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A study of Extended Spectrum ß-Lactamases in Gram-negative Bacterial Isolates in a Tertiary Care Hospital in Mumbai

Anuradha Sharma[1], Leena Mandurke Gajbar[2], Priyanshu Kumar Shrivastava[3]*

Abstract

Background: Injudicious use of antimicrobials for any infection causes microbes to undergo changes through selective pressure and ultimately leads to the development of antimicrobial resistance. The production of ß-Lactamase is one of the most important mechanisms adopted by *bacteria* to evade action by penicillins and cephalosporins. **Materials and Methods:** This prospective *in vitro* study was conducted in a tertiary care hospital in Mumbai over a 6-month period with the aim of identifying extended spectrum b-lactamases (ESBLs) in Gram-negative *bacteria* in clinical isolates, by double disk diffusion test and E test. **Results:** Five thousand five hundred and forty-eight g-negative bacilli were isolated of which 2354 (42.42%) were ESBL producers. Maximum numbers of ESBL production were seen in isolates from the blood sample (53.91%) followed by pus (47%) and urine samples (42.27%). **Conclusion:** ESBL-producing organisms are increasing in the community; therefore, restricted and judicious use of the third generation cephalosporins is to be followed to prevent and control such types of resistance.

Keywords: Antibiotics, Antimicrobial resistance, Beta-lactamase, Extended spectrum b-lactamases, Gram-negative *bacteria*

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Introduction

The pathbreaking discovery of penicillin in 1928 by Alexander Fleming was soon followed by its practical application in 1941. Additional antibiotics were also being discovered at the same time and were called the wonder drugs, to be used as an effective weapon to kill *bacteria*. To everyone's dismay, resistance to them developed in the *bacteria* too soon. "Survival of the fittest" holds good for man, animals as also for *bacteria*. The use of antimicrobials puts selective pressure on microbes whereby they either adapt and survive or succumb to oblivion. This holds true for antimicrobial use in humans, veterinary, or agricultural purposes.[1-3]

Extended-spectrum ß-lactamase (ESBL) enzymes are plasmid-mediated enzymes produced by Gram-negative organisms. They hydrolyze and inactivate the ß-lactams, such as third-generation cephalosporins, penicillins, and aztreonam, but have no detectable activity against cephamycins and imipenem. The name ESBL s came about due to their vast and extended substrate range. An adverse clinical impact on the outcome of the treatment is seen, as these organisms are resistant to other antibiotics such as cephalosporins, aminoglycosides, and quinolones.[4]

The mid-and late 1980 s saw the observation and reporting of the first isolates of ESBLs in Western Europe and the United States of America. Since 1993, numerous outbreaks of infection with ESBL-producing organisms have been observed worldwide. Detection of ESBL-producing organisms is a dilemma for the diagnostic laboratory because monitoring the decrease in susceptibility to oxyimino-cephalosporins and aztreonam has not been sensitive to detect ESBL production.[4,5]

This study was done with the aim of identifying ESBL-producing Gram-negative organisms by double disk diffusion test, among the clinical isolates in our hospital. The objective was to study the antimicrobial resistance pattern, and minimum inhibitory concentration (MIC) of ESBL-producing organisms by E-test, to correlate the double disk diffusion test and the results of the E-test for ESBL detection and to assess the magnitude of the problem posed by ESBL producing organisms.

[1]Department of Microbiology, Faculty of Dentistry, Jamia Millia Islamia, New Delhi, India.

[2]Department of Microbiology, Aditya Microbiology and Molecular Diagnostic Center, Mumbai, Maharashtra, India.

[3]Faculty of Dentistry, Jamia Millia Islamia, New Delhi, India.

