Comparison of Different Phenotypic Methods for the Detection of Extended Spectrum Beta Lactamases in Escherichia coli and in Amp C Co-Producing Phenotypes Through Single Plate Detection Technique

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Abstract

Objectives: The study was done to determine the prevalence of Extended spectrum beta lactamases (ESBL) in urinary isolates of Escherichia coli (E. coli) and to evaluate and compare different phenotypic methods for its detection through single plate detection technique, even in AmpC co-producing ESBL phenotypes of E. coli.

Study design: Cross sectional comparative.

Place and duration of study: The study was done in the Department of Microbiology, Combined Military Hospital, Lahore, Pakistan from January 2016 to June 2016.

Materials & Methods: Out of 945 samples for urine culture, received from various units of CMH Lahore, 70 specimens yielded growth of E. coli. In single plate screening technique, E. coli isolates were tested by Double disk synergy test (DDST) and Modified double disk synergy test (MDDST) using Cefepime (FEP) & Piperacillin-tazobactam (TZP) on a single plate for the detection of ESBL to save time and money. Phenotypic confirmatory disk diffusion method (PCDDT) was taken as “Gold Standard”.

Results: From 70 E. coli isolates, 34 were positive for ESBL production. DDST detected ESBL in 12/34 isolates. MDDST detected ESBL in 33/34 isolates including 14 isolates that were co-producers. Therefore, MDDST proved to be very effective in detecting ESBL in AmpC co-producing E.coli.

Conclusion: The prevalence of ESBL producing E. coli in our hospital was 48.75%. Single plate screening technique seems to be convenient and time saving (within 24 hrs) and increases the probability of detecting ESBL through different methods even in AmpC co-producing E.coli.

Key words: ESBL, Amp C, DDST, MDDST.

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Introduction

E. coli happens to be the commonest isolate in urine samples received at laboratory [1]. It is often been reported of possessing extended spectrum beta lactamases (ESBL). Isolates producing ESBL are associated with increase drug resistance and they have been associated with hospital outbreaks [2], community infections [3] and nursing home infections [4]. Moreover, most of the isolates now do possess AmpC enzymes as well which create problems in detection, by masking ESBL expression [5].

Extended spectrum β-lactamases (ESBLs) and AmpC β-lactamases are most commonly produced by Klebsiella spp and Escherichia coli but may also occur in other Gram-negative bacteria. ESBLs being plasmid mediated typically hydrolyze penicillins, third generation cephalosporins and Aztreonam [6]. Although not active against Cephamycins (Cefoxitin and Cefotetan), these are susceptible to β-lactamase inhibitors (Clavulanic acid). Complexity is generated by AmpC co-producing ESBL phenotypes. AmpC β-lactamases are Cephalosporinases that are poorly inhibited by Clavulanic acid and can be differentiated from ESBLs by their ability to hydrolyze Cephamycins. Furthermore, ESBLs genes are located on plasmids, while genes for AmpC β-lactamases are either plasmid or chromosomally encoded [7].

Routine ESBL testing is no longer necessary, according to CLSI 2015 interpretive criteria, but can be
Phenotypic confirmatory disc diffusion test (PCDDT): The test was applied as per CLSI 2016 guidelines by using control strains of *E. coli* ATCC 25922 (Beta Lactamase negative) and *E coli* ATCC 700603 (ESBL positive). A >=5mm increase in zone diameter for either Ceftazidime or Cefotaxime tested in combination with Clavulanic acid versus its zone diameter when tested alone confirmed ESBL production [10]. This test was taken as “Gold Standard”.

**Double disk synergy test (disk approximation test):** This test was done by using a disc of Augmentin (20ug Amoxicillin + 10ug Clavulanic acid) and discs of Ceftazidime (30ug) and Ceftriaxone (30ug); which were placed around Augmentin disc keeping the distance of 16 to 20 mm from it (centre to centre). The organisms were considered to be producing ESBL when the zone of inhibition around any of these Cephalosporin discs showed a clear-cut increase towards the Augmentin disc [11].

**Modified double disk synergy test (MDDST):** The original double disk synergy test was modified for detecting ESBL for Amp C co-producing clinical isolates. A disc of Piperacillin-tazobactam (100/10ug) was placed at a distance ranging between 16 and 20 mm from the Cefepime disc. The organisms were considered to be producing ESBL when the zone of inhibition around Cefepime showed a clear-cut increase towards the Piperacillin-tazobactam disc [11].

The isolates showing positive results for ESBL production with any of the tests i.e. DDST, MDDST or with any inhibitor combination were taken as ESBL positive and subjected to PCDDTT for confirmation.

**Initial screening with cefoxitin disc (30 μg) for the presence of AmpC β-lactamases:** The presence of AmpC β-lactamases was detected after
initial screening with Cefoxitin (30 µg, Oxoid, Basingstoke, UK) disc. Isolates which showed Cefoxitin zone diameter <18 mm were considered screen positive for AmpC β-lactamase production [12].

