

Prevalence and Identification of *Bla*_{VEB-1}, *Bla*_{SHV} and *Bla*_{CTX-M} in *Pseudomonas Aeruginosa* Isolates from Hospitals in Onitsha Metropolis Using Polymerase Chain Reaction Technique

Ezeador Chika and Agbakoba Nneka R.

Department of Medical Microbiology and Parasitology, Nnamdi Azikiwe University,
Nnewi Campus, P.M.B 5001 Nnewi, Anambra State, Nigeria

Abstract: *Pseudomonas aeruginosa* is widely spread in nature, inhabiting soil, water, plants, animals and humans. It is a frequent cause of nosocomial infections such as pneumonia, urinary tract infections (UTIs) and bacteremia. The aim of this study is to determine the prevalence of ESBL genes in *P. aeruginosa* isolates from hospitals in Onitsha Metropolis, Anambra State, Nigeria. A total of 22 *P. aeruginosa* isolates previously obtained from various clinical and environmental sources were screened for ESBL production and were further subjected to molecular identification by polymerase chain reaction (PCR) to detect the presence of three ESBL genes, *bla*_{VEB-1}, *bla*_{SHV} and *bla*_{CTX-M} genes. Out of the 22 *Pseudomonas aeruginosa* isolates screened for ESBL production, 19 isolates were phenotypically ESBLs positive whereas 3 isolates were negative. Sixteen (16) of the 22 isolates subjected to PCR in order to determine the type of extended spectrum β -lactamases, harbored different *bla* genes. The *bla*_{VEB-1}, *bla*_{SHV} and *bla*_{CTX-M} genes were detected in the frequency of 47.8% (11), 43.5% (10) and 8.7% (2) for *bla*_{VEB-1}, *bla*_{SHV} and *bla*_{CTX-M} genes respectively. A total of 23 *bla* genes were detected from 16 *P. aeruginosa* isolates. From the results of this study, it can be concluded that *bla*_{VEB-1} and *bla*_{CTX-M} genes were the most and the least frequently isolated ESBL genes among the *P. aeruginosa* isolates recovered from hospitals in Onitsha Metropolis.

Key words: PCR • ESBL • Primers • *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic non-fermentative gram-negative bacillus that is responsible for a wide variety of infections in humans ranging from relatively uncomplicated urinary tract infections (UTIs) to severe and life threatening infections including neonatal sepsis and chronic lung infections in patients with cystic fibrosis [1]. *P. aeruginosa* is also a major opportunistic human pathogen notable for its ability to form biofilm and best characterized quorum sensing systems among Gram negative bacteria.

Extended spectrum β -lactamases (ESBLs) were first reported in 1983 in *Klebsiella pneumoniae* from Germany. Typically, ESBLs are mutant plasmid mediated β -lactamases derived from older broad-spectrum

β -lactamases. The mutations alter the amino acid configuration around active site of β -lactamases [2]. The first ESBL to be described in 1983 was actually TEM3 [3] and now over 130 additional TEMs have been isolated. ESBLs have an extended substrate profile that cause hydrolysis of cephalosporins, penicillins and aztreonam and are inhibited by β -lactamase inhibitors, such as clavulanate, tazobactam and sulbactam. ESBLs are commonly produced by *Klebsiella* species and *Escherichia coli*; but also occur in other Gram negative bacteria, including *Enterobacter*, *Salmonella*, *Proteus*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Burkholderia*, *Acinetobacter* species, etc [4].

Pseudomonas aeruginosa is one of the most important microorganisms which causes problems clinically as a result of its high resistance to antimicrobial

agents and is therefore a particularly dangerous and dreaded bug. Despite the discovery of ESBL, Amp C β -lactamases and MBL at least a decade ago [5] there remains a low level of awareness of their importance and many clinical laboratories have problems in detecting ESBL and Amp C β -lactamases. Failure to detect these enzymes has contributed to their uncontrolled spread and commonly to therapeutic failures.

The aim of this study is to determine the prevalence of ESBL genes in *P. aeruginosa* isolates from hospitals in Onitsha Metropolis, Anambra State.

MATERIALS AND METHODS

Bacteria Isolates: This study was carried out in Onitsha, the largest city in Anambra State, South-eastern Nigeria. A total of 22 *P. aeruginosa* isolates previously obtained from various clinical (Swab, wound swab, nasal swab and hand swab) and environmental sources (Patients tables, trolleys, sinks, floors, nurses' trays, sphygmomanometers, theatre beds, patients' beds, buckets, mops, water taps, laboratory work benches and disinfectants) were screened for ESBL production and were further subjected to molecular identification by polymerase chain reaction (PCR).

