function |
|-------------|----------|
| 16s rRNA    | Consensus region |
| PstS        | Enhances virulence of MDR |
**Table 3: Resistance patterns of multidrug-resistant strains of Pseudomonas aeruginosa**

| Resistance pattern | Classification of resistance | No. of isolates |
|--------------------|------------------------------|-----------------|
| PRL, TIC, TJP, CTX, CAZ, MEM, IMP, CN, TOB, CIP | PDR | 4 |
| PRL, TJP, TIC, CTX, CAZ, MEM, CN, TJP | PDR | 1 |
| PRL, TIC, TJP, CTX, CAZ, MEM, IMP, CN, TOB | PDR | 2 |
| PRL, TIC, TJP, CTX, CAZ, MEM, IMP, CN, TOB | PDR | 16 |
| PRL, TIC, TJP, CTX, CAZ, IMP, CN, TOB | PDR | 3 |
| PRL, gTIC, TJP, CTX, CAZ, CN, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, CN, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, CN, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, CN, TOB, CIP | XDR | 17 |
| PRL, TIC, CTX, CAZ, IMP, CN, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, CN, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, CN, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 7 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 4 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 3 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 2 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 7 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 4 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 4 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 8 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 1 |
| PRL, TIC, CTX, CAZ, MEM, IMP, TOB, CIP | XDR | 2 |

**Table 3: (Continued)**

| Resistance pattern | Classification of resistance | No. of isolates |
|--------------------|------------------------------|-----------------|
| TIC, CTX, CN | MDR | 1 |
| TIC, CTX, CAZ | MDR | 1 |
| PRL, TJP, CTX, CN, TOB | XDR | 1 |
| PRL, TJP, CTX, CN, TOB | XDR | 1 |
| PRL, TJP, CTX, CN, TOB | XDR | 1 |
| PRL, TJP, CTX, CN, TOB | XDR | 3 |
| PRL, TJP, CTX, CN, TOB | XDR | 1 |
| PRL, TJP, CTX, CN, TOB | XDR | 1 |
| PRL, TJP, CTX, CN, TOB | XDR | 2 |

**Table 4: MAR indexes of Pseudomonas aeruginosa isolates**

| MAR Index | No. of resistant Pseudomonas aeruginosa isolates | % Prevalence of MAR |
|-----------|-----------------------------------------------|---------------------|
| 0.27      | 18                                           | 13.23               |
| 0.36      | 20                                           | 14.71               |
| 0.45      | 16                                           | 11.76               |
| 0.55      | 17                                           | 12.50               |
| 0.64      | 20                                           | 14.71               |
| 0.73      | 25                                           | 18.38               |
| 0.82      | 19                                           | 13.97               |
| 0.91      | 1                                            | 0.74                |

94°C for 1 min, annealing at 50°C and 55°C for 1 min for 16S rRNA and *PstS* primers, and extension at 72°C for 1 min. This was followed by a final extension step of 5 min at 72°C. The amplification products (amplicons) were separated on a 1.5% agarose gel stained with ethidium bromide and electrophoresis was carried out at 70 volts for 60 min and visualized/illuminated under ultraviolet transilluminator. A 100 bp DNA ladder (Norgen Biotech Corp., Canada) was used as DNA molecular weight marker.

**Data analysis**

Frequency of MDRPA and percentage of resistance to antibiotics were calculated.

**RESULTS**

**Antimicrobial sensitivity**

Of 192 isolates of *P. aeruginosa* that were tested, 136 (70.83%) were resistant to at least two antibiotics. The resistance of the isolates against a panel of 10 antibiotics was cefotaxime, 88.02%; ticarcillin, 87.50%; ceftazidime, 64.96%; ciprofloxacin, 62.50%; piperacillin, 58.33%; tobramycin, 57.29%; gentamicin, 56.25%; piperacillin/tazobactam, 55.73%; meropenem, 24.49%; and imipenem, 12.50%. Evaluation of resistance of the isolates to different classes of antibiotics revealed that 135 (99%) were resistant to at least one antibiotic in three different classes (MDR-PA): 63 (46%) were resistant to all except two or three classes (XDR-PA), while 38 (28%) were resistant to all six classes of antibiotics (PDR-PA), as shown in Table 3. The most prevalent pattern of resistance was (PRL, TIC, CTX, TJP, CTX, CAZ, CN, TOB, CIP).

