Comparison of Two Phenotypic Double Disc Diffusion Test (DDDT) for Detection of Extended Spectrum β -Lactamase Production by *Enterobacteriaceae* and *Pseudomonas* Species at a Tertiary Care Hospital in Bangladesh

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Abstract Background: The detection of extended-spectrum β -lactamase-producing (ESBL) bacteria is of importance for infection control and epidemiological surveillance. Objective: The purpose of the present study was to compare two phenotypic methods for the detection of ESBL-positive *Enterobacteriaceae* and *Pseudomonas* species. Methodology: This cross sectional study was carried out in the Department of Microbiology at Mymensingh Medical College, Bangladesh from January 2011 to June 2011. Patients of all ages and genders presented with UTI or wound infection were taken as study population. Gram negative bacilli (GNB) were analyzed by two methods used for routine susceptibility testing which were Disk diffusion methods and MIC reduction methods. Two methods designed for the detection of ESBL production Ceftazidime and Ceftazidime plus Clavulanic acid, (CAZ/CAZC) and Cefotaxime and Cefotaxime plus Clavulanic Acid (CTX/CTXC) were used and the PCR was considered as gold standard for evaluation of the other test methods. Result: A total number of 300 GNB were isolated and identified of which 214(71%) were ESBL positive. For the disk diffusion method, resistant to third generation Cephalosporins were the highest 87.0%, when tested by ceftazidime and by MIC reduction methods were 67%. For the phenotypic confirmatory methods, specificities were 64% by (CAZ/CAZC), 59% by (CTX/CTXC) and by both method 52%. Among the phenotypic confirmatory ESBL positive strains by Genotypic method ESBL positive were 50.46% TEM, 18.69 % SHV and 46.72% CTX-M gene. Conclusion: Two-step strategies using both DDDT phenotypic methods are useful diagnostic tools for the detection of ESBL from

the Gram negative bacilli.

Keywords Extended-Spectrum B-Lactamase-Producing, ESBL, Phenotypic Detection, Double Disc Diffusion Test

1. Introduction

Antibiotic resistance has been emerged worldwide. It has been reported that the overuse of third generation of cephalosporins has been associated with the emergence of β -Lactamases¹. These enzymes have serine at their active site and attack the amide bond in the β lactam ring of antibiotics². ESBLs are enzymes that mediate resistance to extended spectrum cephalosporins as well as monobactams³.

There are no definite guidelines for the detection of extended-spectrum beta-lactamase (ESBL) among Gram negative bacilli especially *Enterobacteriaceae* as well as *Pseudomonas* species. Though these species may cause hospital associated infection (HAI)⁴⁻⁶ these are developed frequently multidrug resistant (MDR)⁵⁻⁶. These bacteria may constitute a reservoir for plasmid-mediated ESBLs transmission for other *Enterobacteriaceae* species⁷⁻⁹. Phenotypic detection of ESBLs in members of the *Enterobacteriaceae* co-expressing an AmpC beta-lactamase is complex as because AmpC expression may mask the synergy required for ESBL detection between third-generation cephalosporin and clavulanic acid. This problem may be circumvented by detection of ESBL by molecular technique with specific primer¹⁰. Most guidelines

recommend screening isolates based on decreased susceptibility to extended-spectrum cephalosporin in primary susceptibility testing followed by confirmation of ESBL production. However, still there is a controversy about the confirmation of ESBL¹¹. Therefore, detection of ESBL-producing organisms has become a concern for general hospitals and private practice laboratories. The current changes in clinical MIC breakpoints for extended-spectrum cephalosporins and for aztreonam against Enterobacteriaceae by EUCAST and CLSI decrease the likelihood of interpreting an ESBL-producing Enterobacteriaceae as susceptible to extended-spectrum cephalosporins¹²⁻¹⁴. Several phenotypic methods have been developed to confirm ESBL production by Enterobacteriaceae¹⁵⁻¹⁷. Therefore the purpose of the present study was to compare two phenotypic methods for the detection of ESBL-positive Enterobacteriaceae and *Pseudomonas* species.

