Rapid detection of extended spectrum β-lactamases production in *Escherichia coli*

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**ABSTRACT**

Multidrug resistance is a major threat causing a wide range of infections worldwide. The Enterobacteriaceae family is held responsible for many cases of antibiotic resistance, mainly by the means of extended spectrum β-lactamases (ESBLs) production. The modified double disc synergy test (MDDST) is a reliable method for ESBL detection with several drawbacks. The ESBL Nordmann-Dortet-Poirel (NDP) test can be used as a sensitive, specific and rapid alternative method for ESBL detection. This study was designed to evaluate the performance of NDP test for rapid detection of ESBL-producing *Escherichia coli* in comparison with MDDST. The research was carried out on 50 clinical *E. coli* and 25 fecal *E. coli* isolates from healthy controls. The isolates were tested for ESBL production by double disc diffusion test (DDT), MDDST and NDP; comparing the sensitivity and specificity of the three tests. Statistical Package for the Social Sciences (SPSS) version 22 was used for data analyses. P>0.05 was regarded as non-significant, P≤0.05 significant and P≤0.01 highly significant. It was observed that the sensitivity of NDP test was 100% while MDDST’s sensitivity was 83.3%. The specificities of both NDP test and MDDST were 100%, respectively. MDDST yielded results after 3 days while NDP test gave results after 20-45 min. Therefore, NDP test was more rapid and more sensitive than MDDST as regards detection of ESBL producing *E. coli*. It can therefore be used for the detection of ESBL production instead of MDDST as it gave higher positive results than the MDDST and also bypassed the errors produced by unique inhibitor-resistant beta-lactamase.

INTRODUCTION

Multidrug resistance is now emerging worldwide at an alarming rate among Gram negative bacteria, causing both community-acquired and nosocomial infections (Schwaber et al., 2006). One of the most important emerging resistance traits in Enterobacteriaceae family members corresponds to resistance to broad-spectrum β-lactam antibiotics, which is mainly associated with production of extended-spectrum β-lactamase (ESBL) enzymes (Pitout and Laupland, 2008; Coque et al., 2008). ESBLs had emerged by gene mutations that changed the configuration of amino acids around the active site of these β-lactamases. The first ESBL was discovered in 1983 and it was plasmid-encoded (Knothe et al., 1983).

ESBLs are typically plasmid-mediated enzymes that hydrolyze the penicillins, the third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) and the monobactam (aztreonam). They are not active against the cephams (cephoxitin and cefotetan) and carbapenems (imipenem, meropenem) but are susceptible to β-lactamase inhibitors such as clavulanic acid and tazobactam (Pfaller and Segreti, 2006).

Enterobacteriaceae, especially ESBL-producing
Klebsiella, have become a major cause of nosocomial infections since the 1980s. However, during the late 1990s, several community-acquired pathogens have also been found to be ESBL producers, mostly causing urinary tract infections (UTIs) and diarrhea. Knowledge of the antibiotic resistance prevalence is an essential cornerstone for infection control and healthcare policy makers. Currently, a well-known surveillance system is only present in the United States, European Union and Thailand (Soltani et al., 2016).

The ESBL-producing E. coli is now one of the most multi-antibiotic resistant strains emerging worldwide (Piccoli et al., 2013). A variety of ESBLs, mostly of the genotypes CTX-M, TEM and SHV types, have been reported in members of Enterobacteriaceae family, and are often undetectable by the current isolation and susceptibility methods (Nordmann et al., 2012).

Current techniques for detecting ESBL producers are based on the determination of susceptibility to broad-spectrum cephalosporins followed by inhibition of the ESBL activity, mostly by clavulanic acid or tazobactam (Rawat and Nair, 2010). The double-disk synergy test and the E-test were proposed for that purpose with 80 to 90% sensitivities and specificities (Gazin et al., 2012). The previous methods require overnight growth, meaning that up to 24 to 48 h, can elapse before ESBL production is detected once the isolate had grown a delay in the initiation of appropriate antibiotic therapy (Drieux et al., 2008). Molecular detection of ESBL genes by PCR, hybridization, and sequencing is an alternative but remains costly and requires a certain degree of expertise that is not accessible to non-specialized laboratories (Tuine et al., 2014).

