Comparison of phenotypic and genotypic methods for the detection of ESBLs and AmpC producing *Pseudomonas aeruginosa* isolates from intensive care units

Fakhur Uddin, Muhammad Sohail, Qurban Hussain Shaikh, Sagheer Ahmed, Saeed Khan, Kerry Roulston, Timothy D McHugh

Abstract

Detection of AmpC and ESBL producing *P. aeruginosa* by phenotypic methods is challenging, especially in low-income countries such as Pakistan. Therefore, a molecular method was developed for rapid detection of these resistance markers. A total of 303 clinical samples were collected from intensive care units (ICUs) of the Jinnah postgraduate medical centre (JPMC) Karachi, Pakistan. The isolates were identified by traditional and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS). Isolates were phenotypically analyzed for AmpCs and ESBL by D-test and by double disc synergy, respectively. The Check MDR CT103 XL and PCR techniques were used for the detection AmpCs and ESBLs. Out of 303 isolates, 148 (48.8%) were *P. aeruginosa*. The resistance pattern of *P. aeruginosa* against piperacillin, cefatizidime and cefepime was 59.4%, 64.8% and 59.4% respectively. More than 60% isolates were resistant to aminoglycosides and ciprofloxacin. All (148) strains were found sensitive to colistin. Phenotypic ESBL prevalence was 8.8% whereas genotypic resistance was 29.1%. *bla*<sub>VEB</sub> was the most prevalent ESBL. Although 25.67% of *P. aeruginosa* isolates were positive phenotypically for AmpC, microarray (Check-MDR) analysis did not detect chromosomally located AmpC in any of the isolates.

Abbreviations
1. Introduction

*Pseudomonas aeruginosa* commonly causes infections among the immunocompromised patients admitted in intensive care units (ICUs) [1]. The emergence of multi-drug resistant (MDR), extensively drug-resistant (XDR) and pan-drug resistant (PDR) strains of *P. aeruginosa* has serious consequences including therapeutic failure, increase in healthcare cost by 70%, increase in the rates of morbidity and mortality and prolonged hospital stay [2]. The MDR, XDR and PDR strains have evolved through genomic plasticity and accumulation of selected mutations in chromosomal genes and transmission of exchangeable resistance elements [1]. Indeed, the overexpression of the chromosomal AmpC and ESBL production are the two primary mechanisms of resistance against antipseudomonal cephalosporins, penicillins and other β-lactam antibiotics [3, 4]. The *bla*<sub>AmpC</sub> genes, usually located on chromosome but plasmid mediated genes are clinically important [5]. The most common types of plasmid mediated AmpC (pAmpCs) are CMY, ACT, DHA, FOX and MIR in *P. aeruginosa* [5, 6, 7].
Commonly encountered ESBLs are PER, GES, VEB, BEL and PME of class A, while TEM, SHV and CTX-M-type appear less frequently in *P. aeruginosa* [8, 9, 10, 11].

Clinical and Laboratory Standards Institute (CLSI) does not recommend the commonly employed phenotypic methods (double disc diffusion test with clavulanic acid and D-Tests) for the detection of ESBLs and AmpCs in *P. aeruginosa* [12]. Yet these phenotypic methods continue to be used in resource limited settings. A molecular method, Check-MDR CT103 XL (Check-points, Wageningen, Netherlands), has been developed as a rapid and accurate assay for the detection of clinically relevant ESBLs, pAMPCs and carbapenemase variants in a single tube reaction [13]. The prevalence of ESBL-producing strains varies geographically [2] and the prevalence of ESBL and AmpC producing *P. aeruginosa* is partially described in Pakistan.

In the present study we highlight the compromised sensitivity of phenotypic assays used for the determination of ESBL and AmpC types in *P. aeruginosa* as compared to DNA microarray (Check-MDR) and PCR.

2. Materials and Methods

2.1 Setting

The present work was performed in the Department of Microbiology, Basic Medical Sciences Institute (BMSI), Jinnah Postgraduate Medical Centre (JPMC) Karachi, Pakistan (a teaching hospital with >1500 beds) in collaboration with the Department of Microbiology University of Karachi, Dow University of Health Sciences, Karachi, and the UCL Centre for Clinical Microbiology, Royal Free Campus, London.

