

# Investigative analysis of extended-spectrum beta-lactamase (ESBL) producing *Pseudomonas aeruginosa* isolated from hospitals of Islamabad-Rawalpindi

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## ABSTRACT

**Background:** *Pseudomonas aeruginosa* (*P. aeruginosa*) is a ubiquitous gram-negative rod shaped bacteria and a significant nosocomial opportunistic pathogen. Current study aimed at the investigation and determination of extended-spectrum beta-lactamase (ESBL) positive *P. aeruginosa* from clinical samples.

**Materials and methods:** A total of 150 catheter tubes, pus, blood, and sputum samples were collected from three different hospitals in the twin cities of Rawalpindi and Islamabad. The isolates were identified by using standard microbiology procedures. Antibiotic susceptibility of the isolates was done through the disc diffusion method as per the protocol given by CLSI guidelines. Phenotypic characterization of ESBL producers was performed by combination disc test (CDT), double disc synergy test (DDST) and through PCR.

**Results:** A total of 77/218 isolates were identified as *P. aeruginosa*. Among them 47 were resistant to different drugs, while 28 were identified as multidrug resistant. They were resistant to 9 different drugs including Cefotaxime (49%), Cefipime (41%), Cephadrine (32%), Cefotetan (62%), Azetronam (50%), Cofaclor (42%), Ticarcillin (57%), Ciproflaxacin (45%), and Imipenem (36%). CDT and DDST showed that 7/29 isolates produce ESBL. One isolate was positive for VEB and 3 were positive for SHV ESBL genotype.

**Conclusion:** The phenomenon of ESBL production does not only remain in *Klebsiella pneumonia* and *Escherichia coli* but could also be found in *P. aeruginosa*. Moreover, SHV genotype is prevalent in local isolates of *P. aeruginosa*.

## Keywords:

Extended-spectrum beta-lactamases, Multiple-drug resistance, Double disc synergy test, Combination disc test

## INTRODUCTION

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a gram-negative rod shaped bacteria (diameter: 0.5 to 0.8  $\mu\text{m}$ ; length 1.5 to 3.0  $\mu\text{m}$ ).<sup>1</sup> It has low virulence with metabolic flexibility. It is widely present in the soil, water, plants, and animals. This bacterium grows at normal body temperature (37°C but can also survive at (or up to) 42 °C. It is oxidase-positive, has the ability to hydrolyze arginines and reduce nitrates, and produces two types of chromogens pyoverdine and on pyocyanin.<sup>2</sup> The ability of *P. aeruginosa* to survive minimal growth conditions facilitates its persistence receptor activation of the inducer gene which then shows its expression in the form of various virulence factors and biofilm formation.<sup>4</sup> It also overcomes the host immune system to cause mild to serious type of infections in community and hospital environments.<sup>3</sup> The pathogenesis is

mediated by the activity of quorum both sensing and multifactorial processes. It possesses *las* and *rhl* quorum-sensing systems that help the bacterium communicate with other cells. Infections occur by The factors involved in causing infections include adhesion to the surface, type III secretion system, and other proteins which are secreted extracellularly. It can adhere and form biofilms over host cells and/or tissues through the formation of type IV pili-like structures (T4SS).<sup>5</sup> T4SS pili account for 90% of the adherence to host cells and are controlled by 40 different genes.<sup>6</sup>

The control and treatment of *P. aeruginosa* with conventional drugs (antibiotics and disinfectants) are not easy because of the resistance mechanisms adapted/acquired by its strains. Currently, the appearance of resistance of *P. aeruginosa* to various types and classes of antibiotics which includes  $\beta$ -lactamase, fluoroquinolones, and aminoglycosides is a global threat to public health.<sup>7</sup> Aminoglycosides are the drugs that are not recommended in the cases of renal failure because of their nephrotoxicity issues; moreover, aminoglycoside-monobactam-antibiotics especially aztreonam are generally reserved for serious infections