2. Methodology

This cross sectional study was carried out in the Department of Microbiology at Mymensingh Medical College, Mymensingh, Bangladesh from January 2011 to June 2011. Mymensingh is 200 KM away from the capital of Bangladesh which is the largest district of this country and Mymensingh Medical College is the only teaching hospital with more than 500 beds with emergency as well as OPD facilities. Patients presented with UTI or wound infection were at any age with both sexes were taken as study population. Non-repetitive clinical isolates were collected from MMCH both the outpatients and inpatients department over a period of 6 months. Urine and wound infection were used as specimen. Laboratory work was carried out in the department of Microbiology in Mymensingh Medical College, Bangladesh. Specimens were collected aseptically¹⁸. All samples were routinely cultured on MacConkey and blood agar plates at 37°C aerobically for 18 hours. Gram negative isolates were further characterized by standard biochemical tests¹⁹. The susceptibility to antibiotics was determined by Kirby Bauer method according to CLSI 2010 protocols for Gram negative panels²⁰. *E. coli* ATCC 25922 was used as control strains. Three diagnostic tests for ESBL production were performed which were screening test for ESBL by disc diffusion test (DDT) and MIC reduction method, confirmatory test by double disc diffusion test (DDDT) and multiplex PCR for TEM, SHV and CTX-M genes. Isolates were screened for ESBL production by using disc diffusion test on Muller Hinton agar according to the CLSI²¹ recommendations where isolates showing inhibition zone size of ≥ 22 mm with ceftazidime (30 μ g), ≥ 25 mm with ceftriaxone (30 μ g), ≥ 27 mm with cefotaxime (30 μ g), ≥ 27 mm with Aztreonam (30 μ g) were suspected for ESBL production. MIC reduction method $\geq 8\mu$ gm/ml with

Ceftazidime was performed. *E. coli* ATCC 25922 was used as a negative control. Phenotypic confirmatory test for ESBL producers were done by double disc diffusion test (DDDT) by two methods which were ceftazidime and Ceftazidime plus Clavulanic acid (CAZ/CAZC) and Cefotaxime and Cefotaxime plus Clavulanic Acid (CTX/CTXC). Plasmid DNA was isolated from bacterial cells by alkaline lysis method by Medici *et al*²². Supernatant 1 μ l was used as template. The PCR primers and cycling conditions used were previously described²³⁻²⁵. Primers used for TEM gene were TEM F, TEM R, SHV gene were SHV F, SHV R, CTX-M gene were CTX-MU-1, CTX-MU-2. Amplification was performed in an Thermocycler (Eppendorf, Takara, Japan) in 1.0% agarose gel and visualized by staining with ethidium bromide (0.5 mg/ml) running through horizontal electrophoresis. A 100 bp ladder molecular weight marker (Roche, USA) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system (AlphaImager™ 3400, USA) by UV light. The qualitative data were expressed as frequency and percentage. The comparisons of qualitative data were performed by Chi-square test if data were normally distributed. Statistical analysis was performed by using window based computer software devised with Statistical Packages for Social Sciences (SPSS-17) (SPSS Inc, Chicago, IL, USA). 95% confidence limit was taken. The summarized data was interpreted accordingly and was then presented in the form of tables. P value less than 0.05 was taken as level of significance.

3. Result

A total of 300 bacterial isolates were yielded. The specimens were urine 216(72%), wound swab 45(15 %) pus 39(13%). Out of 300 Gram negative isolates in this study majority were *Escherichia coli* (52%) followed by *Proteus* species (18.3%), *Klebsiella* species (15%), *Pseudomonas* species (3%) and others (11.7%). Among 300 GNB 261(87%) were resistant to Ceftazidime, 242(81%) were resistant to Ceftriaxone, 191(64%) were resistant to Cefotaxime and 100% were in aztreonam (Table 1). In MIC reduction method ($\geq 8\mu$ gm/ml) Ceftazidime was resistant in 67% cases. Most of the bacteria showed ESBL positive by both combination (CAZ/CAZC, and CTX/CTXC). *Escherichia coli* and *Klebsiella* species showed maximum ESBLs production in CAZ/CAZC combination. Both the CAZ/CAZC and CTX/CTXC methods were statistically significant. The distribution of ESBLs gene in different isolates rate of TEM, SHV and CTX-M genes present in study population were 50.46%, 18.69% and 46.72% respectively.

Table 1. Third generation Cephalosporin Resistant Isolates

Name of antibiotic	Frequency	Percentage
Ceftazidime	261	87
Ceftriaxone	242	81
Cefotaxime	191	64
Aztrionam	300	100
MIC (≥ 8 μ gm)	202	67

Table 2. Detection of ESBL by Double Disc Diffusion test as confirmatory test

Bacteria Name	CAZ/CAZC	CTX/CTXC	Both
<i>Esch. coli</i> n=105	23 (21.5)	13 (12.3)	69 (65.7)
<i>Klebsiella spp.</i> n=36	5 (13.8)	3 (8.3)	28 (77.7)
<i>Proteus spp.</i> n=40	5 (12.5)	2 (5.0)	33 (82.5)
<i>Pseudomonas spp.</i> n=8	1 (12.5)	1 (12.5)	6 (75.0)
*Others n=25	4 (16.0)	2 (8.0)	19 (76.0)
Total n = 214	38 (17.7)	21 (9.8)	155 (72.4)

*Others = *Enterobacter spp.*, *Citrobacter spp.*; Figure within the parenthesis indicates the percentage.