2.2 Sample size
A total of 303 clinical specimens, that were growth positive, were processed from the patients admitted to ICUs at JPMC, Karachi, Pakistan during November 2015 to May 2016. The duplicated/repeated, Glucose fermenting Gram-negative bacilli and Gram-positive cultures were excluded. The specimens were collected in accordance with the standard operating procedures [14]. The informed consent was obtained from the patient or attendant and ethical approval was taken from the institutional review board (IRB), JPMC Karachi (No. F.2-18/2014-GENL/31649577/JPMC).

2.3 Microbiological assays

Clinical specimens (tracheal aspirates, urine, blood, sputum, pus) were processed using standard techniques and blood specimens were directly inoculated in blood culture bottles according to the standard protocols [14]. *P. aeruginosa* isolates (148) were selected for further analysis.

2.4 Identification of *P. aeruginosa*

Isolates were initially identified by routine cultural characteristics and battery of biochemical tests. The oxidase test was performed by Microbact™ oxidase strips (Oxoid, UK) [15]. These provisionally identified isolates were confirmed by the API 20 NE (Biomerieux, Marcy l’Etoile France) and re-confirmed by the matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS, Microflex, Bruker Daltonics) at the Centre for Clinical Microbiology, UCL, London, UK.

2.5 Antibiotic susceptibility testing (AST)

AST was determined by the disk diffusion technique using Mueller-Hinton agar (MHA), (Oxoid, UK) according to the recommendations of the CLSI. AST results were checked and compared with
the results of micro-broth dilution method and Etest as recommended by CLSI. *P. aeruginosa* ATCC 27853 was used as a quality control strain [12].

2.6 Phenotypic detection of AmpCs and ESBLs in *P. aeruginosa*

AmpCs in *P. aeruginosa* was determined by a diffusion test, D-test [16]. Cephalosporins and penicillin [ceftazidime (CAZ), piperacillin (PIP), and aztreonam (ATM)] were applied as substrate antibiotics (Oxoid, UK) as recommended by the CLSI for AST of *P. aeruginosa* [12]. After incubation at 35°C for 16-18 hours, the growth inhibition zones were determined. A difference of ≥2 mm at inducer side and non-inducer side was interpreted as AmpCs positive strain.

The ESBLs were phenotypically determined by the double disc synergy test (DDST) with clavulanic acid (CA) in accordance with Laudy et al. [11] and CLSI recommendations [12]. The shoulder formation towards the AMC and any of CAZ, CRO, FEP, or ATM was interpreted as ESBLs producer.

2.7 Detection of β-lactamases by DNA microarray

The ESBLs and AmpCs were determined by the Check-MDR CT103XL microarray kit (Check-Points Health BV, Wageningen, Netherlands). This assay was performed at the Centre for Clinical Microbiology, Royal Free Campus, London, UK by following the manufacturer's instructions. The principle of this technique is based on the multiplex ligation detection reaction (LDR). It included two probes for the targeted resistance genes which make it superior to multiplex PCR. This microarray targeted various ESBL and AmpC genes including BEL-CTX-M-groups, GES-ESβL, PER, SHVs, TEMs, VEB variants and ACC, ACT, CMY, DHA, FOX, MIR, MOX respectively [13].
2.8 Detection of \( \text{bla}_{\text{PER}} \) and \( \text{bla}_{\text{VEB}} \) genes by PCR

The primers for the PCR were designed according to the microarray results. PCR was performed using G-Strom-482, (Gene Technologies Ltd, UK) thermocycler and the amplification kit (Qiagen, Germany). PCR reaction cycles and the annealing temperature for VEB gene have been described previously [17]. The optimized PCR cycle time used in this work was 15, 0.5, 0.5, 1 and 10 min for initial, denaturation, annealing, extension and final extension respectively at the different temperature (Table 1).