Recently, a rapid and cost effective biochemical test was developed for the detection of ESBL producers, namely ESBL-NDP test. The ESBL-NDP test is particularly effective for detecting CTX-M producers that currently account for most of the ESBLs identified worldwide but not the TEM and SHV series due to weak hydrolysis of cefotaxime or from low level production of the ESBL related to low MIC values of cefotaxime (CLSI, 2014). This test is based on a technique designed to identify the hydrolysis of the β-lactam ring of a cephalosporin (cefotaxime), which generates a carboxyl group, by acidifying a culture medium. The acidity resulting from this hydrolysis is identified by the color change generated using a pH indicator such as phenol red (CLSI, 2014).

This research was aimed to evaluate the performance of NDP test for rapid detection of ESBL-producing E. coli in comparison with MDDT.

MATERIALS AND METHODS

The study was conducted on 75 E. coli isolates. Fifty (50) isolates of E. coli, isolated from different clinical specimens (24 urine samples isolates, 17 infected wounds isolates, 6 blood culture isolates, 2 sputum sample isolates and 1 Ascetic fluid aspirate isolate). They were obtained from the Central Laboratories of Ain Shams University hospitals. Twenty-five (25) E. coli isolates were also obtained from fecal specimens of healthy adult individual. The study was conducted over a period of 6 months from January 2015 till June 2015. An informed consent was obtained from each of the participants before recruitment in the study. All the study was done in the Microbiology Laboratory of the Faculty of Medicine, Ain Shams University. Lyophilized standard bacterial strains obtained from (Microbiologics, USA) E. coli ATCC 25922 and Klebsiella pneumoniae ATCC 700603 were used as a negative and positive control for ESBL production, respectively (Patricia, 2013).

Stool specimens were collected in clean disposable plastic containers and spread on MacConkey’s agar plates in the conventional manner. The plates were incubated at 37°C overnight. The rose pink colonies suspected to be E. coli were then inoculated onto nutrient agar plates and incubated at 37°C overnight. The colonies then were identified as E. coli using Gram-stained films and conventional biochemical tests; Triple sugar Iron (TSI) agar medium, Indole production test, Methyl Red tests and citrate utilization test (Kaur et al., 2013), supplied by (Oxoid, UK). While the other fifty E. coli strains isolated from clinical samples were inoculated onto MacConkey’s agar plates and identified as previously illustrated. All the 75 E. coli strains were subjected to the following tests.

Disc diffusion test (DDT) (Patricia, 2013)

A direct colony suspension method was used to make a suspension of the tested bacteria, as well as the standard strains, in saline to the density of a McFarland 0.5 turbidity standard. The antibiotic discs (cefotaxime, ceftriaxone and ceftazidime antibiotic discs with concentration of 30 µg for each) were placed on the surface of the inoculated plates using a sterile forceps, leaving about 10-15 mm away from the edge of the Petri dish, and the discs separated from each other by a distance not less than 20 mm to avoid overlapping zones of inhibition. The plates were then incubated at 37°C for 18-24 h. Zones of growth inhibition around the antibiotic disks were measured to the nearest mms and compared with those of E. coli ATCC 25922 and K. pneumoniae ATCC 700603.

Modified double disk synergy test (MDDST) (Dortet et al., 2015)

All E. coli isolates which showed resistance to cefotaxime...
Patterns of synergism of 3rd generation Cephalosporin and 4th generation Cephalosporin with Amoxicillin-clavulanate produced on testing the 49 possible ESBL producing E. coli isolates by MDDST was studied showing, no synergism of 3rd generation Cephalosporin (3GC) and 4th generation Cephalosporin (4GC) with Amoxicillin Clavulanate (AMC) in 24 isolates out of 49 (possible ESBL producers in both clinical and fecal E. coli isolates) (49%), synergism of both 3GC and 4GC with AMC in 10 isolates out of 49 (20%), and synergism of 3GC only with AMC in 15 isolates out of 49 (31%) as shown in Table 3 and Figure 4.
Figure 1. Positive Norman-dortet-poirel test (yellow color).

Figure 2. Negative Norman-dortet-poirel test (red color).

Figure 3. Disc diffusion test by Kirby Bauer method.
Table 1. Frequency of possible extended spectrum β-lactamase producers among *E. coli* isolates detected by disc diffusion test.

<table>
<thead>
<tr>
<th>Total No. of clinical <em>E. coli</em> isolates</th>
<th>Possible ESBL producer (≤20 mm for CTX, ≤19mm for CTR)</th>
<th>Not a possible ESBL producer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of individuals</td>
<td>Percentage (%)</td>
</tr>
<tr>
<td>50 (clinical isolates)</td>
<td>37</td>
<td>74</td>
</tr>
<tr>
<td>25 (fecal isolates from healthy individuals)</td>
<td>12</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 2. Comparison between the results of modified double disc synergy test and Norman-dortet-poirel test as regard extended spectrum β-lactamase detection in *E. coli* isolates.