**Analysis of MAR index**

Analysis of the MAR index showed that the isolates had MAR indexes ranging from 0.27 to 0.91 (Table 4) while evaluation of the MIC of some of the antibiotics against the isolates ranged from 0.12 to 126 µg/ml (Table 5).

**Occurrence of *PstS* gene**

The result of the PCR revealed that of 20 MDRPA isolates that were screened for *PstS* gene, lane 1, 3, 7, 9, 10, and 15 showed positive amplification of the 666 bp *PstS* gene (Fig. 1).

**DISCUSSION**

In this study, 192 clinical isolates of *P. aeruginosa* were screened against a panel of ten antibiotics, representing six classes of antibiotics.
Table 3: The MIC and MBC (µg/ml) of multidrug resistant *Pseudomonas aeruginosa*

| Organisms | MIC (µg/ml) | Ceftazidime (S ≤16 µg/ml and R ≥32 µg/ml)* | Ciprofloxacin (S ≤1 µg/ml, I=8 µg/ml, R ≥16 µg/ml)* | Gentamicin (S ≤4 µg/ml, I=8 µg/ml, R ≥16 µg/ml)* |
|-----------|-------------|------------------------------------------|--------------------------------------------------|-----------------------------------------------|
| ATCC 27853| 0.25        | 1.00                                     | 2.00                                             | 4.00                                          |
| 1         | 8.00        | 4.00                                     | 32.00                                            | 16.00                                         |
| 2         | 0.12        | 2.00                                     | 64.00                                            | 64.00                                         |
| 3         | 0.50        | 64.00                                    | 32.00                                            | 16.00                                         |
| 4         | 1.00        | 16.00                                    | 16.00                                            | 8.00                                          |
| 5         | 8.00        | 32.00                                    | 32.00                                            | 32.00                                         |
| 6         | 0.50        | 4.00                                     | 16.00                                            | 8.00                                          |
| 7         | 2.00        | 16.00                                    | 64.00                                            | 32.00                                         |
| 8         | 8.00        | 64.00                                    | 64.00                                            | 64.00                                         |
| 9         | 32.00       | 128.00                                   | 32.00                                            | 16.00                                         |
| 10        | 2.00        | 32.00                                    | 8.00                                             | 16.00                                         |

Grey shade indicates resistant strains. *CLSIs break points. S: Sensitive, I: Intermediate, R: Resistant, MIC: Minimal inhibiting concentration, MBC: Minimal bactericidal concentration, CLSI: Clinical and Laboratory Standard Institute

Fig. 1: (a and b) Polymerase chain reaction detection of 606 bp amplicons of *PstS* primer for identification of multidrug-resistant *Pseudomonas aeruginosa*, Lane M shows bands for 1kb (1000bp) molecular weight standard. Lanes 1, 3–7, 9, 10, 15, and 16 show a positive amplification band. Indicating the presence of *PstS* gene in *P. aeruginosa* isolates analyzed. Lanes 14, 17, 18, 19, and ATCC 27583 show negative amplification and produced no visible band.

The highest levels of resistance were recorded to the cefpodoxime (cefotaxime and ceftazidime) and β-lactam (ticarcillin) antibiotics, while the lowest resistance levels were recorded to the carbapenems (meropenem and imipenem) and β-lactam antibiotics.

**CONCLUSIONS**

There is the presence of XDR, PDRPA, and *PstS* gene in *P. aeruginosa* strains in Southeast Nigeria. The findings from this study show an alarming degree of resistance among *P. aeruginosa* isolates in Nsukka, Nigeria and an urgent need for healthcare practitioners and policymakers to address the problem of MDR by implementing a more rational and appropriate use of antibiotics. Establishment of an effective surveillance program and strict disinfection policy in hospital
environments would also help to control the spread of MDR, XDR, and PDRPA in hospital settings.

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AUTHORS CONTRIBUTIONS

Martina C. Agbo, as the corresponding author, carried out the experiments and drafted the manuscript. Ifeoma M. Ezeonu conceived, organized, and supervised this research work and reviews the manuscript. Anthony C. Ike helped in review of the work, offered advice, participated in PCR, and read through the manuscript. Celestina C. Ugwu assisted during the sample collection and helped in some bench work.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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