Table 1 The primers and their cycles temperature in this work

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>*In. (°C)</th>
<th>*Det. (°C)</th>
<th>*An. (°C)</th>
<th>*Ext. (°C)</th>
<th>*FE. (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{bla}_{\text{VEB}} )</td>
<td>F: GTTAGCGGTAATTACCAACGATAG&lt;br&gt; R: CGTGGTGCTATGGGCAG</td>
<td>95</td>
<td>94</td>
<td>55</td>
<td>72</td>
<td>72</td>
<td>1070</td>
</tr>
<tr>
<td>( \text{bla}_{\text{PER}} )</td>
<td>F: GCTCCGATAATGAAAAGCGT&lt;br&gt; R: TCCGGCTTAGACTCGGCTGA</td>
<td>95</td>
<td>94</td>
<td>53</td>
<td>72</td>
<td>72</td>
<td>520</td>
</tr>
</tbody>
</table>


2.9 Data analysis

The initial data was recorded in Microsoft Excel version 2010 and statistical analysis was conducted using SPSS (Statistical Package for Social Sciences, Microsoft Inc., USA) software, version 16.0 for Windows. The sensitivity and specificity of phenotypic assays were calculated by using the web calculator (https://www.medcalc.org/calc/diagnostic_test.php).

3. Results

3.1 Frequency of Gram-negative Bacilli

Out of 303 samples 154 (50.8%) were positive for \( \text{Pseudomonas} \) spp., 108 (35.6%) for \( \text{Acinetobacter baumannii} \), and 41 (13.5%) for other Gram-negative bacteria. \( \text{P. aeruginosa} \) was the most prevalent species amongst Pseudomonads (148/154, 96.1%); most of the isolates were
obtained from respiratory (61/148, 41.2%) and blood specimens (53/148, 35.8%), followed by pus samples (Table.2).

3.2 Antibiogram of *P. aeruginosa*

*P. aeruginosa* isolates were phenotypically resistant to most of the antipseudomonal penicillin and cephalosporins but a significant proportion was sensitive to piperacillin/tazobactam (PZT) (58/148, 39.2%). Resistance against the carbapenems (imipenem 74.3%, meropenem, 68.2%), ciprofloxacin (65.5%) and aminoglycosides (64.2%) was also higher (Table.3). All the *P. aeruginosa* isolates were resistant to ampicillin, ampicillin sulbactam and ceftriaxone.
Table 2 Frequency of *P. aeruginosa* in relation to the specimens (n=148)

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Disease</th>
<th>P. aeruginosa</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheal aspirates</td>
<td>VAP/HAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61</td>
<td>41.2</td>
</tr>
<tr>
<td>Blood</td>
<td>Septicemia</td>
<td>53</td>
<td>35.8</td>
</tr>
<tr>
<td>Pus</td>
<td>Diabetic</td>
<td>18</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>Foot/post surgical wound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>UTIs</td>
<td>11</td>
<td>7.4</td>
</tr>
<tr>
<td>Sputum</td>
<td>Pneumonia</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>148</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ventilator-associated pneumonia, Hospital-acquired pneumonia, <sup>b</sup>Urinary tract infections
Table 3 Susceptibility patterns of *Pseudomonas aeruginosa* by combined disc diffusion method and Etest, according to the recommendation of CLSI (n=148)

<table>
<thead>
<tr>
<th>Antibiotics class</th>
<th>Number of isolates (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Penicillins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piperacillin (PIP)</td>
<td>88 (59.45)</td>
<td>8(5.40)</td>
</tr>
<tr>
<td>Piperacillin/tazobactam (PZT)</td>
<td>58(39.18)</td>
<td>11(7.43)</td>
</tr>
<tr>
<td>Cephalosporins 3rd Generation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftazidime (CAZ)</td>
<td>96 (64.86)</td>
<td>2(1.35)</td>
</tr>
<tr>
<td>Cephalosporins 4th Generation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefepime (FEP)</td>
<td>88 (59.45)</td>
<td>-</td>
</tr>
<tr>
<td>Carbapenems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipenems (IPM)</td>
<td>110(74.32)</td>
<td>4(2.70)</td>
</tr>
<tr>
<td>Meropenems (MEM)</td>
<td>101(68.24)</td>
<td>2(1.35)</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin (AK)</td>
<td>96 (64.86)</td>
<td>3(2.02)</td>
</tr>
<tr>
<td>Gentamicin (CN)</td>
<td>95(64.18)</td>
<td>5(3.37)</td>
</tr>
<tr>
<td>Tobramycin (TOB)</td>
<td>95(64.18)</td>
<td>5(3.37)</td>
</tr>
<tr>
<td>Quinolones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>97(65.54)</td>
<td>3(2.02)</td>
</tr>
<tr>
<td>Lipopeptides (Polymyxins)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colistin (CT)*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Broth microdilution, CLSI (2018) guidelines*
3.3 Phenotypic and genotypic detection of ESBLs and AmpCs

Phenotypic assessment identified 8.8% (13/148) isolates of *P. aeruginosa* as ESBL producers, whereas the genotypic prevalence of ESBLs was 43/148 (29.05%). *bla*<sub>VEB</sub> was the most common marker (Fig.1). The microarray method (Check-MDR) did not detect AmpC producers; however, 38 (25.67%) isolates were positive for AmpC by the D-test (Fig. 2).

