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Research Article

PHENOTYPIC EVALUATION OF PREVALENCE OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBLs) PRODUCING UROPATHOGENS USING MODIFIED DOUBLE DISK SYNERGY TEST (MDDST) ALONG WITH THEIR ANTIBIOTIC SUSCEPTIBILITY PROFILE

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Abstract- Urinary tract infections (UTIs) are probably the most common community acquired as well as nosocomial infections, mostly caused by Gram negative bacteria. Treatment of UTI cases is often started empirically, however, with the emergence of drug resistance among uropathogens in the form of extended spectrum beta-lactamases (ESBLs) production, treatment of such isolates has become quite difficult as these enzymes hydrolyze all penicillins, all cephalosporins (except cephamycins) and monobactams. Also, such organisms are often multidrug resistant and as the resistance genes are easily transferred from one organism to another via plasmids, their worldwide spread has become a threat to human population. Hence, the present study was done to determine the prevalence and antibiotic susceptibility profile of extended spectrum beta-lactamases producing uropathogens. A total of 156 consecutive, non duplicate gram negative bacilli recovered from urine samples were identified and their antibiotic susceptibility was tested. These isolates were first screened and then confirmed for ESBL production by modified double disk synergy test. Amongst 156 urinary isolates tested 69 were confirmed ESBL producers, with 8.7% (6/69 isolates) AmpC co-producers. The prevalence of ESBL was found to be 44.2%, with 61.3% among isolates from inpatients and 8.0% from outpatients. Maximum ESBL producers were *Klebsiella pneumoniae* (61.9%) followed by *Escherichia coli* (53.6%). All ESBL producers were 100% sensitive to imipenem, followed by sensitivity to nitrofurantoin (86.6%), piperacillin-tazobactam (81.2%) and norfloxacin (78.3%). To conclude routine ESBL testing for uropathogens along with conventional antimicrobial susceptibility testing should be done for deciding proper treatment of all cases of UTI.

Keywords- extended spectrum beta-lactamases, modified double disk synergy test, prevalence, uropathogens

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Introduction

Urinary tract infections (UTIs) are probably the most common community acquired nosocomial infections. Gram negative bacteria are by far the most common infecting agents in the UTI [1]. Treatment of UTI cases is often started empirically before the laboratory reports of urine culture and antibiotic susceptibility are available [2]. Amoxicillin was traditionally used in the first line therapy for UTIs. However, with the emergence of drug resistance among uropathogens in the form of extended spectrum beta-lactamases (ESBLs) production, treatment of such isolates has become a matter of serious concern [3,4].

ESBLs are plasmid mediated beta-lactamases (enzymes that cleave the beta-lactam ring) which have the ability to hydrolyze penicillins, first-, second- and third-generation cephalosporins and monobactams (aztreonam), but are inhibited by beta-lactamase inhibitors, such as clavulanic acid [5]. However, the cephamycins (cefoxitin and cefotetan) are resistant to the hydrolytic effect of these enzymes, this stability being afforded by their methoxy group. The carbapenems (Imipenem, ertapenem and meropenem) are also not affected by these enzymes [6,7].

ESBLs have emerged due to mutation from pre-existing broad-spectrum beta-lactamases TEM-1, TEM-2 (Temoniera) and SHV-1 (Sulphydryl variable), as a consequence of widespread use of 3rd generation cephalosporins as well as aztreonam [8]. The ESBL producers pose a serious antibiotic management problem as ESBL-producing genes often also carry resistance determinants for aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and even cotrimoxazole, making the microorganisms resistant to wide variety of drugs [9].

As the genes carrying ESBLs are easily transferred from one organism to the other via plasmids, the ESBL producing organisms have spread from hospitals to the community acquired infections, especially those of the urinary tract. ESBL producing organisms most commonly spread via unwashed hands of health care providers [10].

ESBL producers are associated with increased morbidity and mortality, especially amongst patients on intensive care and high-dependency units. The mortality rate in misdiagnosed UTI patients with ESBL producing organisms have ranged from 42-100% [11]. Therefore, accurate laboratory detection is important to avoid clinical failure due to inappropriate antimicrobial therapy, as, resistance to one of the extended-spectrum cephalosporins (ceftazidime, cefotaxime, or ceftriaxone), when mediated by an ESBL, means therapeutic resistance to all cephalosporins, aztreonam and penicillins, even when sensitivity test results may indicate otherwise [6].

