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Original Article

Prevalence of Clinical Strains Resistant to Various β-Lactams in a Tertiary Care Hospital in India

Priya Datta*, Archana Thakur, Bibhabati Mishra and Varsha Gupta[1]

Department of Microbiology, Govind Ballah Pant Hospital, New Delhi and
[1]Department of Microbiology, Government Medical College and Hospital, Chandigarh, India

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SUMMARY: The incidence of infection due to extended spectrum β-lactamases (ESBLs) producing *Enterobacteriaceae* has markedly increased in recent years. The traditional susceptibility methods lack sensitivity and/or specificity and this issue has prompted the search for an accurate test to detect the presence of ESBL. The present study included 300 bacterial strains and was undertaken to determine the prevalence of ESBL-producing strains. Here, compared three tests: a double disk synergy test (DDS), a three-dimensional test (3-D), and an inhibitor potentiated disk diffusion test (IPT); each test employed three different antibiotic discs, i.e., ceftazidime, ceftriaxone, and cefotaxime, in order to screen for ESBL strains. A strain was said to be an ESBL producer if it showed positive result(s) on any one of the three tests. The prevalence rate of ESBL in our hospital was 12.6% (38/300). IPT detected the most strains (34/38), followed by 3-D (23/38), and then DDS (15/38). The ceftriaxone disc was found to detect more ESBLs than either the ceftazidime or the cefotaxime disc.

INTRODUCTION

Extended spectrum β-lactamases (ESBLs) are enzymes that confer resistance to third- generation cephalosporins and to monobactams. The acronym ESBL is usually applied to describe an aspect of *Klebsiella pneumoniae*, which has joined methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) in the 1990s as a cause of important antibiotic-resistant nosocomial pathogen (1). Beginning in Europe in 1983 and in the United States in 1998, increasing reports of nosocomial outbreaks due to strains producing ESBLs have appeared (1). The increased prevalence of ESBL producers among *Enterobacteriaceae* has created a great need for laboratory testing methods that will accurately identify the presence of these enzymes in clinical isolates (2). The traditional susceptibility tests lack sensitivity and/or specificity to detect ESBLs, and thus the search for an accurate test to detect the presence of ESBLs is ongoing (3).

Clinical microbiological tests to detect ESBLs employ a β-lactamase inhibitor, usually clavulanate, in combination with oxyimino-cephalosoporin such as ceftriaxone, ceftazidime, or cefotaxime. In these tests, clavulanate inhibits the ESBLs, reducing the level of resistance to the cephalosporins and thereby increasing the zone of inhibition for the disc diffusion tests. The various tests currently in use include the double disk synergy test (DDS), the inhibitor potentiated disk diffusion test (IPT), the three-dimensional test (3-D), E test ESBL strips, and the MAST double-disk test (MDD) (4-7).

This study was undertaken to determine the prevalence of ESBL-producing strains of *Enterobacteriaceae* in a tertiary care hospital. The National Committee for Clinical Laboratory Standards (NCCLS) recommends the combined disk method and MIC broth micro-dilution for ESBL detection (8). We used three screening methods, DDS, 3-D, and IPT, after carrying out a broth micro-dilution and using three antibiotic discs, namely, ceftazidime, ceftriaxone, and cefotaxime, to compare which of these three methods and antibiotic discs would detect the maximum number of ESBL producers.

*Corresponding author: Mailing address: House No-380, Sector-16, Panchkula, Haryana, India-134113. E-mail: drpriyadatta@hotmail.com

MATERIALS AND METHODS

A total of 300 representative isolates of *Enterobacteriaceae* obtained over a period of 1 year from May 2002 to April 2003 were identified based on colony morphology and biochemical reactions. Various samples included in the study were pus, wound swabs, sputum, endotracheal aspirate, throat swabs, blood, cerebrospinal fluid, ascitic fluid, etc. These samples came from patients admitted to three surgical wards (cardiothoracic-vascular surgery, gastrointestinal surgery, and neurosurgery) and three internal medicine wards (cardiology, gasteroenterology, and neurology). The bacterial isolates came from sporadic cases, and isolates from the same patient were neglected.

