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Detection of Extended Spectrum Beta-Lactamase (ESBL) Producing Strains Of Gram Negative Bacteria among Clinical Samples by Phenotypic Methods: A Hospital Based Study

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Original Research Article

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Abstract: The present study was undertaken to evaluate the prevalence of the extended spectrum β- lactamase (ESBL) producing gram negative strains at our tertiary care hospital by using the Modified Double Disc Synergy Test (MDDST) and CLSI phenotypic confirmatory test (PCT). A total of 300 non-repetitive isolates of enterobacteriaceae from various clinical samples of urine, blood, pus, wound swab, sputum, or intravenous catheter were obtained from inpatient units of various wards of our tertiary care teaching hospital. Gram negative isolates having zone size of <=22mm for ceftazidime and <=27mm for cefotaxime (standard disc diffusion method) were selected as suspicious for ESBL production as recommended by CLSI guidelines. These potential ESBL producing strains were further tested by MDDST and CLSI PCT methods. Among the 300 clinical isolates tested 154 gram-negative isolates were considered suspicious of ESBL production by the initial screening test for ESBL production by the initial screening method. Out of these 154 isolates 148(49.33%) were confirmed to be ESBL producer by phenotypic confirmatory tests. Amongst the various bacterial isolates tested positive for ESBL production maximum percentage of ESBL producer were *Klebsiella spp.* (67.08%) followed by *Pseudomonas spp*. (60%), *Acinetobacter spp.* (54.16%), *Proteus spp.* (40%), *Escherichia coli* (33.63%) and *Citrobacter spp.* (30.78%). Majority of ESBL producing strains were from surgery wards (27.02%) followed by ICUs (22.29%), medicine wards (19.59%). Minimum percentage of ESBL producers were from paediatric wards (5.40%). Both the MDDST and PCDDT methods were highly sensitive and specific in detection of ESBL production. PCDDT was more sensitive in detecting ESBL production in *Klebsiella spp.*, *Pseudomonas spp.* and *Acinetobacter spp*. but the difference is not statistically significant.
Keywords: ESBL, gram-negative bacteria, modified double disk synergy test, phenotypic confirmatory test

INTRODUCTION

Beta-lactamase enzymes produced by several gram-negative bacteria are probably one of the most important reasons for resistance to penicillins and cephalosporins. The extended spectrum β-lactamases (ESBL) are typically plasmid mediated that are able to hydrolyze penicillins, third generation cephalosporins and monbactams [1, 2]. ESBL producing gram-negative bacteria are the most common isolated organisms from health-care associated infections. Owing to very limited therapeutic options available they pose a serious challenge to the clinicians especially among high-risk patients [3].

ESBL are inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam. Various phenotypic methods are available in routine laboratory to detect the ESBL production among gram negative organisms [4]. Although, Double Disc Synergy Test (DDST) is a simple and a reliable method to detect ESBL but it lacks sensitivity because of the problem of optimal disc space and storage of disc of clavulanic acid. Modified Double disc synergy test (MDDST) is similar to DDST where centre to centre distance between β-lactam antibiotics and β-lactamase inhibitor is reduced to increase sensitivity of ESBL detection [5, 6].

ESBL producing isolates, in addition to being resistant to ß-lactam antibiotics, often exhibit resistance to other classes of drugs such as aminoglycosides, cotrimoxazole, tetracycline and fluoroquinolones [7].

Early detection of MBL and ESBL producing organisms is crucial to establish appropriate antimicrobial therapy and to prevent their inter-hospital and intra-hospital dissemination [8]. The present study was undertaken to evaluate the prevalence of the extended spectrum β- lactamase (ESBL) producing gram negative strains at our tertiary care hospital by using the MDDST and Clinical and Laboratory Standards Institute (CLSI) phenotypic confirmatory test (PCT) [9].