The susceptibilities of the bacterial isolates to eleven antimicrobial agents: Piperacillin (30 μ g), cefuroxime (30 μ g), ceftazidime (30 μ g), cefotaxime (30 μ g), cefepime (30 μ g), amikacin (30 μ g), imipenem (10 μ g), ciprofloxacin (30 μ g), chloramphenicol (C:30 μ g), Tetracycline (TE:30 μ g) and amoxicillin - clavulanic acid (30 μ g) (Oxoid Ltd Basingstoke, UK) were determined by the disk diffusion method according to the criteria published by the Clinical and Laboratory Standards Institute (CLSI).

Detection of ESBL: *Pseudomonas aeruginosa* isolates were screened for ESBL production by double disk synergy test (DDST) using ceftazidime (30 μ g), cefotaxime (30 μ g), ceftriaxone (30 μ g) and cefepime (30 μ g) antibiotic on Mueller–Hinton agar as recommended by the Clinical and Laboratory Standards Institute [6]. ESBL production was confirmed in the *P. aeruginosa* isolates by double disk synergy test [7, 8] using ceftazidime (30 μ g) and cefotaxime (30 μ g) antibiotic disks with amoxycillin-clavulanic acid (30 μ g) placed at the centre 15mm apart from them. A ≥ 5 mm increase in the inhibition zone diameter for either of the cephalosporins (ceftazidime and cefotaxime) tested in combination with amoxycillin-

lavulanic acid versus its zone when tested alone confirms ESBL production phenotypically [8, 9].

Molecular Analysis

Polymerase Chain Reaction (PCR): Plasmid DNA isolation was prepared by a rapid alkaline lysis using a commercial plasmid isolation kit (Plasmid Miniprep Kit, Zymogen Co. Ltd. UK) according to the manufacturer instructions but with slight modifications.

All the 22 isolates were examined for the presence of *bla*_{SHV}, *bla*_{VEB-1} and *bla*_{CTX-M} by PCR. Primer sequences for amplification of *bla*_{SHV}, *bla*_{VEB-1} and *bla*_{CTX-M} were listed in Table 1. The SHV, VEB-1 and CTX-M forward/reverse primers were derived from Inqaba Biotechnology Industry Ltd, South Africa. PCRs were conducted under standard conditions using plasmid DNA as templates.

A typical 20 μ l PCR reaction mixture for every primer set was consisted of 1X- PCR reaction buffer (Fermentase, Lithuania), 1.5 μ M MgCl₂ (25mM), 0.7 μ M of each dNTP (10mM), 0.7 μ l of each primer, 1 unit of Dream Taq DNA polymerase 5U/ μ l (Fermentase, Lithuania) and 0.5 μ l of 10 μ g DNA template. Amplification was carried out in a thermocycler (Eppendorf Mastercycler®, Massachusetts, USA). A 15 μ l of the master mix/cocktail was added to the PCR tubes together with 5 μ l of the template DNA and mixed properly. The 20 μ l mixture was vortexed in a Zppy Vortex Vibrator (Perkin Elmer, USA) for 5 seconds to allow for homogeneous mixture. The mixture was centrifuged using e-centrifuge (Zymo R™, UK) for 1 minute and loaded into a PCR well Zymo-Spin™ into the PCR machine. The PCR products were purified using QIAquick PCR Purification Kits (Qiagen, Hilden, Germany). Amplification reactions were run under the following conditions. The initial denaturation at 95°C for 5 minutes under 35 cycles followed by the denaturation temperature at 95°C for 30 seconds and a subsequent annealing at 60°C for 30 seconds for both SHV and CTX-M primers and 55°C for VEB-1 primer for 30 seconds. Extension temperature was 72°C for 30 seconds and final extension was 72°C for 2 minutes.

After the reaction, the PCR products were electrophoresed for 30 minutes with 100 voltages on 1.0% agarose gel stained with 0.5 μ g/ml ethidium bromide (Nippon Genetics, Europe GmbH) solution using 1mM Tris-Borate- EDTA (TBE) buffer. Simultaneously, a positive control was run for each ESBL gene. The gels were observed under U.V. gel documentation (UV DOC, England) at 280nm. 1000-100bp DNA ladder was used to confirm the size of each specific *bla* gene.

Table 1: The Primers and Sequences used for Amplification with respect to ESBL Genes.

