Original Article

Extended-Spectrum Beta-lactamase Producers: Detection for the Diagnostic Laboratory

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Abstract

Background and Objectives: Discovered in 1983, Extended spectrum beta-lactamase (ESBL) producers are still the leading cause of infections in India. Its prompt detection is crucial to the clinical management. The Clinical Laboratory Standards Institute (CLSI) recommends phenotypic screening and confirmatory tests to identify the ESBL producer making it cost and time consuming for the diagnostic laboratory. We compare here the screening and confirmatory tests offering a solution to the CLSI recommendation. **Methods:** Nosocomial isolates *E. coli* (71) and *K. pneumoniae* (25) resistant to cefotaxime and ceftazidime were included. CLSI recommended testing with cefotaxime, ceftazidime and in combination with clavulanic acid by disk diffusion and agar dilution methods were performed. E-test was performed on discrepant results. To determine the genetic relatedness of the organisms, 22 Medical and Surgical ICU isolates were genotyped by PFGE. Dendrogram was constructed using dice co-efficient, UPGMA method with diversity database software. **Results and Conclusions:** Phenotypic screening disk diffusion test versus the confirmatory agar dilution MIC tests with cefotaxime and ceftazidime correlated well with the final ESBL status (kappa 0.852 and 0.905 $P < 0.001$) and (kappa 0.911 and 0.822 $P < 0.001$). The tests show 99-100% sensitivity, 75-83.3% specificity, and positive likelihood ratios between 4.0 -5.9. E-test confirmed 6 of 12 discordant results as ESBLs. Of the 96 nosocomial isolates screened as possible ESBL producers by the Kirby-Bauer disk diffusion test, 86.5% were confirmed ESBL producers. Genotyping on the ICU isolates by PFGE revealed a genetically diverse population suggesting no transmission of phenotypically similar ESBL strains within the ICUs.

Keywords: Extended-spectrum beta-lactamase, laboratory detection of extended-spectrum beta-lactamase, nosocomial extended-spectrum beta-lactamase

Introduction

The war against drug-resistant microbes has been an escalating problem worldwide since the introduction of the first antibiotic in the 1940s.[1] Plasmid-mediated beta-lactamase producing Gram-negative bacilli was discovered in Greece in the 1960s.[2] In 1983, plasmid-mediated beta-lactamases capable of hydrolyzing the third generation cephalosporins, known as the extended-spectrum drugs, were discovered. These enzymes are referred to as extended-spectrum beta-lactamases (ESBLs).[3,4] They confer resistance to most beta-lactam antibiotics including the third generation cephalosporins and monobactam antibiotics sparing the cephamycins.[3,5-8] Infections with these ESBL-producing organisms have been associated with poor outcomes.[7] Currently, carbapenems constitute the best treatment option for infections caused by such organisms causing invasive site infections.[5]

Access this article online

Quick Response Code:

Website: www.jgid.org

DOI: 10.4103/jgid.jgid_49_17

A concern however is the difficulty of reliably identifying ESBL-producing organisms in many clinical laboratories, making it likely that their prevalence is underestimated and knowledge among clinicians still lacking.[2,5]

Phenotypic detection of ESBLs is based on the resistance they confer to oxyimino-beta-lactam substrates and the ability of a beta-lactamase inhibitor, usually clavulanate, to block this resistance. Other acquired enzymes, notably AmpC-type beta-lactamases that are by plasmid as well as chromosomal genes, can provide oxyimino-beta-lactam resistance but are resistant to inhibition by clavulanate and confer resistance to

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How to cite this article: Sahni RD, Mathai D, Sudarsanam TD, Balaji V, Brahamadathan KN, Jesudasan MV, *et al*. Extended-spectrum beta-lactamase producers: Detection for the diagnostic laboratory. J Global Infect Dis 2018;10:140-6.