MATERIAL AND METHODS

Study design

The present study was a hospital based prospective study conducted in the department of Microbiology, Mayo institute of Medical Sciences, Barabanki district of Uttar Pradesh. The study was approved by the institutional ethical committee.

Data Collection

After obtaining informed consent from the patients the demographic(age, sex), clinical conditions data (history of antibiotic usage in the past 2 weeks, duration of hospitalization, history of fever in the past two weeks, site of collecting pus sample or the sample type and the relevant information about the participants was recorded on the pretest proforma.

Sample Collection

A total of 300 non-duplicate gram-negative isolates from various clinical samples of urine, blood, pus, wound swab, sputum, or intravenous catheter were obtained from inpatient units of medicine, surgery, gynaecology and obstetrics, pediatrics, and intensive care unit (ICU). The study included patients of all age groups and both sexes. The samples were processed and isolates identified following standard laboratory procedures [9].

Screening of ESBL producing strains

The isolates were tested for their susceptibility to the third generation cephalosporin (3GCs) i.e. ceftazidime (30µg), cefotaxime (30µg) and ceftriaxone (30µg) by using the standard disc diffusion method as recommended by the CLSI [5]. Gram negative isolates having zone size of <=22mm for ceftazidime and <=27mm for cefotaxime (standard disc diffusion method) were selected as potential ESBL producers as recommended by CLSI guidelines. These potential ESBL producers were further confirmed phenotypically by two different methods.

Phenotypic Confirmation Test

Two methods used for confirmation of ESBL production is mentioned below:

- *Modified Double Disc Synergy Test* (MDDST): Lawn culture of test strain on Mueller Hinton agar (Himedia, Mumbai) was done and discs of cefotaxime (30µg), ceftazidime (30µg), amoxiclav (20µg amoxicillin/10µg clavulanic acid) were applied. The cefotaxime and ceftazidime disc were placed 20 mm center to center from amoxiclav disc and plate was incubated aerobically at 37°C overnight. The test isolate was considered as ESBL producer, if the zone size around the cefotaxime and ceftazidime disc increased towards the amoxiclav disc [5,6].
- *CLSI Phenotypic Confirmatory Test* (PCT): Lawn culture of test isolates was done on Muller Hinton agar. Antibiotics used were ceftazidime (30µg), ceftazidime/clavulanic acid (30µg/10µg). Discs were placed opposite to each other at a distance of 24mm in Muller Hinton agar plate and were incubated aerobically overnight at 37°C. Next day zone of inhibition around ceftazidime and ceftazidime/clavulanic acid were measured. An increase in zone of inhibition around ceftazidime/clavulanic acid by more than 5mm than that of ceftazidime disc alone was confirmatory of ESBL production [9].

Quality control

Klebsiella pneumoniae ATCC 700603 and *Escherichia coli* ATCC 25922 were used as ESBL positive and negative controls, respectively.

Antimicrobial susceptibility testing of gram-negative isolates

The test was done by Kirby-Bauer disc diffusion as per standard CLSI guidelines. Antibiotics used were ampicillin (10µg), amoxiclav(20µg/10µg), cefazolin (30µg), cefepime (30µg), ceftazidime (30µg), nitrofurantoin (30µg) and norfloxacin (10µg) (for urinary isolates only), trimethoprim-sulfamethoxazole (1.25/23.75µg), and ciprofloxacin (5µg), gentamicin (10µg), amikacin (30µg), imipenem (10µg), Meropenem (10µg), Aztreonam (30µg). For *Pseudomonas aeruginosa*, piperacillin-tazobactam (100µg/10µg) was also used.

STATISTICAL ANALYSIS

The findings were statistically analyzed using Chi-square test(X^2).