Primer Name	5'- Sequence – 3'	Detected gene	Molecular weight (bp)	Ann.Temp. (30 sec)
SHV (F)	5'-CGCTGTGTATTATCTCCCT-3'	bla _{SHV}	293	60
SHV (R)	5'-CGAGTAGTCCACCAGATCCT-3'			
VEB-1 (F)	5'-CGACTTCCATTTCCTGATGC-3'	bla _{VEB-1}	643	55
VEB-1 (R)	5'-GGACTCTGCAACAAATACGC-3'			
CTX-M (F)	5'-CGCTTTGCGATGTGCAG-3'	bla _{CTX-M}	550	60
CTX-M (R)	5'-ACCGCGATATCGTTGGT-3'			

F= Forward Primer R= Reverse Primer

RESULTS

Susceptibility pattern carried out in previous work showed that *P. aeruginosa* was most susceptible to only imipenem (100%) and amikacin (86.4%), but showed complete resistance to eight drugs (Piperacillin, cefuroxime, ceftazidime, cefotaxime, cefepime, chloramphenicol, Tetracycline and amoxicillin - clavulanic acid). It was moderately resistant ciprofloxacin (45.5%). All the isolates were MDR (100%). This study reported that out of the 22 *Pseudomonas aeruginosa* isolates screened for ESBL production, 19 (86.4%) isolates were phenotypically ESBLs positive whereas 3 (13.6%) isolates were negative.

In detection of β -lactamases (*Bla*) genes, all the isolates were further subjected to PCR analysis. PCR analysis confirmed that 16 (84.2%) out of 19 ESBL *P. aeruginosa* isolates harbored *bla* genes. A total of 23 *bla* genes were detected from the 16 *P. aeruginosa* isolates (Table 2). The frequency of *bla* genes producers based on specimen sources is summarized in Table 2.

Detection of *bla* genes showed that 43.5%, 47.8% and 8.7% of isolates respectively carried SHV, VEB-1 and CTX-M genes that could be observed on agarose gel electrophoresis at 293bp for SHV, 643bp for VEB-1 and 500bp for CTX-M genes (Figures 1, 2 and 3). The prevalence of *bla* genes in the 22 *P. aeruginosa* isolates from various sources is summarized in Table 3. *P. aeruginosa* isolate (P₉) obtained from a floor swab was found to harbor the 3 (13.0%) *bla* genes tested (SHV, VEB-1 and CTX-M genes) whereas isolates obtained from theatre bed, lab. bench, sphygmomanometer, mop and trolley swabs harbored 2 (8.7%) out of the 3 *bla* genes tested (SHV and VEB-1 genes). Three *P. aeruginosa* isolates (P₃, P₇ and P₂₁) were phenotypically positive for ESBLs on DDST but was negative for *bla*_{SHV}, *bla*_{VEB-1} and *bla*_{CTX-M} genes on PCR. Other isolates showed only the presence of 1 (4.3%) of the three *bla* genes (Table 3). There was no statistical significant difference between the number of ESBLs producer *P. aeruginosa* isolates and the number of *bla* genes produced at $p < 0.05$.

Table 2: The frequency of *bla* genes producers based on specimen sources

Gene source	ESBLs Isolate	No. of <i>bla</i> genes	SHV	VEB-1	CTX-M
			No. (%)	No. (%)	No. (%)
Theatre bed	1	2	1 (10)	1 (9.1)	0 (0)
Sink	3	2	1 (10)	1 (9.1)	0 (0)
Patients bed	0	0	0 (0)	0 (0)	0 (0)
Mops	1	2	1 (10)	1 (9.1)	0 (0)
Hands swab	0	0	0 (0)	0 (0)	0 (0)
Nasal swab	1	1	1 (10)	0 (0)	0 (0)
Nurses' tray	1	0	0 (0)	0 (0)	0 (0)
Floor	1	3	1 (10)	1 (9.1)	1 (50)
Disinfectant	0	0	0 (0)	0 (0)	0 (0)
Patients' table	1	1	0 (0)	1 (9.1)	0 (0)
Trolley	2	3	1 (10)	2 (18.2)	0 (0)
Sphygmomanometer	2	3	1 (10)	2 (18.2)	0 (0)
Water tap	0	0	0 (0)	0 (0)	0 (0)
Buckets	2	2	0 (0)	1 (9.1)	1 (50)
Lab work bench	2	3	2 (20)	1 (9.1)	0 (0)
Ear swab	1	0	0 (0)	0 (0)	0 (0)
Wound swab	1	1	1 (10)	0 (0)	0 (0)
TOTAL	19	23	10 (43.5)	11 (47.8)	2 (8.7)