cephamycins, which ESBLs do not. The Clinical laboratory Standards Institute (CLSI) recommends screening isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *and Proteus mirabilis* by disc diffusion (DD)[4,5,8,9] and dilution antimicrobial susceptibility tests.[4,5,10] The phenotypic confirmatory tests for ESBL production include cephalosporin/clavulanate combination discs[4,5,8-10] and the broth/agar dilution method demonstrating a synergistic activity between a cephalosporin and a beta-lactamase inhibitor.[2,4,5,11] Other methods of ESBL detection are the double-DD test[4,12,13] the agar supplemented with clavulanate,[4] the disk replacement method,[4,14] and the three-dimensional test.[15] Commercially available methods for ESBL detection include the *E*-test for ESBL:[16] AB Biodisk (Solna, Sweden), chromogenic media, and the automated susceptibility systems.

Nosocomial-resistant bacterial infections are a major focus of concern for infection control programs.[17] Such infections may occur as an outbreak or may become established as a regular occurrence.

Although bacterial resistance as understood currently may be conferred by the presence of either single or multiple mechanisms, the understanding of an ESBL organism and its detection is still poorly understood by many laboratories and clinicians.

The present study looks at the phenotypic and molecular characteristics of ESBL producing nosocomial bacterial infections with a view to gain a wider understanding of easier laboratory diagnosis and policies that may help reduce nosocomial transmission of *E. coli* and *Klebsiella* species. We compare here the sensitivity of the DD screening versus the confirmatory method and Agar dilution method for the detection of an ESBL producer and the E-Test method. We also determine the genetic relatedness of the ESBL-producing strains of *E. coli* and *Klebsiella* spp among samples received from the Medical and Surgical Intensive Care Units' patients in a 6-month period by Pulsed-field gel electrophoresis (PFGE).

Materials and Methods

This prospective diagnostic study was done in the Department of Clinical Microbiology, Christian Medical College, an over 2000-bedded tertiary care teaching hospital in Vellore South India from March 2005 to February 2006. Consecutive isolates of *E. coli* and *Klebsiella* spp isolated from the sources mentioned below which were resistant to both cefotaxime (CTX) (30 μg) and ceftazidime (CZD) (30 μg), i.e., showing zones of inhibition ≤27 mm and ≤22 mm, respectively, were identified in the laboratory in accordance with the CLSI 2005 DD guidelines. Blood culture isolates were identified both retrospectively and prospectively from February 2005 to February 2006 while CSF, exudate, and sterile body fluid isolates were identified prospectively from August 2005 to February 2006. The isolates which met the CDC definition of being nosocomial[18] were selected by patient chart review and were included for the study; these were non-expectorated respiratory samples (Broncho alveolar lavage, tracheal aspirate, suction tip) from patients with nosocomial pneumonias, IV Catheter tips or postoperative nosocomial pyogenic isolates, deep abscess or CSF infections which are nosocomial and blood isolates from patients with any nosocomial infection. We excluded samples from patients in whom the data were insufficient to confirm the isolate as being nosocomial and more than one sample per patient per admission.

Confirming an extended-spectrum beta-lactamase production

The ESBL production among the isolates was performed by the Kirby-Bauer DD method using CTX 30 μg and CZD 30 μg with and without clavulanic acid (4 μg/ml) and confirmed by the Minimum inhibitory concentration (MIC) agar dilution method with the same antimicrobial combinations to demonstrate the effect of clavulanic acid in combination with CTX and CZD (0.25–256 μg/ml/4 μg/mL) and compare the results of those with the DD results.

ESBL positive isolates show an increase of 5 mm zone of inhibition with clavulanic acid as compared to the zone size for CTX and CZD alone and the MIC agar dilution test done with CTX and CZD show a decrease in three or more doubling dilutions with CTX and CZD in combination with clavulanic acid.

The *E*-test was done on all samples showing discrepant results between the screening and confirmatory phenotypic tests (CTX 0.025-16/CTX with clavulanic acid 0.016–1.0 μg/ml and CZD 0.5–32/CZD with clavulanic acid 0.064–4.0 μg/ml).

Test controls ESBL-producing ATCC 700603 *K. peumoniae* and ESBL-nonproducing ATCC 25922 *E. coli* were included in the testing of each batch. Sensitivity, specificity and likelihood ratios were calculated for each method of ESBL detection.