Detection of ESBL producing organisms from samples such as urine may be important because this represents an epidemiological marker of colonization and the potential for transfer of such organisms to other patients. Therefore, it is imperative that microbiology laboratories should detect such infections promptly and with accuracy [7]. Molecular methods can accurately detect ESBL but facilities for them are not available in most of the laboratories especially in developing countries. The phenotypic method employs a beta-lactamase inhibitor, usually clavulanate, in combination with third generation cephalosporins for routine detection of ESBL production in Gram negative bacilli [9]. But the inhibitor-based confirmatory test approach is most promising for isolates that do not co-produce an inhibitor-resistant beta lactamase like AmpC [12]. A high-level production of AmpC may prevent the detection of an ESBL. This problem is frequently observed in tests with species or strains that produce a chromosomally encoded inducible AmpC beta-lactamase (*e.g., Enterobacter spp., Citrobacter spp., Serratia spp., Proteus spp.* and *Pseudomonas aeruginosa*). Plasmid mediated AmpC are found in *K. pneumoniae, E. coli, Salmonella spp.* and *Shigella spp*. Plasmid mediated AmpC genes were initially thought to be non-inducible, but inducible Amp C genes on plasmids have been reported [13].

In these organisms, clavulanate may act as an inducer of high-level AmpC production resulting in an increase in the resistance of the isolate to other screening drugs, producing a false-negative result in the ESBL detection test. Tazobactam and sulbactam are much less likely to induce AmpC beta-lactamases and are, therefore, preferable inhibitors for ESBL detection tests with these organisms [14]. Also, high-level AmpC production has a minimal effect on the activity of cefepime (4th generation cephalosporin), making this drug a more reliable detection agent for ESBLs in the presence of an AmpC beta-lactamase [15]. Hence, the present study was undertaken to determine the prevalence of ESBL production among Gram negative uropathogens using modified double disk synergy test (MDDST) along with their antimicrobial susceptibility pattern from urine samples collected from patients suspected of urinary tract infection at a tertiary care hospital in North India.

Materials and Methods

A hospital based cross-sectional study was done over a period of 4 months from January to April 2017. The study was approved by the Institutional Ethics Committee. Informed consent was taken prior to collection of urine samples from clinically suspected cases of UTI from both inpatients and outpatients and processed immediately in the clinical bacteriology laboratory of Microbiology Department. All the samples were processed by culturing on Blood agar and MacConkey agar and incubating aerobically at 37°C for 24 hours. The growth was identified as per the standard microbiological protocols and procedures [16]. The diagnosis of urinary tract infection was based on microscopic findings of significant pyuria with 10^4 leucocytes per ml (*i.e.*, 1 leucocyte per 7 high power fields on urine microscopy) and significant bacteriuria with colony count of >10^5 colony forming units per ml of single pathogen [17]. A total of 156 consecutive, non-duplicate Gram-negative bacilli recovered from urine samples were included, whereas, samples showing no growth or yielding growth of Gram positive bacteria and yeast isolates were excluded from the study.

Antibiotic Susceptibility testing

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) by Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines using antibiotic disks (HiMedia Laboratories, India) such as, ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), cefepime (30µg), aztreonam (30µg), piperacillin-tazobactam (100/10µg), norfloxacin (10µg), ciprofloxacin (5µg), trimethoprim-sulfamethoxazole (cotrimoxazole, 1.25/23.75µg), imipenem (10µg), meropenem (10µg), and nitrofurantoin (300µg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as standard quality control strains [18]. When an isolate was found to be resistant to ≥3 antimicrobial agents, it was considered as multidrug resistant [19]. The screening was done as per CLSI guidelines and all the isolates showing resistance to 3rd generation cephalosporins, namely ceftazidime, ceftriaxone and cefotaxime by Kirby-Bauer disk diffusion method, were considered as potential ESBL producers [18]. These isolates were further tested for confirmation of ESBL production by phenotypic method by putting modified double disk synergy test (MDDST).