The antibiotic susceptibility pattern was tested by the disc diffusion method on Mueller-Hinton media and the results were interpreted according to the NCCLS criteria (9). The following antibiotics were used; ampicillin (10 μg); ciprofloxacin (1 μg); gentamicin (10 μg); cefuroxime (30 μg); cefotaxime (30 μg); amikacin (30 μg); netilmicin (10 μg); cefoperazone (30 μg); ceftriaxone (30 μg); cotrimoxazole (25 μg); and oflaxacin (10 μg).

Minimum inhibitory concentration (MIC) determination: The minimum inhibitory concentrations of ceftazidime (Glaxo Ltd., Nasik, India) were determined for each of the 300 strains by the broth micro-dilution method. All of the strains, with a MIC of ≥2 μg/ml were further screened for potential ESBL production according to the three chosen methods, DDS, 3-D, and IPT (10). A strain was said to be an ESBL producer if it showed positive result(s) on any one of the three tests.

DDS: The test inoculum (0.5 McFarland turbidity) was streaked onto Mueller-Hinton agar (Hi Media, Mumbai, India). A disk of amoxicillin-clavulanate (augmentin, 20/10

Table 1. Percentage of antimicrobial resistance in clinical isolates

Antibiotic	Bacterial isolates					
	E. coli	*Klebsiella*	*Citrobacter*	*Proteus*	*Enterobacter*	*Salmonella*
Ampicillin	95.4	94.1	97.4	93.5	95.0	20.0
Netilmicin	41.3	60.5	89.7	70.9	65.0	0.0
Amikacin	16.0	28.5	15.3	51.6	20.0	0.0
Cephalexin	94.2	93.2	94.8	100.0	75.0	0.0
Ceftazidime	78.1	81.5	79.4	70.9	85.0	0.0
Cefotaxime	87.3	83.1	87.1	64.5	80.0	0.0
Ceftriaxone	87.3	76.4	76.9	58.0	95.0	0.0
Cotrimoxazole	94.2	93.2	94.8	93.5	85.0	0.0
Gentamicin	64.3	68.0	64.1	61.2	65.0	0.0
Oflaxacin	93.1	94.1	94.8	93.5	65.0	0.0
Cefuroxime	91.9	85.7	87.1	83.8	95.0	0.0
Cefoperazone	100.0	99.1	100.0	100.0	100.0	0.0
Ciprofloxacin	90.8	74.7	71.7	50.0	50.0	0.0

μg) was placed at a distance of 20 mm (center to center) from ceftazidime (30 μg,) cefotaxime (30 μg), and ceftriaxone (30 μg). The Mueller-Hinton agar plate was incubated at 37°C for 24 h. Enhancement of the zone of inhibition of ceftazidime, cefotaxime, and/or ceftriaxone towards the augmentin disc was reported as a positive result (4).

3-D: A Mueller-Hinton (Hi Media) plate was streaked with a strain of *Escherichia coli* that was sensitive to ceftazidime, ceftriaxone, and cefotaxime according to the Kirby-Bauer method. A hole (diameter, 4 mm) was punched into this agar plate. Thirty microliters of test inoculum (0.5 McFarland turbidity) was placed into the hole using a micropipette. A disc of ceftazidime was placed 2 mm away from the hole. The test was repeated with discs of ceftriaxone and cefotaxime. The Mueller-Hinton agar plate was incubated at 37°C for 24 h. Distortion or discontinuity in the expected circular zone of inhibition was considered as a positive test (11).

IPT: Two flasks of Mueller Hinton were prepared. In one flask, 4 mg of clavulanic acid was added to 1 liter of Mueller-Hinton agar. The test inoculum (0.5 McFarland turbidity) was streaked onto Mueller- Hinton agar plates, one with clavulanic acid and one without clavulanic acid. Ceftazidime, cefotaxime, ceftriaxone, and aztreonam were placed on both of these plates. The agar plates were then incubated at 37°C overnight. Inhibition zones for the four discs were compared between the plates with and without clavulanic acid. A difference of ≥10 mm was taken as presumptively positive for ESBL (5).