Table 3. Cross tabulation between CAZ/CAZC and CTX/CTXC of ESBL detection

ESBL	CAZ/CAZC	Combined	CTX/CTXC
Positive	193	155	176
Negative	21	59	38
Total	214	214	214

* χ^2 value 22.1 at df 3, $p < 0.001$, highly significant; χ^2 value 5.8 at df 3, $p < 0.01$, highly significant; Note: Figures in parentheses represent percentage; Others - *Enterobacter spp.*, *Citrobacter spp.*

Table 4. Pattern of distribution of TEM, SHV and CTX-M genes among phenotypic confirmed ESBL producers

Name of Gene	Frequency	Percentage
TEM	54	50.46
SHV	20	18.69
CTX	50	46.72

4. Discussion

Detection of ESBLs are a great challenge in the clinical microbiology laboratory²¹⁻²⁵. The CLSI guidelines regarding the sensitivity breakpoints designated for *Klebsiella* species and *Escherichia coli* against cefotaxime, ceftriaxone, and ceftazidime are inefficient for the detection of ESBLs²⁶⁻²⁷. Failure to detect ESBL production by routine disc-diffusion tests has been well documented²⁶. Highly informative studies were performed by Tenover *et al*²⁶ in the United States and Livermore *et al*²⁸ in Europe and were reported that ESBL mediated resistance are frequently encountered difficulties with both automated and disk diffusion methods. This might be due to the variable affinity of enzymes for different substrates and inoculum effect²⁹. Furthermore, ESBL-producing organism gives the treatment failure with third generation cephalosporin if the infection is outside the urinary tract²⁷. In addition to that reports of failures in both animal models and clinical settings are well documented when third-generation cephalosporins are used to treat ESBL

infections³⁰⁻³¹. The sensitivity of any particular extended-spectrum cephalosporin used as a screening test can be dependent on the geographic variation and the resistance patterns of these organisms²⁰. More resistant to ceftazidime is reported and SHV-3 like ESBLs has been found which is 100.0% resistant to ceftriaxone; whereas only 50.0% are resistant to ceftazidime³⁰. In this study resistant to Ceftazidime is found in 87.0%. Moland *et al*³¹ has used ceftazidime resistance as marker for ESBL production which is 78.0% in a broth dilution format. Similarly it has been found statistically more significant by these combinations. Though ceftazidime plus clavulanic acid (CAZ/CAZC) is the best single disc diffusion test for the detection of ESBL²⁵, it cannot cover all the strain. Use of only one combination may fail to detect ESBL positive strains; thus this might give underestimation of prevalence of ESBL. In this study both combinations were found statistically significant. The use of cefotaxime, ceftazidime and ceftriaxone as the only indicator of ESBLs screening can no longer be recommended. If only one indicator antibiotic would be used for screening,

cefpodoxime has proven to be the best molecule for screening all types ESBLs producers in clinical sample and this also produces AmpC β -lactamase²³. In this study two combinations with clavulanic acid like CAZ/CAZC and CTX/CTXC are tested and have been found that *E. coli* and *Klebsiella* species showed maximum ESBLs production in CAZ/CAZC combination which correlates with other studies³¹. However, the combination of ceftazidime and cefotaxime still missed two strains producing the SHV-5 and SHV-7 ESBLs³⁰. If the both combination does not use the actual figure may not appear and may show low prevalence rate. These types of discrepancies between susceptibility data and disc diffusion results have increased the need for an improved method of ESBL detection which can be incorporated into the routine susceptibility procedure.

This study should be viewed in the context of several limitations. Firstly, because of constraints of time and budget, this was a single centered with relatively small sample size; therefore, the results cannot be generalized to the whole country. Furthermore, antibiotic history of the patients before and after *in vitro* sensitivity test was not considered and only *in vitro* drug response was taken as the result. PCR-based molecular assays have their own limitations. These need expensive equipment and reagents and expert personnel which are not always available to the diagnostic laboratory. In addition, primers have to be designed within low mutation rate regions and the use of specific primers hinders the identification of novel resistance genes giving false negative results. However, so far this type of study has not been performed in Bangladesh. Further study is needed to correlate between *in vitro* and *in vivo* drug response among the patients infected with ESBLs producing bacteria.

5. Conclusions

In conclusion routine used of both DDDT combinations is useful for the detection of ESBL in clinical samples. This is a better method for the detection of ESBL producing bacteria especially enterobacteriaceae and *Pseudomonas* species.

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