RESULTS

Among the 300 clinical isolates tested, 154 gram-negative isolates were considered potential ESBL producer by the initial screening test for ESBL production. All these isolates showed a zone diameter of <22mm for ceftazidime. All of these 154 isolates were further tested with two additional phenotypic methods MDDST and PCT for confirmation of ESBL

production. Out of the total 300 isolates tested maximum number of isolates were obtained from urine (31%) followed by pus (20.33%), blood (21%), sputum (13%), body fluids (9.66%) and stool (5%).

Table-1: **Specimen wise distribution of Gram Negative Isolates (N=300)**

Sample	No. of isolates
Urine	93
Pus	61
Sputum	39
Body fluid	29
Blood	63
Stool	15
Total	**300**

Amongst the various bacterial isolates tested positive for ESBL production maximum percentage of ESBL producer were *Klebsiella spp*. (67.08%) followed by *Pseudomonas spp.* (60%), *Acinetobacter spp.* (54.16%), *Proteus spp*. (40%), *Escherichia coli* (33.63%) and *Citrobacter spp*. (30.78%).

Table-2: **Distribution of ESBL producing gram-negative isolates (N=300)**

Isolates	**No. of isolates**	**ESBL producer**
E. coli	110	37
Klebsiella spp	79	53
Acinetobacter spp.	48	26
Pseudomonas spp	40	24
Citrobacter spp.	13	4
Proteus spp.	10	4
Total	**300**	**148**

Maximum number of ESBL producing strains were from surgery wards (27.02%) followed by ICUs (22.29%), medicine wards (19.59%). Minimum percentage of ESBL producers were from paediatric wards (5.40%).

Table-3: **Ward-wise Distribution of Gram Negative isolates (N=300) and ESBL Producers (N=148)**

Ward	Gram-negative Isolates	Isolate%	ESBL Producer	ESBL%
Medicine	51	17	29	19.59
Surgery	84	28	40	27.02
Paediatrics	30	10	8	5.40
Obs/gynae	45	15	15	10.13
Orthopaedics	24	8	15	10.13
ICU	46	15.33	33	22.29
CCU	20	6.66	8	5.40
Total	**300**	**100**	**148**	**100**

Both the MDDST and PCT methods were highly sensitive and specific in detection of ESBL production. PCT was more sensitive in detecting ESBL production in *Klebsiella spp*., *Pseudomonas spp.* and *Acinetobacter spp.* but the difference is not statistically significant.

Table-4: **ESBL producer among gram-negative isolates in various clinical samples**

Organism	ESBL Producers	
	MDDST	**PCDDT**
E. coli	37/110(33.63%)	37/110(33.63%)
Klebsiella spp	51/79(65.51%)	53/79(67.08%)
Acinetobacter spp.	24/48(50%)	26/48(54.16%)
Pseudomonas spp	23/40 (57.50%)	24/40(60.0%)
Citrobacter spp.	4/13(30.76%)	4/13(30.76%)
Proteus spp.	4/10(40%)	4/10(40%)

The antibiotic sensitivity pattern revealed that the maximum sensitivity among ESBL producers was seen for imipenem (100%), followed by meropenem (88.88%), amikacin (93.33%), piperacillin/tazobactam (77.77), cefipime (66.66%), amoxiclav (55.55%), aztreonam (55.55%) and gentamicin (37.77%). Among the urinary ESBL producing strains, nitrofurantoin showed reasonably good sensitivity (55.55%) while only 18.1% of the total urinary isolates were sensitive to norfloxacin.

Table-5: **Comparative Study of Antibiotic Sensitivity Patterns of both ESBL Producers and Non ESBL Producers**