Confirming genetic relatedness among the nosocomial isolates

PFGE method was carried out on all the isolates from the medical and surgical Intensive Care Units (ICUs) over a 6-month period including a random computer selected control group to confirm genetic relatedness among the isolates causing infections in closed spaces. Genomic DNA was isolated and digested with XbaI (New England Biolabs, Beverly, Mass.). PFGE was performed with the CHEF II system (Bio-Rad, Hercules, CA). Analysis of results was performed based on the characteristics of the molecular weight bands generated. Numerical analysis of the PFGE patterns was done by the Dice coefficient method. Dendrogram was constructed by the unweighted pair group method with arithmetic averages. The analysis and construction of dendrogram was done by use of the Diversity database software version 2.2 (Bio-Rad, CA, USA). The study data were analyzed using the SPSS for windows, Version 15, Chicago, SPSS Inc.

Results

The STROBE figure reveals the recruitment and laboratory analysis of the study samples [Figure 1]. We worked with 96 isolates determined as nosocomial isolates. Most patients were under general surgery (28%), Hematology (12.4%), Gastroenterology (11%), and Internal medicine departments (10%). Most of them were admitted in the intensive care units.

The mean age of the patients was 40.7 years (standard deviation [SD] 19.1), ranging from neonates to 82 years old; 65.2% were male. The mean duration of hospital stay was 31.5 days (SD 18). The average duration of stay of all patients admitted to the hospital during this time period was 6.69 days. There were no gender differences for age or duration of stay.

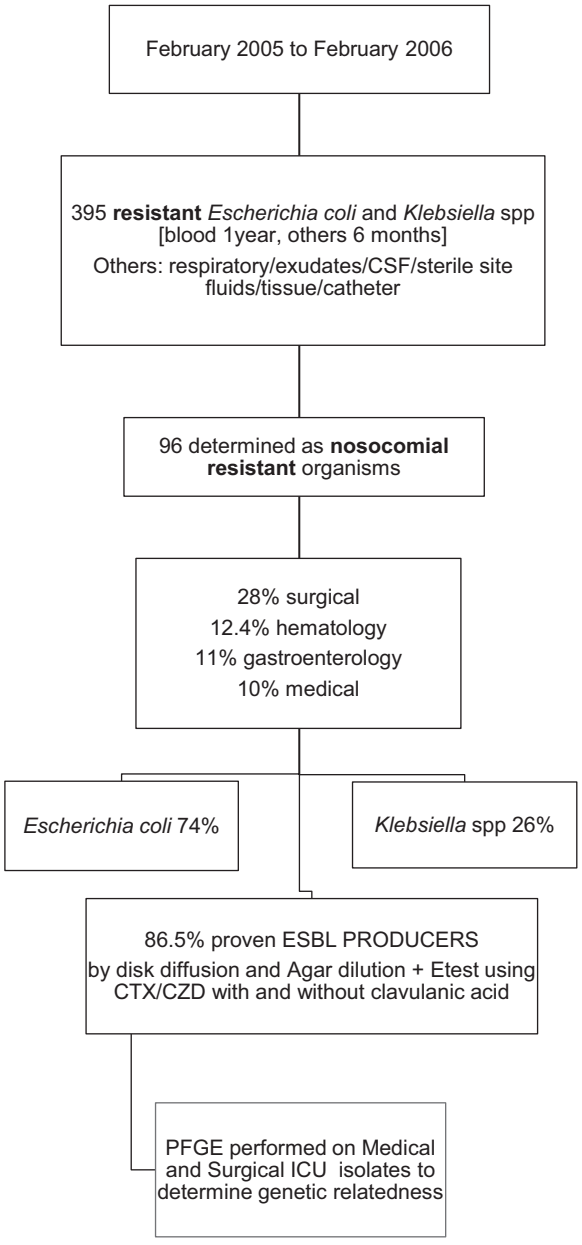

Figure 1: Study flow diagram (STROBE figure)

Of the 62 patients for whom outcome status at discharge was available, 22 had expired (35%); the concomitant overall hospital inpatient mortality was 10%–12%.