Corresponding Author: Priyanshu Kumar Shrivastava, Faculty of Dentistry, Jamia Millia Islamia, Maulana Mohammad Ali Jauhar Marg, Jamia Nagar, New Delhi, India. E-mail: priyanshushri25@gmail.com

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Materials and Methods

This prospective *in vitro* study was done in a tertiary care hospital in Mumbai. The detailed history of the patients was taken and all samples were collected using sterile precautions. The sample types were blood, urine, peritoneal and pleural fluid, cerebrospinal fluid, sputum, pus, wound cast, and different catheters. The samples were accepted from all different wards and all clinical specialties including the critical care unit.

Identification of the Organism

The samples were processed by making a primary Gram's smear, followed by culturing on MacConkey's and Blood agar. The culture was observed the next morning for growth and the required biochemical tests were put in to identify the organisms.[6,7]

Screening for ESBL Production

The antibiotic sensitivity was done on the Mueller-Hinton agar plates (HI-MEDIA). A well-isolated colony identified by the above

method was inoculated in 4 mL of sterile peptone water. The inoculated test tube was incubated for 2–4 h at 37°C. Turbidity of the test tube was matched with the 0.5 Mac Farland standard, which corresponds to the 1.5 × 10° CFU/mL. A sterile cotton swab was taken and dipped in the broth and the surplus broth was removed by rolling the cotton swab by the sides of the test tube. Later, the Mueller-Hinton agar plate was inoculated by evenly streaking the swab in three planes at a 60° angle to each other and lastly sweeping the entire circumference of the agar plate to form a lawn culture. After the inoculated plates were dried, antibiotics disks were applied to the lawn culture with the help of sterile forceps, as recommended by CLSI guidelines.[8] Later, the plates were incubated for 16–18 h at 37°C, after overnight incubation, the zones of inhibition were measured with the help of a scale to the nearest millimeter. The zones of inhibition of different antibiotics were interpreted as per the CLSI guidelines.[8]

Detection of ESBL by Double Disk Diffusion Test

A well-isolated colony was inoculated in 4 mL of sterile peptone water. The inoculated test tube was incubated for 2–4 h at 37°C. Turbidity of the test tube was matched with the 0.5 Mac Farland standard, which corresponds to the 1.5 × 10° CFU/mL. A sterile cotton swab is taken and dipped in the broth and the surplus broth was removed by rolling the cotton swab by the sides of the test tube. Later, the Mueller-Hinton agar plate was inoculated evenly by streaking the swab in three planes at a 60° angle to each other and lastly sweeping the entire circumference of the agar plate to form a lawn culture. After the inoculated plates were dried, antibiotics (HI-MEDIA) were applied to the lawn culture with the help of sterile forceps. The ceftazidime disk was placed 20 mm from the ceftazidime + clavulanic acid combination disc. This was followed by incubation for 16–18 h at 37°C, after which the zones of inhibition were measured to the nearest millimeter. Enhancement of the zone of inhibition towards ceftazidime + clavulanic acid combination disk by more than 5 mm was considered as ESBL-producing organisms.

E-test

A well-isolated colony was inoculated in 4 mL of sterile peptone water. The inoculated test tube was incubated for 2–4 h at 37°C. The turbidity of the test tube was matched with the 0.5 Mac Farland standard, which corresponds to the 1.5 × 10° CFU/mL. A sterile cotton swab is taken and dipped in the broth and the surplus broth was removed by rolling the cotton swab by the sides of the test tube. The Muller-Hinton agar plate was inoculated evenly by streaking the swab in three planes at a 60° angle to each other and lastly sweeping the entire circumference of the agar plate to form a lawn culture. E-strip containing ceftazidime (TZ)/ceftazidime + clavulanic acid (CA) (TZ/TZL) (MIC 0.5–32/0.064 – 4 µg/mL + 4 µg/mL CA) and cefotaxime (CT)/cefotaxime + clavulanic acid (CT/CTL) (MIC of 0.25–16/0.016–14 g/mL + 4 µg/mL CA) was applied on the lawn culture plate. The plates were incubated for 16–18 h at 37°C and MIC values were read where the inhibition ellipses intersected the strips. Growth along the entire gradient, that is, no inhibition ellipse indicates that the MIC is greater than the highest value on the reading scale. An inhibition ellipse below the gradient indicates a MIC less than the lowest value on the scale. The ratio of MICs of TZ and TZ/TZL and CT and CT/CTL was calculated. The ratio of TZ and TZ/TZL and CT and CT/CTL, if more than 8, was considered ESBL-producing organism. The presence of any phantom zone or deformation of the ellipse also indicates the production of ESBL.