Disc approximation techniques to detect inducible/ Plasmid mediated AmpC activity: The use of single plate detection technique made detection of inducible Amp C activity easy. Already applied 10 µg Imipenem, 30 µg Cefoxitin, and 20/10 µg Amoxicillin- clavulanate discs act as the inducing substrates and 30 µg Ceftazidime discs (all from Oxoid, Basingstoke, UK) as the reporter substrate (Diag :1). Discs were applied at a distance of 16-20 mm, and any obvious blunting or flattening or D-Zone formation of the zone of inhibition between the ceftazidime disc and the inducing substrates was interpreted as a positive result for plasmid mediated AmpC.

All susceptibility testing and MICs was performed using 0.5-McFarland-standard suspensions prepared from overnight cultures, applied to Mueller-Hinton agar (Becton Dickinson), and incubated at 35°C for 16 to 18 hrs. Zones of inhibition were measured to the edge of obvious inhibition, ignoring any micro colonies present within a clear zone of inhibition.

E. coli ATCC 700603 was used as a positive control and E. coli ATCC 25922 as a negative control with each batch of tests[12,13].

Data Analysis: Data was analysed using SPSS version 21. Keeping PCDDT as Gold Standard. Other tests were compared using Chi-Square test. p-value, sensitivity, specificity, PPV and NPV of the tests were calculated using appropriate formulae. Overall efficiency of each test was also calculated.

RESULTS

A total of 945 urine samples for culture received at Combined Military Hospital Lahore, during a period of February 2016 to July 2016 were included in this study.

Out of 945 urine samples, pure growth was obtained in 177 samples ,713 samples yielded no growth and 55 samples yielded growth of mixed organisms, later put into the category of repeat sample. Among these 177 samples, E coli were isolated in 70 (table :1). From the susceptibility testing of 70 E coli, ESBLs were confirmed in 34 samples as per DDST and MDDST. These isolates were then confirmed for the production of ESBLs by Phenotypic confirmatory disc diffusion method (PCDDT) as per CLSI criteria.

Out of these 34 ESBLs, 14 had been screened positive for Amp C beta lactamases also. 04 Isolate showed Carbapenems resistance, so in short, all three enzymes were detected (ESBL + Amp C + Carbapenemase) through single plate detection technique.
Comparison of different phenotypic methods for the detection of extended spectrum beta lactamases in *Escherichia coli* and in Amp C co-producing phenotypes through single plate detection technique

DDST detected ESBL in 12/34 isolates. MDDST detected the same in 33/34 isolates, including 14 isolates having Amp C enzyme as well (co-producers). 03 isolates through Disc approximation techniques using Imipenem & Ceftazidime found to have inducible/Plasmid mediated AmpC activity. (table:2)

The ability to detect ESBLs by these methods against PCDDT as gold standard was as follows; MDDST technique had an overall sensitivity and specificity of 97.05% and 100%, while positive predictive value (PPV) and negative predictive value (NPV) were found to be 100% and 97.29% respectively. Contrarily DDST had shown an overall sensitivity and specificity of 35.29% and 100% with PPV and NPV of 100% and 75%.

Overall efficiency of MDDST was 98.75% and DDST was 68.75% respectively.

Figure-1: Single plate detection technique.

Showing positive double disk synergy test for ESBL giving synergy between Amoxicillin-clavulanic acid (AMC) & Ceftazidime (CAZ). Positive modified double disk synergy test for ESBL showing synergy between Cefipime (FEP) & Piperacillin-tazobactam (TZP). Positive Amp C screening by exhibiting resistance to Cefoxitin (FOX) zone size < 18mm.

Figure-2: Single Plate Detection Technique (in isolate producing Amp C and ESBL).

**Positive Amp C screening test** showing resistance to Cefoxitin (FOX), Ceftazidime (CAZ), Ceftriaxone (CRO), and AMC Positive modified double disk synergy test for ESBL showing synergy between Cefipime (FEP) & Piperacillin-tazobactam (TZP).

<table>
<thead>
<tr>
<th>Table-1: Percentage of Organisms isolated from Urine samples for C/S.</th>
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<tbody>
<tr>
<td><strong>Organism isolated (n=177)</strong></td>
</tr>
<tr>
<td>Staph saprophyticus</td>
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<tr>
<td>Klebsiella oxytoca</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Acinetobacter jhonsonii</td>
</tr>
<tr>
<td>E. coli</td>
</tr>
<tr>
<td><strong>Candida species</strong></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
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<tr>
<td><strong>Enterococcus fecalis</strong></td>
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<tr>
<td>Enterococcus facium</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
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</tbody>
</table>
Table-2: Percentage detection of ESBL producing E coli by different methods n= 34, Prevalance (48.75%)