The resistance patterns of the nosocomial-resistant *E. coli* and *Klebsiella* spp reveal high-level resistance to ampicillin, 1st and 2nd generation cephalosporins, co-amoxiclav, quinolones, and piperacillin-tazobactam. Carbapenems had near 100% sensitivity with only 2 strains (one *E. coli* and one *Klebsiella* spp strain from the same patient) resistant to meropenem. Overall, beside the carbapenems, amikacin demonstrated higher susceptibility trends over the study period.

MIC_{50} and MIC_{90} values for *E. coli* to CTX were 32 and 64 ug/ml, while for *Klebsiella* spp, the common MIC_{50} and MIC_{90} values are 64 and 128 ug/ml. Correspondingly, the most common MIC values for CZD for *E. coli* and *Klebsiella* spp are 16, 32, and 64 ug/ml.

Susceptibility testing to cefepime done by E test shows high MIC values of MIC_{50} 8.0 ug/ml and MIC_{90} 96.0 ug/ml (range 0.023- >256 ug/ml) while the isolates demonstrate high susceptibility of MIC_{50} 0.047 ug/ml and MIC_{90} 0.25 ug/ml (range 0.06–2.0 ug/ml) to ertapenem.

Extended-spectrum beta-lactamase detection screening versus confirmatory tests

Comparison of the screening and confirmatory tests for ESBL testing as seen in Table 1 shows a good correlation between the different methods. The correlation between the CTX plus clavulanic acid MIC method and DD method was Kappa = 0.95, $P < 0.001$ and for CZD, Kappa = 0.75, $P < 0.001$.

All the four tests offer a high sensitivity and moderately high specificity. They offer good positive and negative predictive values. There does not appear to be any trend to any one method being more efficient than the others [Table 2].

The *E*-test done on 12 isolates (for which the ESBL test results were discordant) revealed six ESBL, three non-ESBL producers, while two were suspected to be Amp-C producers and one was a possible CTX-M producer.

The final ESBL status of each organism is shown above in Table 3. As we can see of the 96 screened strains using the simple Kirby-Bauer DD testing method, that could be analyzed, 83 were confirmed to be ESBL producers (86.5%).

The results of the PFGE are seen in Figure 2a (medical ICU [MICU]) and Figure 2b (surgical ICU). The isolates show high diversity, indicating no genetic relatedness with each other. The data from this procedure were used to create the dendrogram seen in Figure 3. Most strains appear to arise from a cluster with 30% similarity into two main clusters of organisms, with similarity ranging from 50% to 70%. However, in the lower cluster formed by only *E. coli* isolates, two strains bear a 70% similarity with one another. These two strains were from nonrelated patients and the specimens were received 3 months apart from each other.Of the 2 isolates, one was isolated from a MICU male patient

Table 1: Different methods of extended-spectrum beta-lactamase testing compared with the different organisms

Organism	Number that were resistant to CTX/CZD on screening	Number that were confirmed as nosocomial isolates	ESBL by CTX plus clavulanic acid by disk diffusion method	ESBL by CTX plus clavulanic acid MIC testing method	ESBL by CZD plus clavulanic acid by disk diffusion method	ESBL by CZD plus clavulanic acid MIC testing method	*E*-test done*	ESBL status based on the *E*-test	Number confirmed as ESBL
Escherichia coli	395	71	65	64	62	63	8	4	63
Klebsiella spp.		25	21	21	21	20	4	2	20
Total	395	96	86	85	83	83	12	6	83

**E*-test was performed on isolates that showed discrepant results between CTX and CZD by the CLSI recommended methods (i.e., a third generation cephalosporin with a beta-lactamase inhibitor-clavulanic acid) to confirm the status as ESBL/non-ESBL. ESBL: Extended-spectrum beta-lactamase, CTX: Cefotaxime, CZD: Ceftazidime, CLSI: Clinical and Laboratory Standards Institute, MIC: Minimum inhibitory concentration