Modified Double Disk Synergy Test (MDDST) for ESBL

All the potential ESBL producers were subjected to MDDST for phenotypic confirmation of ESBL production. The test inoculum (0.5 McFarland turbidity) was spread onto Mueller-Hinton agar using a sterile cotton swab. A disk of augmentin (20 µg amoxicillin and 10 µg clavulanic acid) was placed in the centre of MHA (HiMedia Laboratories, Mumbai, India); then disks of ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), aztreonam (30ug) and cefepime (30µg) were kept 16 to 20 mm (centre to centre) around augmentin disk. From the cefepime disk at a distance of 22 to 25 mm centre to centre disk of piperacillin-tazobactam (100/10µg) was placed. The plate was incubated at 37°C overnight. The organisms were considered to be ESBL producer when the zone of inhibition around cefepime or any of the extended-spectrum cephalosporin disks showed a clear-cut increase towards the piperacillin-tazobactam disk or augmentin disk [12,20,21].

Detection of AmpC among co-producers

All the isolates which showed a synergistic effect with piperacillin-tazobactam in MDDST were further tested for the AmpC enzyme production by AmpC disk test after an initial screening with a cefoxitin (30 µg) disk (HiMedia Laboratories, Mumbai, India). A lawn culture of a 0.5 McFarland's suspension of *Escherichia coli* ATCC 25922 was prepared on a Mueller-Hinton agar plate. A cefoxitin (30µg) disk was placed on the inoculated surface of the agar. A sterile plain disk (6mm) which was inoculated with several colonies of the test organism was placed beside the cefoxitin disk, almost touching it. The plates were examined after an overnight incubation at 37°C for either an indentation or a flattening of cefoxitin's zone of inhibition, indicating its enzyme inactivation (positive result), or an absence of distortion indicating no significant inactivation of cefoxitin (negative result) [22].

Statistical Analysis: Data were statistically analyzed using SPSS Data Editor Software, Chicago, version 20. The association between ESBL production and resistance to antibiotics was analyzed using Chi-square test and p value < 0.05 was considered as statistically significant.

Result

The distribution of 156 Gram negative uropathogens is shown in [Fig-1]. *Escherichia coli* constituted the most frequent isolate (44.2%), followed by *Klebsiella pneumoniae* (26.9%), *Pseudomonas aeruginosa* (12.2%), *Proteus mirabilis* (10.3%), *Citrobacter spp.* (5.8%) and *Acinetobacter spp.* (0.6%). Out of 156 Gram negative bacilli tested, 84 (53.8%) isolates were found to be potential ESBL producers by screening method. Amongst these 69 (44.2%) isolates were found to be confirmed ESBL producers by giving positive results in modified double disk synergy tests [Fig-2 and Fig-3]. The remaining 15 (9.6%) isolates gave negative results in MDDST. The distribution of various uropathogens on the basis of ESBL production is shown in [Table-1]. Of these 69 isolates, 6 (8.7%, 6/69) isolates were found to be AmpC beta-lactamase co-producers by MDDST. All these 6 isolates (3 *Escherichia coli,* 1 *Klebsiella pneumoniae,* 1 *Citrobacter spp.* and 1 *Pseudomonas aeruginosa*) were found to be resistant to cefoxitin (30µg) disk and positive for AmpC production by AmpC disk test [Fig-4]. Hence, the prevalence of ESBL producers and ESBL + AmpC beta-lactamase co-producers among urinary isolates was found to be 44.2% (69/156) and 3.8% (6/156) respectively.

Maximum ESBL production was seen among isolates of *Klebsiella pneumoniae* (61.9%), followed by *Escherichia coli* (53.6%). This finding was found to be statistically significant (p < 0.001). The prevalence of ESBL production was found to be 44.2% (69/156), with 61.3% (65/106) from inpatients and 8.0% (4/50) from outpatients [Table-2]. This difference was found to be statistically significant (p < 0.001).