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caused by bacteria resistant to other beta-lactam drugs.<sup>8</sup> Carbapenem drugs are increasingly used for these bacteria which leads to the production of mutation by resistance genes. It results in the biosynthesis of  $\beta$ -lactamases (carbapenemase enzymes) that are equipped with the capacity to degrade all antibiotics containing  $\beta$ -lactam rings including carbapenem.<sup>9</sup> Carbapenem has high permeability across the cell membranes and is most stable against the activity of beta-lactamases. Recently carbapenem drugs had been prescribed intensively for the eradication of diseases caused by Gram-negative bacteria which led to the emergence of carbapenem resistance in *P. aeruginosa*. Carbapenem resistance of *P. aeruginosa* is hypothesized as low or insufficient membrane permeability, modification in proteins of penicillin binding sites, efficient efflux pumps in the bacterium, and enzymes like carbapenemase produced by bacteria to hydrolyze carbapenem.<sup>10</sup> Resistance to broad-spectrum antibiotics is developing because of the over-expression of Ampc enzymes along with pseudolysin, i.e., metallo-beta-lactamases, which help bacteria to invade and colonize host cells and cause severe inflammation.<sup>11</sup>

Extended-spectrum beta-lactamase (ESBL) positive *P. aeruginosa* species pose a major threat globally in health care infections. Generally, hydrolysis of broad-spectrum beta-lactams occurs due to plasmid-mediated enzymes known as ESBLs; clavulanate is a substance that strongly inhibits these enzymes. Transmission of these enzymes among various bacteria is occurred by plasmids. Failure of clinical treatment generally occurs with improper antimicrobial treatment for ESBL-producing bacteria. Therefore, the proper examination and prediction of the infection by clinical methods can aid in the proper selection of antibiotics and hence results in an improved health state.<sup>12</sup>

### MATERIALS AND METHODS

The current study was performed in the twin cities of Islamabad-&-Rawalpindi. Samples (catheter tubes, pus, blood, and sputum) were collected from different hospitals in sterile bags and transported to the lab at COMSATS University Islamabad where they were kept at refrigerated temperature (4°C) for further analysis.

#### Isolation and biochemical identification of *P. aeruginosa*

The collected samples were inoculated on Blood and MacConkey agar plates, and incubated at 37°C overnight under aerobic conditions. *P. aeruginosa*

colonies were identified by colony morphology, Gram staining, urease, Triple Sugar Iron (TSI), oxidase, and indole production tests.

#### Antibiotic susceptibility testing

Antibiotic susceptibility of *P. aeruginosa* was done through the disc diffusion method as per the protocol given by CLSI guidelines. Mueller-Hinton (MH) medium was used to test the antimicrobial susceptibility of bacterial strains. Ticarcillin-clavulanate, Cefepime, Ceftazidime, Aztreonam, Gentamicin, Ciprofloxacin, Levofloxacin, Tobramycin, Ofloxacin, Amikacin, Cefotaxime, Meropenem, and Imipenem discs (Oxoid, England) were used.

#### ESBL phenotypic confirmation

Phenotypic characterization of ESBL producers among the *P. aeruginosa* isolates was performed by combination disc test (CDT) and double disc synergy test (DDST). CDT was performed using cephalosporins including cefotaxime, ceftazidime, and cefepime combined with clavulanic acid.<sup>13</sup> Clavulanate was used to estimate the inhibition of ESBL. The zone of inhibition around the cephalosporin discs alone and in presence of clavulanic acid was measured. DDST was performed to confirm the keyhole phenomenon.<sup>14</sup> Four (30 µg) cephalosporin discs (cefotaxime, ceftriaxone, ceftazidime, cefepime) were placed next to the amoxicillin (30µg) + clavulanic acid (10µg) disc. The distance between cephalosporin discs and clavulanic acid discs was kept to 20 mm center to center.

#### Molecular analysis of ESBL positive *P. aeruginosa* strains

Molecular evaluation of the strains which were positive for both, CDT and DDST were selected for this test by isolating the DNA of bacteria and subjecting them to PCR mediated in vitro amplification. The DNA was extracted through the ‘phenol-chloroform’ method and a PCR was carried out under standard conditions for 30 cycles. Five different primers were used (Table 1).<sup>15</sup>

Table 1: Sequences for forward and reverse primers.