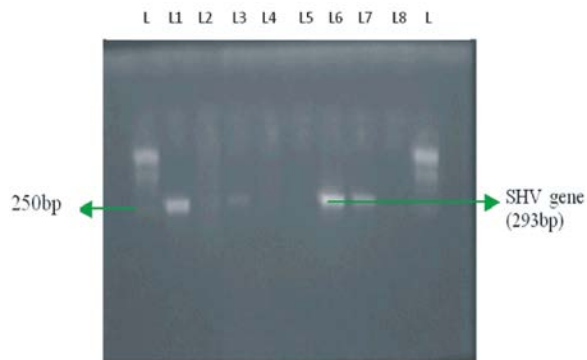


Fig. 1: Agarose gel electrophoresis showing PCR detection of *bla_{SHV}*. L: DNA size marker (1kb ladder), L1, L3, L6 and L7 represents SHV positive isolates while other lanes represents SHV negative isolates.

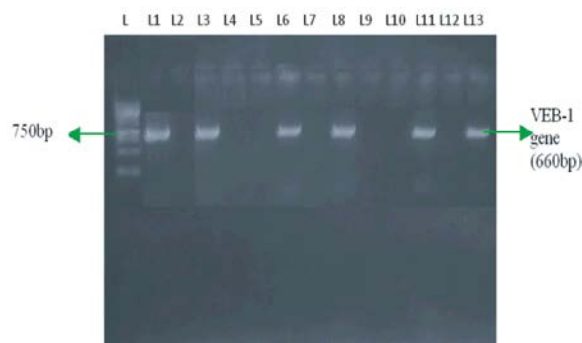


Fig. 2: Agarose gel electrophoresis showing PCR detection of *bla_{VEB-1}*. L: DNA size marker (1kb ladder), L1, L3, L6, L8, L11 and L13 represents VEB positive isolates while other lanes represents VEB negative isolates.

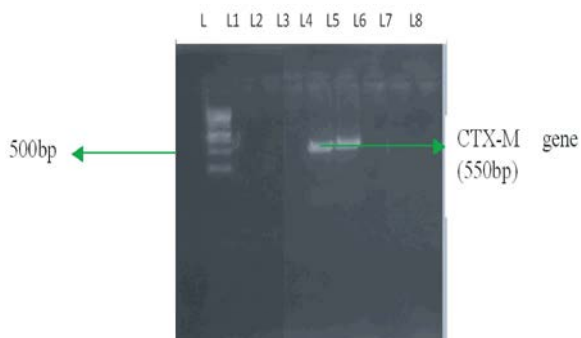


Fig. 3: Agarose gel electrophoresis showing PCR detection of *bla_{CTX-M}*. L: DNA size marker (1kb ladder), L4 and L5 represents CTX-M positive isolates (P₉ and P₁₅) while other lanes represent CTX-M negative isolates.

Table 3: The prevalence of *bla* genes in 22 *P. aeruginosa* isolates from various sources.

Isolates No.	Source	No. of <i>bla</i> genes isolated	Description of the <i>bla</i> genes isolated
P ₁	Sink	1	VEB-1
P ₂	Sink	1	SHV
P ₃	Sink	0	*
P ₄	Mop	0	-
P ₅	Disinfectant	0	-
P ₆	Theatre bed	2	VEB-1, SHV
P ₇	Nurses' tray	0	*
P ₈	Nasal swab	1	SHV
P ₉	Floor	3	VEB-1, SHV, CTX-M
P ₁₀	Patients' table	1	VEB-1
P ₁₁	Sphygmomanometer	1	VEB-1
P ₁₂	Bucket	1	VEB-1
P ₁₃	Lab. Work bench	2	VEB-1, SHV
P ₁₄	Sphygmomanometer	2	VEB-1, SHV
P ₁₅	Bucket	1	CTX-M
P ₁₆	Lab. Work bench	1	SHV
P ₁₇	Mop	2	VEB-1, SHV
P ₁₈	Trolley	2	VEB-1, SHV
P ₁₉	Trolley	1	VEB-1
P ₂₀	Patients' table	0	-
P ₂₁	Ear swab	0	*
P ₂₂	Wound swab	1	SHV
Total		23	

*Isolate No. 3, 7 and 21 were phenotypically positive on DDST but negative on PCR showing the absence of *bla* genes.