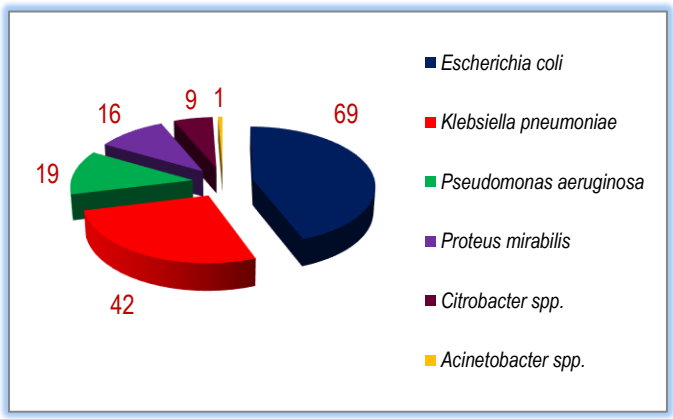

Fig-1 Distribution of various gram-negative bacilli isolated from the urine samples

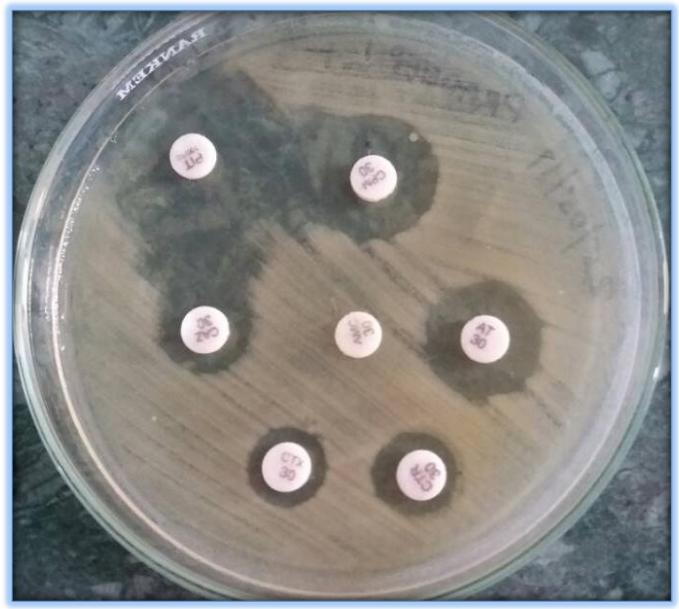

Fig-2 Modified double disk synergy test (MDDST) showing positive ESBL production as there is synergy between cefepime (CPM) and piperacillin-tazobactam (PIT) as well as between ceftazidime (CAZ) and PIT.

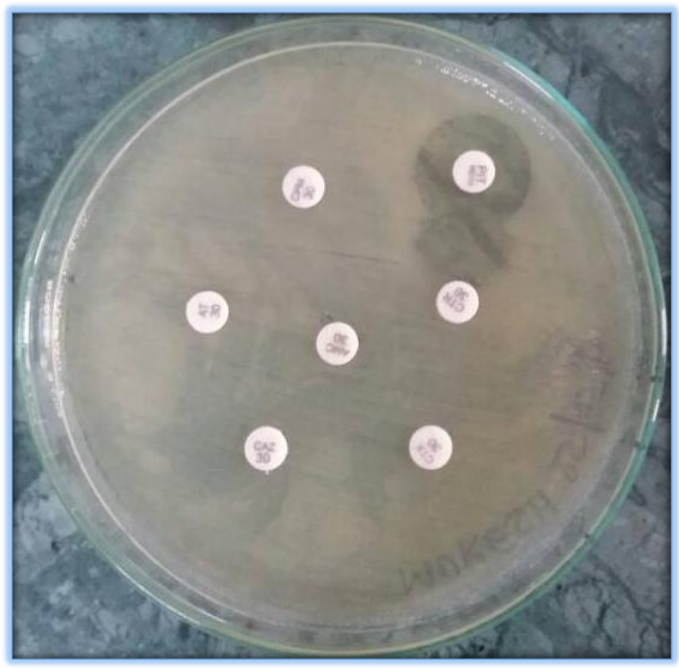

Fig-3 MDDST showing positive ESBL production as there is synergism between piperacillin-tazobactam (PIT) and ceftriaxone (CTR)

The antimicrobial susceptibility profile of all the 156 urinary isolates showed that ESBL producers possessed higher degree of resistance towards most of the commonly used antibiotics for the treatment of UTI. It was found that most of the tested drugs showed statistically significant correlation ($p < 0.05$) as regards to percentage of susceptible isolates among ESBL producers as compared to those of non-ESBL producers [Table-3].