Table 2: Comparison of 4 methods for extended-spectrum beta-lactamase detection as compared to the final extended-spectrum beta-lactamase status

Method	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio
CTX-clavulanic acid disc diffusion	100	75	97	100	4	0
CZD-clavulanic acid disc diffusion	99	83.3	98	91	5.9	0.01
CTX-clavulanic acid MIC	100	83	98	91	5.9	0
CZD-clavulanic acid MIC	99	83	98	90	5.8	0.01

MIC: Minimum inhibitory concentration, CTX: Cefotaxime, CZD: Ceftazidime

Table 3: Final extended-spectrum beta-lactamase status of all the organisms selected as possible extended-spectrum beta-lactamase producers

Resistant organisms causing the nosocomial infection	Final confirmation of ESBL status		Total
	ESBL	Non-ESBL	
Escherichia coli	63	8	71
Klebsiella spp.	20	5	25
Total (%)	83	13	96 (86.5)

ESBL: Extended-spectrum beta-lactamase

and the second was a computer generated study control of a non-MICU isolate which was isolated from a child admitted in the neonatal ICU. Overall, the dendrogram suggests that there is no genetic relatedness among the nosocomial isolates from the intensive care wards.

Discussion

ESBL infections have been on a steady rise despite major improvement and awareness in health-care systems over the last 10 years. The detection and case management of an ESBL infection is relevant today as ESBL infections continue to be the leading cause of the infections caused by resistant organisms.

The occurrence of an ESBL infection causing a nosocomial infection in turn increases hospitalization time. *E. coli* and *Klebsiella* spp remain the *main* causative agents of hospital-acquired infections caused by members of the Family *Enterobacteriaceae* followed by *Enterobacter* spp.

Nosocomial infections with ESBL *E. coli* and *Klebsiella* spp affect all age groups.

Our study reveals that many require intensive care therapy, particularly, the postsurgical patients. Previous studies document a higher mortality, morbidity, and prolonged hospital stay in those with infection due to ESBL-producing *K. pneumonia.*[19,20]

Among the nosocomial acquired ESBL *E. coli* and *Klebsiella* spp studied, the predominant site infected was the respiratory tract (34.6%) with a predominance of *E. coli* causing infections. While some centers in India have reported a predominance of *E. coli,* others have reported primarily *Klebsiella* spp.[12,21-37]

The current CLSI recommendation for the phenotypic confirmation is the DD test and the MIC method using more than one, third or fourth generation cephalosporin in combination with clavulanic acid. Overall, the CTX and CZD DD and MIC for confirmation of an ESBL producer have excellent sensitivity of 99%–100%. CTX DD method alone appears to have a lower specificity than the CZD DD, and the MIC methods with CTX and CZD in the detection of an ESBL producer. As seen, the positive likelihood ratios for all four methods range between 4 and 5.9 and the negative likelihood ratios are near zero indicating that they are all excellent tests in our clinical situation. A recent study comparing automated susceptibility testing systems with conventional testing methods[38] found that the DD test had the highest sensitivity and positive predictive values 97% and 98%, respectively, which compares well with the results of this study. Current evidence seems to suggest that the DD test is better than automated systems for the confirmation of ESBL