Primer	Primer Sequence	Amplified band
VEB-1	Forward: CGACTTCCATTTCCCGATGC Reverse: GGACTCTGCAACAAATACGC	640 kb
PRE-1	Forward: ATGAATGTCATTATAAAAGC Reverse: AATTTGGGCTTAGGGCAGAA	930 kb
TEM	Forward: GAGTATTCAACATTTCCGTGTC Reverse: TAATCAGTGAGGCACCTATCTC	1075 kb
SHV	Forward: AAGATCCACTATCGCCAGCAG Reverse: ATTCAGTTCGGTTTCCAGCGG	865 kb
CTX-m1	Forward: GACGATGTCAGTGGCTGAGC Reverse: AGCCGCCGACGCTAATACA	870 kb

## RESULTS

A total of 150 pus, blood, sputum and catheter samples were collected from three different hospitals in the twin cities of Rawalpindi and Islamabad. The samples yielded 218 different bacterial isolates which were further sub-cultured on selective growth mediums to isolate *P. aeruginosa*. A total of 77 (51.3%) *P. aeruginosa* isolates were further identified using series of biochemical testing.

### Antimicrobial susceptibility testing

The positive samples for *P. aeruginosa* were subjected to drug profiling to determine the antimicrobial susceptible pattern of the isolates. The complete statistics along with selected images of the drug susceptibility assays are shown in Figure 1. Among a total of 77 isolates, 47 were resistant to various drugs. Among them, 18 were resistant to only single type of drug and 29 were resistant to multiple drugs (i.e. MDR) (Figure 1C). The isolates were resistant to nine different classes of drugs including, Cefotaxime (49%), Cefipime (41%), Cephadrine (32%), Cefotetan (62%), Azetronam (50%), Cofactor (42%), Ticarcillin (57%), Ciproflaxacin (45%), and Imipenem (36%) (Figure 1D).

Total 27/29 MDR isolates produced zone of inhibition smaller than 27mm against cefotaxime (30 µg) and aztreonam (30 µg) therefore, they were further

tested for ESBL production. Two phenotypic (combination disc test and Double Disc Synergy Test) and a genotypic molecular confirmatory test were used to differentiate between ESBL positive and negative isolates of *P. aeruginosa*.

### Combination disc test (CDT)

In the CDT, the two combinations of antibiotics, i.e. cefotaxime (30 µg) alone and with clavulanic acid (10 µg); ceftazidime (30 µg) and alone with clavulanic acid (10 µg) were tested. The results showed that 7 out of the 29 MDR isolates of *P. aeruginosa* were positive for ESBL production (Figure 2).

### Double Disc Synergy Test (DDST)

The DDST is another phenotypic analysis that can distinguish between the ESBL positive and negative bacterial isolates. In this test, cefotaxime, ceftriaxone (30 µg), ceftazidime (30 µg), and cefepime (30 µg) along with a disc of amoxicillin (30 µg) plus clavulanic acid (10 µg) were used. In this study, 7 out of the 29 MDR isolates of *P. aeruginosa* produced the effect and were confirmed to be ESBL positive. Figure 2B shows a keyhole (like features) appeared, i.e. the cephalosporin had a clear zone expansion towards the disc which had a combination of amoxicillin and clavulanic acid.