<table>
<thead>
<tr>
<th>ESBL Detection Methods</th>
<th>Percentage detection of ESBL</th>
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</thead>
<tbody>
<tr>
<td>PCDDST</td>
<td>34 (100) %</td>
</tr>
<tr>
<td>DDST</td>
<td>12 (35.29) %</td>
</tr>
<tr>
<td>MDDST</td>
<td>33 (97.05) %</td>
</tr>
<tr>
<td>MDDST+DDST</td>
<td>11 (32.35) %</td>
</tr>
<tr>
<td>Fox screening disk for AMP C</td>
<td>14</td>
</tr>
</tbody>
</table>

DISCUSSION

Escherichia coli has emerged as the main etiologic agent causing Urinary tract infections (UTIs) with an estimated global incidence of at least 150 million cases per annum[14]. Misuse or over use of antimicrobial agents have invariably resulted in the development of antibiotic resistance in the form of ESBL production by most of these isolates, which in recent years, has become a major public health problem worldwide [15].

In our study, only 34 E coli were detected to be ESBL producers. The prevalence of ESBLs among the clinical isolates varies worldwide and are rapidly changing over time [16]. Prevalence of ESBL production amongst our isolates found out to be 48.75%, while various studies like Arindam Chakraborty et.al prevalence was 70.5% [17], Kateregga JN et al it was 70.7% [18], with highest prevalence rate 89.8% given by Mohammad Soltan Dallal et.al from Iran [19]. Decreased prevalence rate of ESBL in our study as compared to above mentioned studies attributed to educated patient stream who do not believe in misuse of drugs and strict antibiotic policy of the hospital, restricting over the counter availability of drugs. Another factor is easy access of microbiological set up, convincing clinicians to seek microbiologist advice first, through culture and sensitivity before starting antibiotics.

In our study E coli (39.5%) identified to be the commonest isolate in urine samples, Candida (21.4%) and Enterococcus fecalis (11.86%) secured subsequent positions respectively. Amongst all the organisms Gram negative bacilli proved to be frequent culprits (table 1). A 6 years study done by Michael Osthoff, 10974 (72.5%) of the total isolates were identified as E coli [20]. Review article by Lindsay E Nicolle showed that E coli proved to be the most frequently isolated Species to catheter-acquired urinary tract infection related bacteremia [21]. Another study by Augusti Kako Gomes, reported prevalence of E coli in urine samples to be 72% [22].

Evaluation of various methods had been done for optimum detection of ESBL in E coli. MDDST was identified as most sensitive and specific method by detecting ESBL in co-producers also. likewise, DDST, using Amoxicilav as inhibitor of ESBL, showed decrease sensitivity. This was also shown by the study done by Jaspal Kaur et al [23]. He explained reason of decreased sensitivity of DDST to strains producing both the enzymes (ESBL + Amp C), thus masking ESBL detection. Decreased sensitivity of DDST in my study was attributed to same co-producing strains. Second reason is Clavulanic acid which was used in the standard tests for ESBL detection (PCDDT & DDST) itself act as inducer of high level AmpC production. Thus, resulting in false negative result despite the presence of ESBL [24]. In MDDST, Tazobactam was less likely to induce AmpC B-lactamase so, simultaneous Amp C induction did not occur in co-producers. Piperacillin-tazobactam remained sensitive in susceptibility testing and synergy could be seen between PTZ and 4th generation cephalosporins (FEP) [24]. MDDST had identified 33/34 (97.05%) ESBL positive isolates. A study of MKR Khan et al on co-producers had similar findings, in which DDST was positive in 25/40 isolates, MDDST was positive in 40/40 isolates, PCDDT was positive in 39/40 isolates [11]. A study carried out by
Modi Dhara et al at Ahmadabad in 2012 had shown that DDST had identified 36/44 (81.81%) and MDDST had identified 44/44 (100%) which was well correlated with our study [7]. Thus, suggesting that PCDDT and DDST should be used only in ESBL producing isolates but not useful for detection of ESBL in isolates co-producing AmpC enzyme, like *Enterobacter*, *Serratia*, *Citrobacter* and now with *E. coli* and *Klebsiella species* also.

The presence of AmpC enzyme in 14 isolates requires confirmation by Modified three-dimensional test (M3DT) using 10μg Cefoxitin disc as screening method. one of the study appreciating M3DT as the most sensitive phenotypic method to identify Amp C in ESBL co-producer isolates among all other test. As M3DT was out of scope it was not performed on Amp C positive isolates, but in future test will be performed to correlate the results.

CONCLUSION

Today, the significant challenge for diagnostic laboratories is the detection of extended-spectrum β-lactamases in the presence of AmpC β-lactamase enzymes. Modification of double disc synergy test that combines Piperacillin-tazobactum with Cefepime not only enhances the possibility of ESBL detection in co-producers but gives a significant room to other antibiotic discs to be applied on the same plate for the detection of other enzymes as well.

AUTHORS CONTRIBUTION

Qanita Fahim: Entire research work, write up.
Fatima Hameed: Helped in data collection.
Ayesha Khalid: Helped in sample analysis
Muhammad Saeed Anwar: Overall supervision

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