Antibiotic Susceptibility Test

Antibiotic susceptibility of other antimicrobial agents was done by modified Kirby-Bauer diffusion method as per CLSI guidelines.[8] The medium used was Mueller Hinton agar (Hi media). A well-isolated colony was inoculated in 4 ml of sterile peptone water. The inoculated test tube was incubated for 2–4 h at 37°C. Turbidity of the test tube was matched with the 0.5 Mac Farland standard which corresponds to 1.5 × 10° CFU/mL. A sterile cotton swab was taken and dipped in the broth and the surplus broth was removed by rolling the cotton swab by the sides of the test tube. Mueller-Hinton agar plate was inoculated evenly by streaking the swab in three planes at a 60° angle to each other and lastly sweeping the entire circumference of the agar plate to form a lawn culture. After the inoculated plates were dried, the antibiotics disks mentioned in the following table were applied to the lawn culture with the help of sterile forceps. Plates were incubated for 16–18 h at 37°C, after overnight incubation, the zones of inhibition were measured with the help of a scale to the nearest millimeter. The zones of inhibition of different antibiotics were noted as per CLSI guidelines. The organisms' sensitivity pattern was noted by the following zone of inhibition produced by the organisms. First-line antibiotics used for the urine sample were amoxycillin (20 µg), cefuroxime (30 µg), ceftazidime (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), amikacin (30 µg), and ciprofloxacin (5 µg). The second-line drugs used were piperacillin-tazobactam (100/10 µg), imipenem 10 µg, meropenem 20 µg, nalidixic acid 30 µg, and netilmicin 30 µg. For other samples, the first line antibiotics used were piperacillin (100 µg), ampi-sulbactam (10/10 µg), amoxy-clav (20/10 µg), cefuroxime (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg) and the second-line drugs were piperacillin-tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), and netilmicin (30 µg).[8]

Results

A total of 5548 g-negative bacilli were isolated. Two thousand three hundred and thirty (41.99%) were *Klebsiella* spp. 1286 (23.17%) were *Escherichia coli* and 1932 (34.82%) were other g-negative *bacilli*. Two thousand three hundred and fifty-four (42.42%) of the Gram-negative isolates were ESBL producers with the maximum number of ESBL production seen in isolates from the blood sample (53.91%) followed by isolates from pus (47%) and urine samples (42.27%). Approximately 85% of the ESBL producers were from the inpatients with a maximum from the wards (86%) followed by the neonatal intensive care unit (6%), medical intensive care unit (5%), and integrated physical care unit (3%). Only 15% of the patients were from the outpatient department [Table 1].

ESBL production was seen in 891 (38%) of the 2330 isolates of *Klebsiella* spp., 839 (65%) of the 1286 *E.coli* isolates, 169 (21%) of the 819 *Acinetobacter* spp. isolated, 162 (28%) of the 574 isolates of *Proteus* spp., 150 (56%) of the 267 *Citrobacter* spp. isolated, 105 (58%) of the 182 isolates of *Enterobacter* spp. isolated, 36 (54%) of the 67 isolates of *Providencia* spp., and 02 (9%) of the 23 *Morganella* spp. isolated [Table 2].