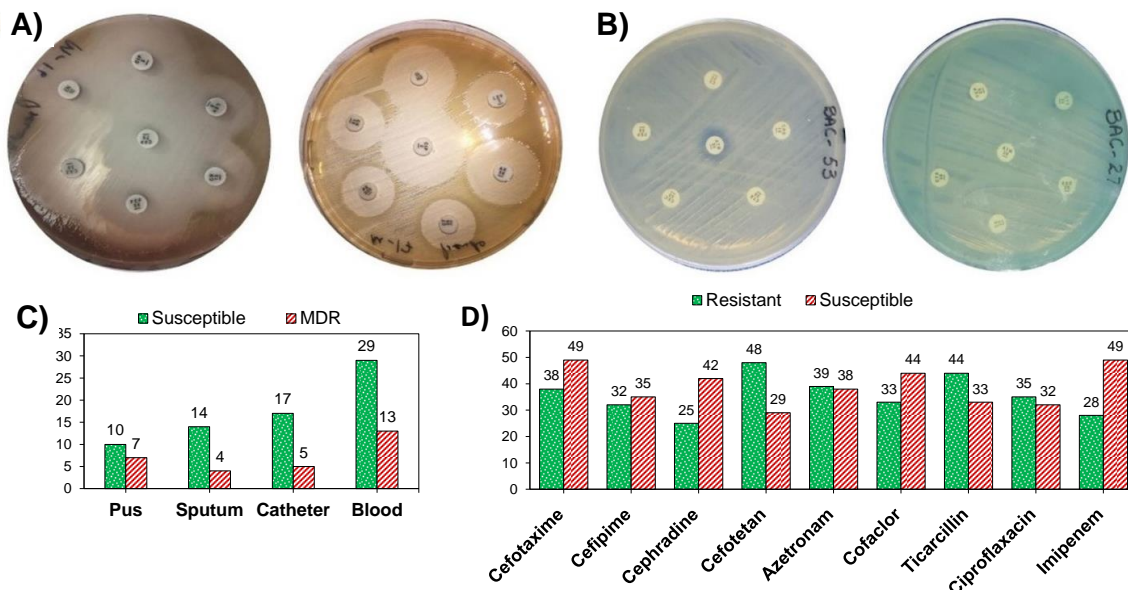


Figure 1: Antimicrobial susceptibility testing using disc diffusion assay on Mueller Hinton Agar (MHA) medium. A) The clear zones of growth inhibition around the disc of inhibition around the discs for susceptible isolates. (B) Isolates resistant to different antimicrobials. (C) Number of susceptible vs. multidrug-resistant *P. aeruginosa* isolates from different types of samples. (D) The number of resistant vs. susceptible isolates to different antibiotics with the percent resistance pattern.

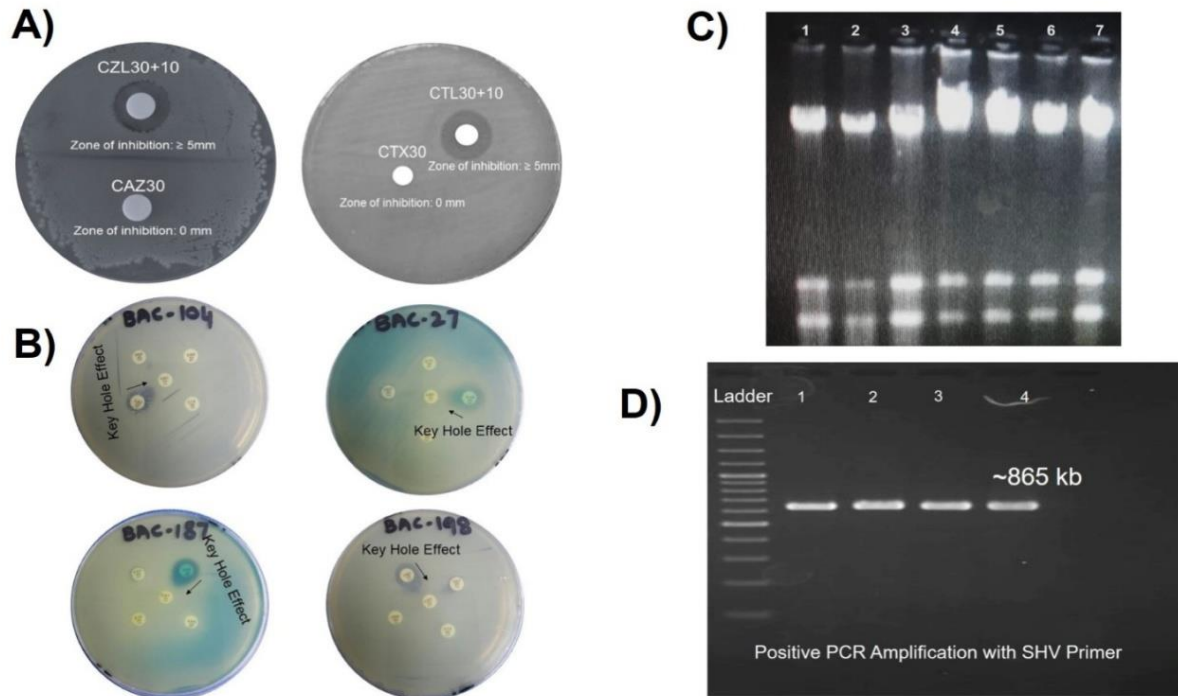


Figure 2: Phenotypic confirmation of ESBL production in *P. aeruginosa* through (A) CDT and (B) DDST; (C) DNA isolated from the 7 samples confirmed to be ESBL by phenotypic evaluation and (D) positive amplification of PCR for SHV genes' primers

**Molecular Investigation through PCR**

The 7 isolates showing ESBL activity were further used for molecular investigation through PCR. The DNA from these 7 isolates was extracted and target sequences were amplified using specific primers (Table 1). The isolated DNA was over 300 ng/μL in concentration (Figure 2C). The results showed that for the 7 samples, 1 was positive for VEB and 3 were positive for SHV gene target (Table 2).