<table>
<thead>
<tr>
<th>Total No. of <em>E. coli</em> isolates</th>
<th>MDDST</th>
<th>NDP test</th>
<th>Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ESBL +ve</td>
<td>ESBL -ve</td>
<td>ESBL +ve</td>
</tr>
<tr>
<td>25 (fecal isolates from healthy individuals)</td>
<td>9 (36%)</td>
<td>16 (64%)</td>
<td>10 (40%)</td>
</tr>
<tr>
<td>50 (clinical isolates)</td>
<td>16 (32%)</td>
<td>34 (68%)</td>
<td>20 (40%)</td>
</tr>
</tbody>
</table>

Tazobactam was added to inhibit the ESBL production to all the tested isolates showing 100% inhibition to all ESBL producers detected by NDP test with highly significant P-value = 0.000 as shown in Table 4. A comparison was made between MDDST and NDP tests and NDP test showed higher sensitivity (100%) and shorter time for ESBL detection as shown in Table 5.

DISCUSSION

The infections that ESBL-producing *E. coli* can cause range from urinary tract infections, to cases where they enter the bloodstream and cause bacteremia and sepsis. Infections with ESBL-producing *E. coli* are most common amongst the elderly, or those who have recently been in hospital or received antibiotic treatment. In a worldwide survey conducted in 2004, 10% of the *E. coli* strains were found to be ESBL producers (Rossi et al., 2006). Patients with an infection caused by ESBL-producing bacteria are at risk for therapeutic failure or even death because there is often a delay before the correct antibiotic treatment is given (Paterson et al., 2000). ESBLs are usually plasmid-encoded and frequently coexist with the resistance to other antibiotic family, mainly the fluoroquinolones (93%) and the aminoglycosides (78%) (Parveen et al., 2010). This significant resistance to the non β-lactam antibiotics widely limits the treatment choices to the carbapenems as a last resort, contributing to the emergence of carbapenem-resistant bacterial strains overtime (Al-Zarouni et al., 2008). In this study the frequency of ESBL positive *E. coli* isolated from pathological specimens screened by DDT and confirmed by MDDST was 32% and 40% by NDPT. Comparatively, in a study conducted in UAE, the frequency of ESBL +ve *E. coli* isolated from pathological specimens screened by DDT and confirmed by BD Phoniex ESBL system and cephalosporin/clavulante combination was 38.5% (Ashrafian et al., 2015). In Iran, the frequency of ESBL +ve detected by DDT among *E. coli* isolated from clinical specimens obtained from inpatients and outpatients was 26.5% (Tham et al., 2103). In Southern Sweden, 5.3% of *E. coli* isolated from different pathological samples gave positive ESBL results when tested by Chrome IDTM ESBL (Bouchillon et al., 2004). The frequency of ESBL producing bacteria in Greece, Portugal, Germany and Netherland was 27.4, 15.5, 2.6 and 2% respectively (Kaur et al., 2013). Also, Soltani et al. (2016) reported that resistance rates of *E. coli* and *K. pneumonia* to beta-lactam antibiotics were parallel to ESBL production rates. The variations in the above results may be attributed to differences in time of collection of specimens, differences in tests used for detection of ESBLs and differences in study population, design and number of cases (Hawkey, 2008).

In this study, the frequency of ESBL detection among *E. coli* isolated from the stools of normal persons was 36% by MDDST and 40% by NDPT, respectively. The ESBL fecal *E. coli* carriage was 10% in Asia (Valverde et al., 2004), 5.5% in Spain (Paterson and Bonomo, 2005) and 17.7% in Saudi Arabia (Kader et al., 2007). The high frequency of ESBLs producing *E. coli* in certain areas can be due to the selective pressure created by the frequent use and over use of the 3rd generation cephalosporins (Dhara et al., 2012).

The MDDST was first used to bypass the errors resulting from the use of the original DDST, which depends on the use of 3rd generation cephalosporine alone. The use of a 4th generation cephalosporine (cefepeime) in the MDDST had largely helped in detecting...
Figure 4. Modified double disc synergy test showing distortion towards the disc of amoxicillin-clavulanate (synergism).