DISCUSSION

This study observed 86.2% ESBLs producing *P. aeruginosa* isolates phenotypically. The studies conducted by others depicted low rates, 3.7% [10] 4.2% [11] and 8.7% [12] respectively, of ESBL production in *P. aeruginosa*. While moderately high rates 20.3%, 35.85% and 39.41% were recorded by Aggarwal *et al.* [13], Mirsalehian *et al.* [14] and Ullah *et al.* [15]. This could be due to sample size or other environmental factors.

Polymerase chain reaction assay was used to determine the prevalence of genes encoding three different ESBLs (SHV, VEB-1 and CTX-M) among 22 isolates of *P. aeruginosa* cultured from both clinical and environmental samples in Onitsha hospitals. This study revealed the frequency of *bla_{SHV}*, *bla_{VEB-1}* and *bla_{CTX-M}* genes among the isolates as 43.5%, 47.8% and 8.7% respectively. A total of 23 *bla* genes were detected and only 16 (84.2%) out of 19 *P. aeruginosa* isolates phenotypically positive for ESBLs harbored *bla* genes. This is similar to a study by AL-Marjani *et al.* [16] in Baghdad, Iraq who reported detecting *bla_{CTX-M}* genes from *P. aeruginosa* but *bla_{SHV}* gene did not appeared in any isolate of ESBLs and MBLs producers and the latter is

contrary to 43.5% of *bla*_{SHV} genes obtained in this study. A study by Al-Grawi [17] revealed that among isolates of *P. aeruginosa* which were cefotaxime resistant, 72% were harboring CTX-M gene, whereas another study in Baghdad revealed a percentage of 83.3% for *bla*_{CTX-M} gene and 100% for *bla*_{TEM} gene [18]. Furthermore, a study by Al Naiemi *et al.* [19] also showed the presence of the *bla*_{CTX-M-1}, *bla*_{SHV-1} and *bla*_{TEM-116} genes in the *P. aeruginosa* strains and *bla*_{CTX-M-1} and *bla*_{SHV-1} in the *S. maltophilia* strain. Of these β -lactamases, only CTX-M-1 has been shown to produce a transferable ESBL phenotype.

Isolates containing these three *bla* genes were resistant to almost all previously tested antibiotics except imipenem and this agrees with the result of Shacheraghi *et al.* [20] who reported that their isolates containing *bla*_{VEB-1} gene were resistant to almost all tested antibiotics and had a frequency of 24% and 22% for *bla*_{SHV} and *bla*_{VEB-1} respectively. The three *bla* genes (*bla*_{SHV}, *bla*_{VEB-1} and *bla*_{CTX-M}) tested for in this study were all detected in a floor swab specimen whereas two *bla* genes (*bla*_{SHV} and *bla*_{VEB-1}) were commonly detected from mop, trolley and lab bench respectively. Only a *bla* gene (*bla*_{SHV}) was detected in the wound specimen. The most prevalent *bla* gene in this study was *bla*_{VEB-1} whereas *bla*_{CTX-M} gene was least prevalent.

The most resistance rates in isolates carrying SHV genes respectively belonged to piperacillin, amoxicillin-clavulanic acid, ceftazidime, cefuroxime, cefotaxime, cefepime, amikacin, ciprofloxacin, tetracycline and chloramphenicol. Also, the most resistance rates in isolates carrying VEB-1 genes respectively belonged to piperacillin, amoxicillin-clavulanic acid, ceftazidime, cefuroxime, cefotaxime, cefepime, ciprofloxacin, tetracycline and chloramphenicol. On the other hand for the isolates carrying the CTX-M gene, the most resistance rate belonged to piperacillin, amoxicillin-clavulanic acid, ceftazidime, cefuroxime, cefotaxime, cefepime, ciprofloxacin, tetracycline and chloramphenicol.

CONCLUSION

The concern with ESBL infections or ESBL-producing bacteria is that ESBL-mediated form of resistance renders virtually all available antibiotics (Especially the beta-lactams) ineffective in therapy and they can easily spread to other clinically important pathogens thereby compounding and perpetuating the problem of antibiotic resistance in a given community or hospital environment.

From results of this present investigation, it can be deduced that *bla*_{VEB-1} and *bla*_{CTX-M} genes were the most and the least frequently isolated ESBL genes among the *P. aeruginosa* strains detected from clinical and environmental isolates of *P. aeruginosa*. It is therefore necessary to focus on tracing the source of infections, control of nosocomial infections and therefore, design strategies to diminish the nonspecific use of broad spectrum antibiotics in the hospitals.

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