3.4 Sensitivity and specificity of phenotypic methods

The sensitivity and specificity of the double disc synergy method for ESBLs in *P. aeruginosa*, was 30.2% and 100%, respectively, compared to the microarray (Check-MDR). The data was also analyzed for the co-production of AmpC in genotypically VEB positive and 11.6% (5/43) isolates were found D-test positive for AmpC.
Figure 1  PCR results of $bla_{VEB}$ gene (1070 bp), L, DNA ladder (1kb), N negative control, P, positive control, sample #. 5, 10, 30, 32, 74 were positive and 1, 4, 11, 12, 13, 31, 37, 39, 50, 51, and 55 were negative.

Figure 2  Phenotypic and genotypic compression of ESBLs and AmpC in *P. aeruginosa*
4. Discussion:

The environment in ICUs favors survival of *P. aeruginosa* due to its ability to grow in nutritionally poor environments, resistance to commonly used disinfectants and other environmental factors such as favorable temperature and humidity. This increases the risk of transmission of multidrug resistant and carbapenem resistant *P. aeruginosa* to immunocompromised and critically ill patients [18, 19, 20]. The present study corroborated previous findings that the resistance to imipenem and meropenem was more prevalent than the antipseudomonal penicillins (piperacillin and piperacillin/tazobactam) and cephalosporins including ceftazidime and cefepime in *P. aeruginosa* [21]. The resistance may be attributed to the alteration of porins or repression of OprD, presence of intrinsic AmpCs and over-expression of MexAB-OprM and modification in efflux pump activity [22, 23]. Among these phenotypically tested strains, AmpCs producing *P. aeruginosa* showed higher prevalence (26.0%) than ESBLs (8.8%) producers in present study. However the results of microarray (Check-MDR) were in contrast to the phenotypic results, showing the higher prevalence of ESBL producing *P. aeruginosa*. A similar pattern of ESBLs and AmpC has been reported from Pakistan but at lower frequencies [24]. Lower prevalence of ESBLs (7.4%) in *P. aeruginosa* has also been found in other countries, such as in Egypt [13]. The AmpCs were not found by the microarray (Check-MDR) in all the *P. aeruginosa* isolates as the microarray (Check-MDR) detects only plasmid mediated AmpC (pAmpCs). Previously microarray (Check-MDR) has been reported as 100% specific and sensitive for pAmpC targets [13].

VEB-1 was initially discovered in *E. coli* from Vietnam (1996), and then it was widely reported in *Pseudomonas* spp. [25]. In the present study, VEB appeared as the only ESBL in *P. aeruginosa* (28.4%) and it is may be endemic in this geographic location. Other variants of ESBLs
(CTX-M-15 and OXA-10) have also been reported from this region [26]. The ESBL in *P. aeruginosa* is not restricted to some types but it varies from VEB to OXA-like, GES, PER and CTX from various geographic regions [11, 15, 26, 27]. The higher frequency of *bla*\textsubscript{VEB} carrying *P. aeruginosa* in this study may be due to clonal spread in the ICUs of this hospital or these strains are present in environment. Woodford et al. also found higher prevalence of VEB harboring *P. aeruginosa* from the UK [25].

In summary, the sensitivity of phenotypic methods is compromised due to the accumulation of different resistance mechanisms against the extended cephalosporins, which are used as substrate in the phenotypic test for ESBLs detection.

5. **Conclusion**

The phenotypic methods for the detection of ESBLs and AmpC have good specificity but poor sensitivity than genotypic tests. The VEB-type producing *P. aeruginosa* strains are common in this hospital. Other than ESBLs, resistance mechanisms are present in the *P. aeruginosa* which may be investigated at local level to understand the molecular mechanisms of resistance.

**Declarations**

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**
The authors declare that they have no competing interests.

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**References**