Table 3. Different patterns of synergism of 3rd generation Cephalosporin and 4th generation Cephalosporin with Amoxicillin Clavulanate produced on testing 49 possible ESBL producing E. coli isolates by modified double disc synergy test.

<table>
<thead>
<tr>
<th>Pattern of synergism</th>
<th>Number</th>
<th>Percentage (%)</th>
<th>Cause of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No synergism of 3GC and 4GC with AMC</td>
<td>24</td>
<td>49</td>
<td>Non-ESBLs</td>
</tr>
<tr>
<td>Synergism of both 3GC and 4GC with AMC</td>
<td>10</td>
<td>20</td>
<td>ESBLs</td>
</tr>
<tr>
<td>Synergism of 3GC only with AMC</td>
<td>15</td>
<td>31</td>
<td>ESBLs</td>
</tr>
</tbody>
</table>

Table 4. Effect of tazobactam on extended spectrum β-lactamases production.

<table>
<thead>
<tr>
<th>Total number of E. coli isolates</th>
<th>No. of ESBL +ve by NDP</th>
<th>No. of ESBL +ve by NDP inhibited by tazobactam</th>
<th>Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>30</td>
<td>30 (100%)</td>
<td>22.5</td>
</tr>
</tbody>
</table>

Table 5. Comparison between sensitivity, specificity and time factor of modified double disc synergy test and Norman-dortet-poiirel test.

<table>
<thead>
<tr>
<th></th>
<th>MDDST</th>
<th>NDPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of true ESBL +ve</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Number of false ESBL +ve</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of true ESBL -ve</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Number of false ESBL -ve</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>83.3%</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Positive predictive value (PPV)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Negative predictive value (NPV)</td>
<td>90%</td>
<td>100%</td>
</tr>
<tr>
<td>Time elapsed to show results</td>
<td>Average 72 h</td>
<td>Average 20-45 min</td>
</tr>
</tbody>
</table>
additional ESBL-producing bacteria that secrete both ESBLs and AmpC β-lactamase not detected by the DDST (Tsakris et al., 2009), yet it has some drawbacks as it takes plenty of time to show the results, the strains that showed possibility for ESBL production on DDT had to be subjected for testing on MDDST to confirm ESBL production (Kaur et al., 2013).

This study shows that NDP test gives higher positive ESBL results than ESBL confirmatory MDDST. This can be explained by the presence of inhibitor–resistant ESBL variants (Jacoby, 1997), or by the expression of TEM-1 or SH 1 β-lactamas es in association with alteration in the outer membrane permeability (CLSI, 2012).

The NDP test has multiple advantages over the MDDST in detection of ESBL production. This test is rapid as it can be used to search for ESBL producers among bacterial colonies isolated prior to any antibiotic susceptibility testing (Schwaber and Carmeli, 2007) or even directly from different pathological specimens. This would greatly save time as bacterial isolation is not a necessity to perform this test (yet the protocol of NDP test differs in this case). It usually takes around 20-45 min for NDP test to show a result after incubation of cefotaxime-phenol red-bacterial suspension mixture. The NDP test is easy to prepare with cheap in-hand kits, making it a better alternative for ESBL detection in small laboratories (Nordmann et al., 2012).

It is also observed that the sensitivity of NDP test was 100% in comparison to MDDST which had a sensitivity of 83.3%. This was proven by comparing the results of the standard control positive and negative ESBL-producing strains using the NDP test and the MDDST and the inhibition of the NDP reaction in all the ESBL-positive strains, including the five strains which were positive by NDP test but negative by MDDST, using tazobactam.

Isolation, identification and antimicrobial susceptibility of pathogens is crucial in restricting the antimicrobial use. So it is important to implement an antibiotic policy in everyday clinical practice to prevent selective pressure and the further development of resistance in these pathogens (Poorabbas et al., 2015).

Conclusion

The ESBL NDP test can detect both ESBLs that are inactivated or not inactivated by beta lactamase inhibitors. NDP test is more rapid and more sensitive than MDDST as regard detection of ESBL producing bacteria.

RECOMMENDATIONS

NDP test can be used instead of the MDDST for confirmation of the detection of ESBL production as it gives higher positive results than the MDDST and bypass the errors produced by unique inhibitor-resistant beta-lactamase in a short time (20-45 min). This may have an important value for prevention of outbreaks, particularly in high-risk units (that is, intensive care units, etc.). In addition, the ESBL NDP test used as a screening test is much cheaper than other phenotypic methods.

REFERENCES