RESULTS

Of the 300 strains isolated, *K. pneumoniae* (35.7%) was the most common isolate, followed by *E. coli* (29.0%). Other species isolated were *Citrobacter freundii* (6.7%), *Proteus mirabilis* (6.3%), *Citrobacter koseri* (6.0%), *Enterobacter aerogenes* (6.0%) *Proteus vulgaris* (4.0%), *Klebsiella oxytoca* (4.0%), *Salmonella* Typhi (1.0%), *Enterobacter cloacae* (0.6%), and *Salmonella* Paratyphi A (0.3%).

The antibiotic susceptibility pattern of 300 bacterial strains was studied using the Kirby-Bauer method (Table 1). Among the β-lactam antibiotics, the maximum degree of resistance was seen to cefoperazone. Resistance shown by various strains to ceftazidime, ceftriaxone, and cefotaxime, commonly used for ESBL detection, varied from 58% in the case of *Proteus* to 95% in the case of *Enterobacter*.

Table 2. Number of ESBL producers detected by each method

Bacterial strains	Screening Methods		
	DDS	3-D	IPT
E. coli	6	8	12
Klebsiella spp.	3	7	12
Citrobacter spp.	2	3	5
Proteus spp.	4	5	5
Total	15	23	34

DDS, double disk synergy test; 3-D, three-dimensional test; IPT, inhibitor potentiated disk diffusion test.

MICs of test strains for ceftazidime: Among the 300 strains, 249 (83%) had a MIC of ≥2, and these strains were further screened for ESBL. None of the *Salmonella* spp. had a MIC of ≥2.

ESBL detection: Among the 300 strains, we isolated 38 (12.6%) ESBL producers. Of these, 16.1% (14/87) *E. coli* and 12.5% (13/107) *K. pneumoniae* produced ESBLs. Of these, 36.8% of *P. mirabilis*, 20.0% of *C. freundii*, 16.6% of *K. oxytoca* and 5.3% of *C. koseri* were ESBL producers. None of the *Enterobacter* spp. or *P. vulgaris* strains was found to be an ESBL producer.

Of the 38 ESBL-producing strains, IPT detected a maximum of 34 ESBLs, while 3-D detected 23 and DDS detected only 15 ESBL producers (Table 2). Four strains were detected only by 3-D; these four stains were missed by DDS and IPT. IPT detected 13 strains that DDS and 3-D were unable to detect.

Of the 15 ESBL strains detected by DDS, 7 ESBL strains were detected with the ceftazidime disc and 13 ESBL strains each were detected with ceftriaxone and cefotaxime. 3-D detected 23 ESBL strains; the ceftazidime disc detected 12, the cefotaxime disc detected 16, and the ceftriaxone disc detected 18 strains. Out of the total 34 ESBLs detected by IPT, 30 strains were detected by using the ceftazidime disc, 26 strains were detected using the cefotaxime disc, and 31 strains were detected by using the ceftriaxone disc. The use of an aztreonam disc did not prove to be of any additional help in IPT.

DISCUSSION

For a number of reasons, the detection of ESBL-producing

strains is of significant importance for all major hospitals worldwide. First, these strains are most likely even more prevalent than is currently recognized. Due to the difficulty of their detection by the current clinical methods, many of these strains have been reported to be susceptible to widely used and tested broad-spectrum β-lactams (12). Second, ESBLs constitute a serious threat to current β-lactam therapy. Treatment of ESBL infection is difficult as the NCCLS recommends that all expanded-spectrum cephalosporins be taken resistant in ESBL producers (9). Third, institutional outbreaks are increasing because of selective pressure due to the heavy use of expanded-spectrum cephalosporins and also due to lapses in effective infection control measures (13).

ESBL-mediated resistance poses many problems for in vitro susceptibility testing and reporting. The optimal substrate profile varies from one ESBL enzyme to another (14). For this reason, susceptibility panels with only one extended-spectrum cephalosporin cannot predict resistance to other extended-spectrum cephalosporins (15). In addition, ESBL-producing organisms may go undetected because they are associated with MICs as low as 0.5 μg/ml for ceftazidime, cefotaxime, ceftriaxone, and aztreonam (16). These enzymes do not always increase the MIC to a high enough level to be considered resistant by the NCCLS interpretation guideline (12). Although the NCCLS recommends both the broth microdilution method and the disk diffusion method, we compared DDS, 3-D, and IPT after carrying out the broth microdilution method, in order to determine which of these screening tests would be able to detect the maximum number of ESBLs.