Cefotaxime resistance was seen in approximately 100% of the ESBL producers. Ceftriaxone resistance varied from approximately 95–100% of the ESBL producers. One thousand seven hundred

Table 1: Distribution of Gram-negative *bacterial* isolates and ESBL producers in clinical samples

Sample	*Numbers of isolates*	*Klebsiella spp.*	*Escherichia coli*	*Others (Acinetobacter spp., Proteus spp., Citrobacter spp., Enterobacter spp., Providencia spp., Morganella spp.)*	*ESBL producers (%)*
Pus	2609	1142	251	1216	1224 (47)
Urine	1476	696	754	26	624 (42.27)
Blood culture	594	143	128	323	179 (53.91)
Respiratory	332	187	42	103	113 (34.03)
Fluids	176	69	33	74	51 (28.97)
Catheter	361	93	78	190	163 (45.51)
Total	5548	2330	1286	1932	2354 (42.42)

Table 2: Comparison of the double disk diffusion test (DDDT) with the resistance patterns of third-generation cephalosporins

Organisms	*Total samples*	*DDDT (%)*	*Resistance to third-generation cephalosporins*	
			Cefotaxime (%)	*Ceftriaxone (%)*
Klebsiella spp.	2330	891 (38)	886 (99.43)	891 (100)
Escherichia coli	1286	839 (65)	839 (100)	828 (98.68)
Acinetobacter spp.	819	169 (21)	167 (98.81)	169 (100)
Proteus spp.	574	162 (28)	162 (100)	162 (100)
Citrobacter spp.	267	150 (56)	150 (100)	143 (95.33)
Others	272	143 (58)	143 (100)	140 (97.90)

and thirty isolates showed ESBL production in samples other than the urine sample. For the first line of drugs, they showed the highest sensitivity to amikacin (79.72%), followed by ciprofloxacin (47.06%), piperacillin (35.9%), and gentamicin (34.22%). About 100% of resistance was seen to ampicillin-sulbactam, amoxyclav, cefuroxime, ceftriaxone, and cefotaxime. Five thousand and sixteen isolates were tested for the second-line drugs, out of which, they were most sensitive to imipenem (100%), followed by meropenem (99.62%), piperacillin-tazobactam (94.97%), and netilmicin (21.52%). About 100% of resistance was seen to second-line drugs, ceftazidime, and cefepime.

Antibiotic sensitivity testing of urine isolates with ESBL production (n = 624), to first line drugs, showed the highest sensitivity to amikacin (77.25%), followed by nitrofurantoin, cotrimoxazole (19.72%) and norfloxacin (12.99%). About 100% of resistance was seen to amoxicillin, cefotaxime, and ceftriaxone. In the second line of drugs, 100% of sensitivity was seen to imipenem, followed by meropenem (98.54%), piperacillin-tazobactam (81.27%), netilmicin (11.36%), and nalidixic acid (6.6%). About 100% of resistance was seen to ceftazidime.

ESBL-producing isolates by double disk diffusion test (DDDT) were tested with the E-test. Ninety-five isolates were positive by cefotaxime (CT)/cefotaxime + clavulanic acid (CT/CTL) strip and 93 isolates were positive using ceftazidime (TZ)/ceftazidime + clavulanic acid (TZ/TZL) strip. Using both strips, 96 organisms showed a MIC ratio of >8.1 organism showed a non-determinable zone which can be due to the MIC of the organism being more than the concentration of the antibiotic provided in the E test strip. Approximately 72% of the ESBL had a MIC of more than 32 µg/mL and more than 96% had a MIC of more than 16 µg/m.