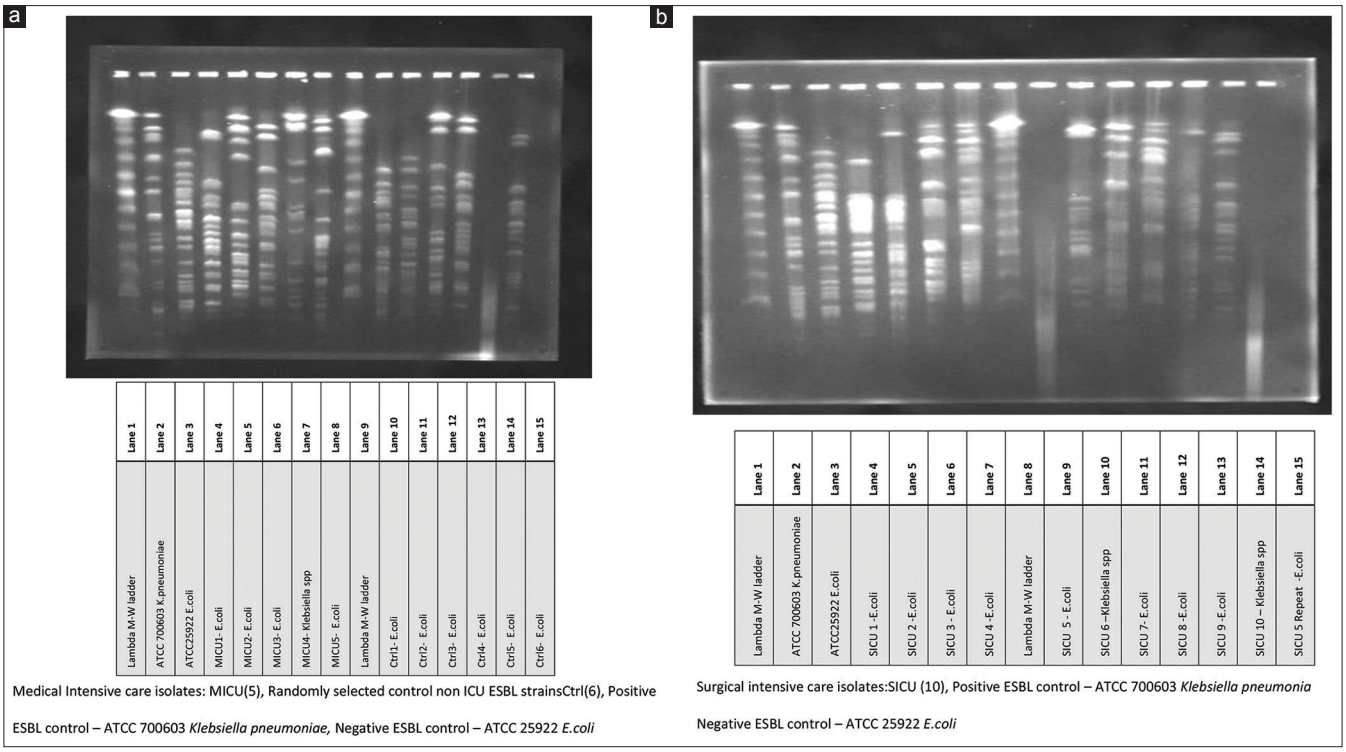

Figure 2: (a) Pulsed-field gel electrophoresis image 1: medical intensive care isolates. (b) Pulsed-field gel electrophoresis image 2: Surgical Intensive Care Isolates

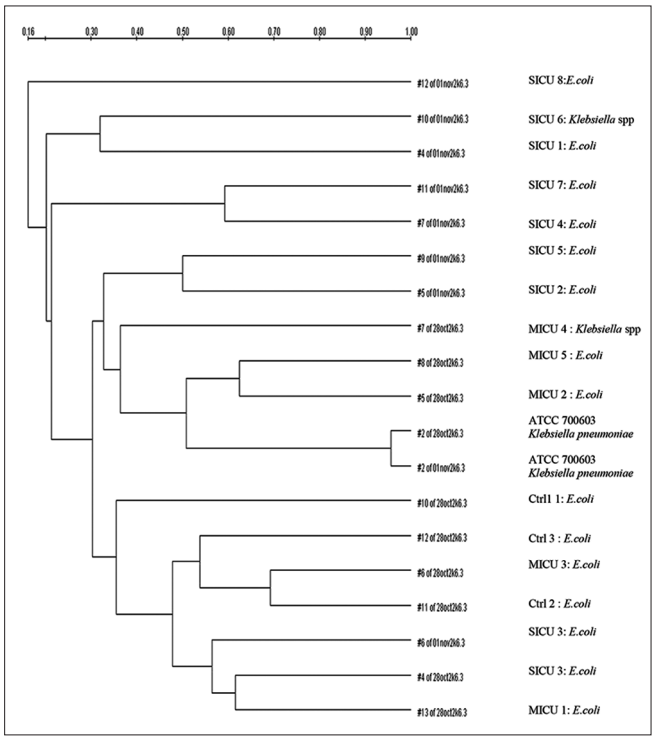

Figure 3: Dendrogram of the Intensive Care Units strains constructed using Dice coefficient correlation and the UPGMA methods and the Diversity database version 2.2