Our study demonstrated a high level of resistance to cephalosporins; resistance varied from 99 - 100% to cefoperazone, 78 - 85% to ceftazidime, 64 - 87% to cefotaxime, and 58 - 87% to ceftriaxone. This high level of resistance agreed well with that observed in other studies (17-19). This high level of resistance is due to the rampant and injudicious use of newer antibiotics, especially such use as practiced in intensive care units and in the treatment of post-operative patients.

Of 300 strains, 38 were found to be ESBL producers. Our institution thus has a prevalence rate of 12.6% for ESBLs among *Enterobacteriaceae*. In the United States, the occurrence of ESBLs in *Enterobacteriaceae* ranges from 0 to 25%, and the national average is 3%. In India, the prevalence rate varies in different institutions from 28 to 84% (20). The prevalence of ESBL producers is lower at our institute due to our hospital infection control cell, which advises periodic antibiotic rotation (every 6 monts).

IPT was found to be the best screening method, followed by 3-D and then by DDS. IPT detected the maximum number of ESBL-producing strains. Ho et al. showed that the sensitivity of IPT was 100%, as compared to a 96% sensitivity rate for the DDS (5). In our study, DDS missed 23 ESBL producers. Thomson and Saunders (17) showed DDS to be 79% sensitive, whereas Vercauteren et al. (11) found the sensitivity to be 93%. This lack of sensitivity results from the fact that DDS is not a standardized procedure. Moreover, the choice of drug and disc distance varies from study to study. Here, 3-D was shown to be more sensitive than DDS. Other groups have reported the sensitivity of 3-D to be between 93 - 96% (11). Ceftriaxone detected the maximum ESBL rate in DDS, 3-D, and IPT, followed by cefotaxime and lastly ceftazidime. Coudron et al. found the sensitivity of ceftriaxone to be 88%, and that of ceftazidime to be 79% for these ESBL screening methods (21).

In *K. oxytoca*, the hyper-production of chromosomal K-1 β-lactamase can arise via mutation. These strains show decreased susceptibility or frank resistance to aztreonam, ceftriaxone, and cefopodoxime; ceftazidime and cefotaxime typically retain susceptibility against these strains. In principle, K-1 strains of *K. oxytoca* should be distinguished from ESBL producers on the basis of their susceptiblility to ceftazidime and cefotaxime, but difficulties can arise if ESBL screening is based only on reduced susceptibility to aztreonam, ceftriaxone, or cefopodoxime (8). Thus, in our study, we differentiated K-1 β-lactamase from ESBLs of *K. oxytoca* by using both ceftazidime and cefotaxime as indicator drugs.

ESBL testing in AmpC-producing species of *Enterobacteriaceae* is an unresolved issue in the field of ESBL testing. In *Enterobacter* spp. and *Citrobacter* spp., clavulanic acid may induce the expression of high levels of AmpC production and then antagonize rather than protect the antibacterial activity of parent β-lactam, thus masking any synergy arising from the inhibition of an ESBL. Much better inhibition is achieved with the sulfones, such as tazobactam and sulbactam, which are preferable inhibitors for ESBL detection tests with these organisms. Another approach is to rely on cefepime as an indicator drug. High-level AmpC production has a minimal effect on the activity of cefepime, making this drug a more reliable detection agent for ESBLs in the presence of an AmpC β-lactamase (8). We will be using this approach in our future studies of ESBL detection, whereby cefepime will be emplyoed for the detection of ESBL in *Citrobacter* spp. and *Enterobacter* spp. in order to separate ESBL producers from overproducers of AmpC.

Definitive identification of ESBL is possible using molecular detection methods. However, the techniques required for the task of identifying the exact ESBL subtype (e.g., DNA probing, polymerase chain reaction, restriction fragment length polymorphism, and isoelectric focusing) are available only at research facilities. Perhaps with the advent of gene chip technology in the near future, the subtype identification of ESBL will be performed more routinely in our laboratory (8).

In conclusion, the prevalence of ESBL-producing strains (12.6%) was found to be lower at our hospital than in the rest of India (28 - 84%). The inhibitor-potentiated method was found to be the best screening method when combined with the use of a ceftriaxone disc.

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