Discussion

The injudicious use of antimicrobials has led to the acquisition of plasmids in Gram-negative *bacteria*, to overcome the antibiotic pressure, even though it has been a burden on the *bacteria*. The production of ESBL has been a diagnostic as well as a therapeutic challenge since 1993. In our study, there were 5548 isolates of Gram-negative bacilli, out of which *Klebsiella* spp. were 2330, *E. coli* 1286, and other Gram-negative organisms were 1932. Morosini *et al.* in 2006 isolated *E. coli* (172), *Klebsiella pneumoniae* (75), *Klebsiella oxytoca* (9), *Enterobacter cloacae* (16), *Enterobacter aerogenes* (03), *Enterobacter gergoviae* (01), *Citrobacter freundii* (03), *Citrobacter amalonaticus* (01), and *Salmonella* spp. (05) in their study on antibiotic coresistance in extended-spectrum-ß-lactamase-producing *Enterobacteriaceae*.[9]

About 42.42% of the total clinical samples were observed to be ESBL producers. Maximum numbers of ESBL producers were seen in samples of blood for blood culture (53.91%) followed by pus sample (47%) and urine sample (42.27%). Mathur *et al.* in the year 2002 reported 68% of ESBL production among Gram-negative *bacteria* from a tertiary care hospital. Hosoglu *et al.* reported 72.1% as ESBL producers in their study which is higher than our study. Gupta *et al.* observed an increase in the prevalence, around 66.8 to 71.5%, of infections due to ESBL-positive *bacteria* in their institute.[10-12]

Morosini *et al.* used specimens such as urine (141), feces (39), respiratory samples (33), wound (23), blood (21), catheter (7), intra-abdominal (7), rectal (4), skin (4), and other (6) sources in their study. In our study, the urine samples showed 42.27% of isolates to be ESBL producers. A study by Tankhiwale *et al.* reported that 48.3% of isolates in urine were ESBL producers. This is similar to our study. Table 2 shows the distribution of the ESBL producers in different organisms. The prevalence of ESBL-producing *Klebsiella* and *E. coli* was 38.24% and 65.24%, respectively. Navon-Venezia *et al.* found that 42.5% of ESBL producers in the Gram-negative isolates of which *Klebsiella* spp. were 79%, *Proteus* spp. 62%, *E. coli* 53%, *Enterobacter* spp. 42%, *Serratia* spp. 14%, *Citrobacter* spp. 24%, *Providencia* spp. 24%, and *Morganella* spp. 5%. Memon *et al.* reported a 20% occurrence of ESBL producers among the *Enterobacteriaceae*, with 28.5% *Enterobacter*, 21.2% *Klebsiella,* and 19.2% *E. coli* as elaborate ESBL producers.[9,13-15]

About 86.6% of *Klebsiella* spp., 73.4% of *Enterobacter* spp., and 63.6% of *E. coli* were reported by Jain *et al.* as ESBL producers from cases of neonatal septicemia. Babypadmini *et al.* in their study reported 40% and 41% of ESBLs among *K. pneumoniae* and *E. coli,* respectively. In the present work, cefotaxime resistance is seen in approximately 100% of the ESBL producers. Resistance to ceftriaxone varies from approximately 95–100% in the ESBL producers. Shubha *et al.* reported 63.3% of ESBL-producing organisms to be resistant to cefotaxime and 74.4% of resistance to ceftriaxone. Hope *et al.* reported screening with cefotaxime and ceftazidime to be showing better specificity for the identification of ESBL-based resistance.[16-19]

The antibiotic sensitivity pattern of the ESBL-producing organisms to the first line of antibiotics showed 79.72% sensitivity

to amikacin. In the second line of antibiotics, imipenem (100%) and meropenem (99.62%) were the most sensitive antibiotics followed by piperacillin + tazobactam (94.97%). Similar results were seen in a study made by Hosoglu where the most active antibiotic was meropenem (none of the isolates was resistant to this drug) followed by piperacillin-tazobactam. Morosini *et al.* found 100%, 100%, 97.5%, 93.3%, and 93% sensitivity to meropenem, imipenem, tigecycline, amikacin, and piperacillin-tazobactam, respectively.[9,11]

In our study, all the ESBL isolates showed the highest sensitivity to imipenem and later to Meropenem. Carbapenems have been the most effective drug in many of the published reports and are the most promising antibiotic for the treatment of infections caused by ESBL producers. Chaudhary *et al*. also reported 100% of sensitivity to imipenem in the study at Haryana.[4]