Table 2: In vitro PCR amplification results

ESBL positive samples	VEB	PRE-1	TEM	SHV	CTX-m1
#1 (BAC27)	-	-	-	+	-
#2 (BAC63)	-	-	-	-	-
#3 (BAC104)	-	-	-	+	-
#4 (BAC149)	-	-	-	+	-
#5 (BAC174)	-	-	-	-	-
#6 (BAC187)	+	-	-	-	-
#7 (BAC198)	-	-	-	-	-

**DISCUSSION**

The advent of antibiotics had revolutionized medicine for many years, but not more than a decade had passed when it was observed that bacteria developed resistance to these antibiotics. Bacteria are now resistant to almost every antibiotic they had been exposed to. ESBL is one of the resistance patterns in *E. coli* and *K. Pneumoniae*, however, it is not only limited to them but can also be found in *P. aeruginosa*.<sup>16</sup> The

percentage of the prevalence of ESBL though is very low its presence still triggers the alarm on industrial and scientific community to develop, design, and discover new-and-novel antimicrobial agents. *P. aeruginosa* strains showed higher resistance towards cefotetan (a 2<sup>nd</sup> generation cephalosporin); which indicate the overexpression of AmpC in these bacteria which hydrolyzes the ureido-cephalosporin resulting in increased resistance against these drugs.<sup>17</sup> Increased resistance to other classes of antibiotics like ticarcillin and cefotaxime is due to the overproduction of proteins responsible for efflux of the drugs out of the bacterial system, uptake of resistant gene, and overexpress it and also by overproduction of the acquired beta-lactamases.<sup>18</sup> Beta-lactamases produced by the bacteria have the ability to hydrolyze cefotaxime and ceftazidime (3<sup>rd</sup> generation cephalosporins) but the activity of beta-lactamases can be inhibited by the presence of clavulanic acid. It acts as an inhibitor by binding to active serine sites in beta-lactamases resulting in enzyme inactivation. It does not work as an antibiotic itself but works synergistically.<sup>19</sup>

In this study 29 out of 77 positive samples of *P. aeruginosa* were found to be multiple drugs resistant and 7 out of 29 MDR isolates were positive for ESBL. Out of these 7 positive ESBL samples 4 were also positive through PCR. The SHV amplification was seen

through PCR *in vitro* amplification. Among the most important beta-lactamases are SHV and TEM. The results indicate that the prevalence of SHV gene is higher suggesting that this type of beta-lactamase is a major problem in the area (Rawalpindi & Islamabad) where this investigation was conducted. In *Enterobacteriaceae*, SHV genes have emerged, the blaSHV-1 gene was identified in the 1970s in *E. coli* for the first time and it had been causing infections in the last decades of the 20<sup>th</sup> century and were detected in various regions worldwide.<sup>20</sup> The enzyme encoded by bla<sub>SHV-1</sub> gene is SHV-1 (sulfhydryl reagent variable) that had reported activity against the drugs, penicillins, and cephalosporins of the 1<sup>st</sup> generation.<sup>21</sup> Several allelic variants for SHV beta-lactamases have been already reported. They have evolved from a narrow to extended-spectrum of hydrolyzing activity. SHV-ESBLs are mainly expressed by self-transmissible extra-nuclear DNA (plasmids), intermittently carrying resistant genes to various classes of antibiotics. Due to this these have become widespread worldwide in several *Enterobacteriaceae*, emphasizing their clinical significance. A research study from Iran was conducted to identify the prevalence of SHV type ESBL and it was reported to be 10.57%.<sup>22</sup> In *K. pneumoniae* and *E. coli* from Vietnamese patients plasmid- and integron-located bla<sub>VEB-1</sub> gene had been reported too.

In this study, the prevalence of SHV-ESBLs is 13.7% which is similar to the reports published in Iran. This points to an event of gain of function, that is, horizontal gene transfer events from other bacteria to *P. aeruginosa*.

## CONCLUSION

*P. aeruginosa* is among the hospital-acquired infections in Pakistan which has not only become resistant to a variety of antibiotics but has also become an ESBL producer – a mechanism for which it is not conventionally known. Thus, detection of ESBL genes using molecular methods, and patterns of antimicrobial resistance can provide us with useful information and risk factors associated with *P. aeruginosa* infections.

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