	ESBL Producer (%) N=148			ESBL Non-Producer (%) N=152		
Antibiotics	**Sensitive**	**Intermediate**	**Resistant**	**Sensitive**	**Intermediate**	**Resistant**
Ampicillin	0	0	100	55.96	0	44.036
Cotrimoxazole	8.88	0	91.12	50.45	0	58.71
Amoxiclav	55.55	11.11	33.33	67.88	0	32.11
Cefazolin	55.55	11.11	33.33	73.39	0	26.60
Nitrofurantoin(U)	74.07	3.70	22.22	96.42	0	3.57
Norfloxacin(U)	18.51	0	81.48	85.71	0	14.28
Ciprofloxacin	0	0	100	55.04	0	44.95
Gentamicin	37.77	0	62.22	92.66	0	7.33
Amikacin	93.33	0	6.66	91.74	5.50	2.75
Ceftazidime	22.22	0	77.77	89.90	0	10.09
Cefepime	66.66	11.11	22.22	86.23	0	13.76
Imipenem	100	0	0	100	0	0
Meropenem	88.88	2.22	8.88	99.08	0.91	0
Aztreonam	55.55	0	44.44	87.15	2.75	10.09
Piperacillin/tazobactam	77.77	0	22.22	100	0	0

In this study maximum numbers of the ESBL producing gram negative isolates were obtained from urine specimen (54.72%), followed by sputum (20.94 %), pus (14.86 %), and body fluids (9.46 %). No ESBL production was detected in gram negative isolates recovered from blood and stool samples.

Table-6: **Distribution of ESBL Producing Isolates in Various clinical Specimens**

Isolate	Clinical Specimens						
	Urine	Sputum	Pus	Body fluid	Blood	Stool	Total
Escherichia coli	27	6	4	-	-	-	37
Klebsiella spp	31	15	1	6	-	-	53
Acinetobacter spp	8	5	10	3	-	-	26
Pseudomonas spp	10	5	6	3	-	-	24
Citrobacter spp	2	-	-	2	-	-	4
Proteus spp.	3		1	-	-	-	4
Total	81	31	22	14	0	0	148

DISCUSSION

Antimicrobial resistance is a problem which was faced by clinicians very soon after its discovery. Development of resistance not only increases the duration of treatment but also its cost effectiveness. Resistance among bacteria causing nosocomial infections is a great matter of concern as it may lead to transmission of infection to susceptible patients. Prevalence of extended spectrum beta-lactamase producing strains is increasing day by day in various health care facilities. The present study was also aimed to determine the prevalence of ESBL producing Gram-negative isolates among the patients admitted to our hospital.

In the present study 49.33% gram-negative isolates were found to be producing ESBL. This finding was consistent with the study conducted by Sharma M *et al.* [10] which has found a prevalence of 52.49% of ESBL producing isolates. A higher (61.6%) prevalence of ESBL producing isolates was found in a study conducted by Dalela G *et al.* [11]. However a lower prevalence of 18% ESBL producing isolates was found by Shrestha S *et al*. [12].

Among the isolated ESBL producer, maximum percentage were of *Klebsiella spp.* (67.08%) followed by *Pseudomonas spp.* (60%), *Acinetobacter spp.* (54.16%), *Proteus spp*. (40%), *Escherichia coli* (33.63%) and *Citrobacter spp.* (30.78%). Similar finding with highest ESBL production by *Klebsiella*

spp. (67.04%) followed by *Escherichia coli* (56.92%), *Proteus spp.* (46%), *Pseudomonas spp.* (41.89%), *Citrobacter freundii* (27.59%), *Salmonella typhi* (26.31%), *Acinetobacter spp.* (11.11%) and *Salmonella paratyphi* A (5.56%) was found by Sharma M *et al.* [10]. However Dalela G *et al.,* has found that the isolates of *Escherichia coli* (73.5%) were the most common ESBL producers, followed by *Proteus vulgaris* (60%), *Klebsiella pneumoniae* (58.1%) and others. Similarly Shrestha S *et al.,* has found that among ESBL producing isolates *Escherichia coli* was 53.7%, *Klebsiella pneumonia* (14.8%), *Proteus mirabilis* (12.9%) and others (7.4%) [11, 12].