Antibiotic sensitivity patterns of urine isolates showed 77.25% of sensitivity to amikacin followed by nitrofurantoin (54.17%) among the first line of antibiotics. In the second line of antibiotics, imipenem (100%) and meropenem (98.54%) were the most sensitive antibiotics followed by piperacillin + tazobactam (81.27%). Babypadmini reported the susceptibility of ESBL producers in urine samples to imipenem, nitrofurantoin, and amikacin at 100%, 89%, and 86%, respectively. They found associated resistance with cotrimoxazole (74%), gentamicin (75%), and fluoroquinolones (96%). A study done by Tankhiwale *et al.* in Nagpur reported significant multidrug-resistance in ESBL-producing isolates than non-ESBL producers, in urine samples. Mendonça *et al.* reported that all strains of ESBL producers were sensitive to carbapenems and multidrug-resistance phenotype was seen in 92% of the strains.[13,17,20]

ESBL-producing isolates by DDDT were tested with the E-test. Ninety-three isolates were positive using ceftazidime (TZ)/ceftazidime + clavulanic acid (TZ/TZL) strip. Ninety-five isolates were positive for cefotaxime (CT)/cefotaxime + clavulanic acid (CT/CTL). Using both strips, 96 organisms showed a MIC ratio of >8, and one organism showed no determinable zone. It can be due to the MIC of the organism being more than the concentration of the antibiotic provided in the E-test strip. CLSI recommends the use of both the strips for detection of ESBL. This increases the sensitivity of the test as seen in our study. Linscott and Brown reported the sensitivity of the E-test to be 99% in their study.[8,21]

The MIC of ceftazidime by ceftazidime E test showed that approximately 72% of the ESBL isolates had a MIC of more than 32 µg/mL. 32 µg/mL is the highest concentration of the antibiotic on the strip. MIC of cefotaxime by cefotaxime E-test was more than 16 µg/mL in approximately more than 96% of the ESBL producers. 16 µg/mL is the highest concentration of the antibiotic on the strip. Similar results were observed by Kumar *et al*. who increased resistance of *K. pneumoniae* and *E. coli* to cefotaxime as compared to ceftazidime.[22]

About 85% of the ESBL producers were from the IPD while only 15% of the patients were from the OPD. In the OPD patients, most of the samples were urine isolates, with a history of complicated urinary tract infection. Woodford *et al.* reported 24% of ESBLs from community-acquired infection in patients in the UK, most of which were multidrug-resistant.[23]

ESBL producers were found to be the maximum from the wards (86%), followed by NICU (6%), MICU (5%), and IPCU (3%). In the critical care unit, NICU had the maximum number of ESBL organisms. All of them were critically ill with a history of low birth weight, and respiratory distress, and were on life support. In a study by Vinod Kumar and Neelagund, ESBL-mediated resistance was found in 13.54% of their isolates which is higher than our study.[24]

Summary and Conclusion

The present study was carried out on 5548 patients samples received in our laboratory of a tertiary care teaching hospital. About 42.42% were ESBL-producing Gram-negative isolates. With the use of reliable and easy techniques, ESBL-producing organisms can be detected among the Gram-negative organisms in our laboratory. All the ESBL producers were resistant to many ß lactams and non-ß lactam antibiotics. All ESBL-producing organisms are 100% of sensitive to imipenem and meropenem. Therefore, imipenem can be given in life-threatening and serious infections by these organisms. There is an increase of these organisms in the community; therefore, restricted and judicious use of the third generation of cephalosporins is to be followed, to prevent and control such types of resistance. Health-care facilities must have a functional hospital infection control committee along with appropriate hospital antibiotic policies with regular updates. To conclude, regular monitoring of ESBL production in Gram-negative organisms is the need of the hour.

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