producers.[38] Since there is excellent correlation between the methods, laboratories are encouraged to detect the presence of an ESBL, which may be done easily by just the simple DD screening test using more than one drug The choice of method however could be based on cost, availability and level of skill and burden of work in individual laboratories.

PFGE analysis of ICU strains suggests two main clusters with <30% similarity among which were two strains with a 70% similarity. However, the two strains were not related to each other, indicating that the transmission of these strains did not take place in the intensive care units. Harris *et al*.[39] prospectively evaluated 23 patients who developed nosocomial ESBL-producing *E. coli* infection in intensive care PFGE. They found that 3/23 (13%) patient acquisitions were defined as patient-to-patient transmission. A recent study found no evidence of environmental transmission of ESBL-producing pathogens among 7651 medical and surgical intensive care patients.[40] Perhaps, nosocomial horizontal transmission is not a major problem in most ICU settings. However, as bacteria such as *Klebsiella* spp can survive relatively longer on hands and environmental surfaces, adequate precaution for prevention of horizontal transmission is a must.[20] Recent studies of transmission dynamics of ESBL organisms have found nosocomial transmission rates between 1.5 and 8%.[41,42]

Based on the cost-effectiveness, some studies have suggested that genotypic assays may be cost-effective in their setting.[43]

The phenotypic method, namely, the disk diffusion test as described in this study is both cost-effective and a highly sensitive test in the detection of ESBL producing pathogens. Therefore, in view of the low transmission rates, genotypic

methods would be considered unnecessary in smaller hospitals in need of a practical solution to detect and provide immediate clinical management of an ESBL infection. Genotyping can be reserved to answer research queries and in outbreak scenarios.

Limitations of the study

The specimens included in this study were those resistant to CTX and CZD by the Kirby Bauer DD method. We did not include strains that were susceptible or intermediate susceptible by this method due to financial limitations. This is an area requiring further work. In addition, the type of the ESBL by PCR method as defined by the Bush and Jacoby classification was not determined. There are more than 900 types of these enzymes, and the practical use of this typing is limited and it was outside the purview of this study which was to compare the recommended methods for detection of an ESBL producer and its utility for smaller laboratories.

Conclusions

The screening disk diffusion test using cefotaxime and ceftazidime compares well with the phenotypic confirmatory tests involving the addition of clavulanic acid and with the MIC methods to reliably identify an ESBL producer. The simple disk diffusion test has a high positive and low negative likelihood ratios. This indicates its usefulness as a stand-alone test in detecting ESBL producers in a diagnostic laboratory. It is cost effective in resource limited setting.

As ESBL producers are still our main pathogens today, carbapenems are the drug of choice for ESBL-producing *E. coli* and *Klebsiella* spp however, other drugs such as amikacin stand as a good alternative for invasive site infections.

Finally, although nosocomial transmission of ESBL organisms is of concern especially in closed wards and ICUs, the general transmission rate when infection control practices are in place is found to be low.

Acknowledgments

We would like to acknowledge the financial support and ethical clearance by the Institutional Review Board, Christian Medical College, Vellore, India and the Surveillance of antimicrobial resistance in India (SARI) project for the funding the molecular work of this project.

Dr. Mary S. Mathews, Ms. S. K. Kavitha, Ms. Ismath Jahan, Mr. Aaron Jeyseelan and staff of the Department of Microbiology, CMC, Vellore for their support and guidance.

Financial support and sponsorship

We would like to acknowledge the financial support from the Fluid research grant, Christian Medical College, Vellore and the SARI project.

Conflicts of interest

There are no conflicts of interest.

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