Among ESBL producing isolates 54.72% isolates were obtained from urine samples followed by sputum (20.94%), pus (14.86%) and body fluid (9.45%). Similar findings was observed by Sharma M *et al.,* who has found that ESBL producing isolates were isolated in maximum number from urine (57.2%) followed by blood (31.07%), pus (48.03%), respiratory tract (63.83%), body fluid (52.17%) and stool samples (59.29%) [10]. This finding is also consistent with study conducted by Dalela G *et al.,* which has found that higher number of isolates were obtained from urine (66.4%), followed by pus (57.3%) and others (54.2%) [11].

Table-7: **Comparative studies in different regions of India**

Author	**Year**	**Place**	**Prevalence**
Subha A *et al*.[13]	2002	Chennai	6.6
Babypadmini S *et al.* [14]	2004	Coimbatore	40.3
Rodrigues C *et al*. [15]	2004	Mumbai	53
Shukla *et al*.[6]	2004	New Delhi	30.18
Singhal S *et al*.[16]	2005	Gurgaon	64
Mangaiyarkarasi *et al.* [17]	Oct2008- Apr2010	Pondicherry	60.86
Shaswati *et al*.[18]	2011-12	Bhopal	48.27
Singh *et al*.[19]	2012	Imphal	27.7
Rao et al.[20]	2014	Bellary, Kolar	57.5
Agarwal *et al*.[21]	2014	Rohtak	36
Wadekar *et al*.[22]	2013	Mysore	43
Mathur P *et al.* [23]	2002	New Delhi	68
Present Study	2015	Barabanki	49.33

In *Pseudomonas aeruginosa*, the ESBL production was 60% that is far more than *Escherichia coli* (33.63%). The high prevalence of ESBL production is probably because of the larger number of *Pseudomonas spp.* being isolated from the samples received from the ICUs and the surgical wards where the prevalence of ESBL producer isolates was 27.02% and 22.29% of the total ESBL isolates that is much higher when compared to the prevalence of ESBL producers in other wards.

In the present study the antimicrobial susceptibility pattern showed that all the ESBL producing isolates were sensitive to imipenem with a higher degree of susceptibility to amikacin (93.33%), meropenem (88.88%), piperacillin/tazobactum (77.77%) and nitrofurantoin (74.07%). Dalela G *et al.* has also found that all the ESBL producing isolates isolates were susceptible to imipenem followed by piperacillin/tazobactum (69.9%) [11]. in the present study all the ESBL producing isolates were resistant to ampicillin and ciprofloxacin. Similar findings were observed by Shrestha S *et al.* who have also found that all the ESBL producing isolates were resistant to ampicillin and ceftazidime whereas 65% were resistant to ciprofloxacin and 70% were resistant to gentamicin [12]. These findings showed that ESBL producing isolates were multi-drug resistant as compared to ESBL non-producing isolates. Thus transmission of ESBL producing strains among the patients admitted to hospital may be an important reason for treatment failure.

In present study it was found that PCT was more sensitive than MDDST in detection of ESBL production in *Klebsiella spp*, *Pseudomonas spp.* and *Acinetobacter spp*. Dalela G *et al.,* has also found a similar finding with PCT being more sensitive and cheaper alternative as compared to DDST [11]. PCT was technically simpler and is also less expensive. The MDDST test was used in place of DDST and it was found to be an inexpensive alternative for the DDST, for the detection of ESBL producers. The DDST lacks sensitivity because of the problem of optimal disc space and the correct storage of the clavulanic acid containing discs.

CONCLUSION

A committee must be formed at all hospitals, which should provide guidelines for the judicious use of antibiotics and should formulate policies which will help in minimizing the emergence of resistant bacteria among the patients. There is a possibility that the restricted use of antibiotics can lead to the withdrawal

of selective pressure and that the resistant bacteria will no longer have a survival advantage against these antibiotics.

In the end, it has been felt that there is a need to formulate strategies to detect and prevent the emergence of ESBL producing strains for the effective treatment of infections which are caused by them.

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