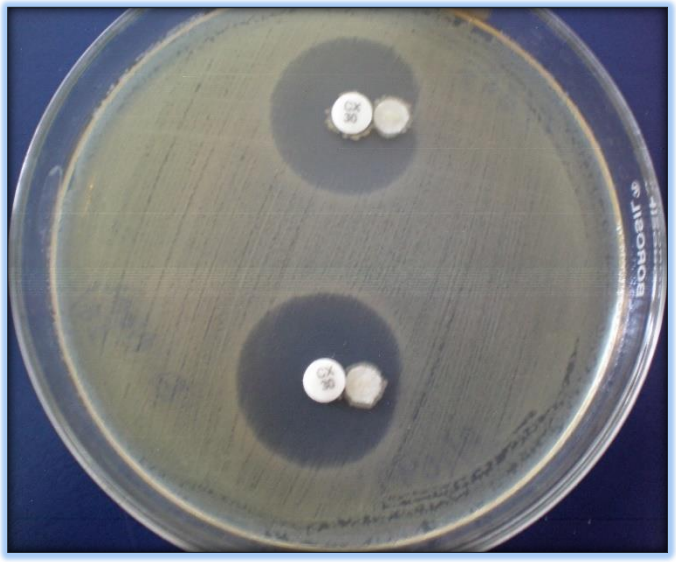

Fig-4 Positive AmpC disk test. In the upper one slight indentation is present and in the lower one flattening of zone of inhibition of cefoxitin disk is present

Multidrug resistance was more common in ESBL producers (62.2 %) as compared to non-ESBL producers (37.8%). The ESBL producers showed highly decreased susceptibility to all the third generation cephalosporins, as well as to ciprofloxacin and co-trimoxazole as compared to non-ESBL producers. These differences were highly statistically significant ($p < 0.001$). All the ESBL producers were found to be highly sensitive to imipenem (100%) and meropenem (100%), followed by sensitivity to nitrofurantoin (86.6%), piperacillin-tazobactam (81.2%) and norfloxacin (78.3%). Both the ESBL producers and non-ESBL producers were found to be equally sensitive to nitrofurantoin (86.6% & 91.3% respectively) and piperacillin-tazobactam (81.2% & 87.4% respectively) with no statistically significant difference between their susceptibility pattern ($p = 0.378$ and $p = 0.287$ respectively).

Table-1 *Distribution of various uropathogens on the basis of their ESBL producing status (N = 156)*

Isolated Uropathogens	ESBL producers N = 69 (44.2%)	Non-ESBL producers N = 87 (55.8%)	Total isolates tested N = 156 (100%)	Chi- Square (χ^2) value & *p value
Escherichia coli	37 (53.6%)	32 (46.4%)	69 (100%)	$\chi^2 = 25.543$, $p < 0.001$
Klebsiella pneumoniae	26 (61.9%)	16 (38.1%)	42 (100%)	
Pseudomonas aeruginosa	2 (10.5%)	17 (89.5%)	19 (100%)	
Proteus mirabilis	3 (18.8%)	13 (81.2%)	16 (100%)	
Citrobacter spp.	1 (11.1%)	8 (88.9%)	9 (100%)	
Acinetobacter spp.	0 (0.0%)	1 (100%)	1 (100%)	

*N = Number of isolates. * $p < 0.05$ was considered as statistically significant*

Table-2 Distribution of organisms according to their ESBL producing status and source of patients whose urine samples were tested (N = 156)

Source of Patients	ESBL producers N (%)	Non-ESBL producers N (%)	Chi- Square (χ^2) value & *p value
Inpatients (N = 106)	65 (61.3%)	41 (38.7%)	$\chi^2 = 39.158$, $p < 0.001$
Outpatients (N = 50)	4 (8.0%)	46 (92.0%)	
Total (N = 156)	**69 (44.2%)**	**87 (55.8%)**	

*N = Number of isolates. * $p < 0.05$ was considered as statistically significant*

Discussion

Urinary tract infection is among the most prevalent infectious disease in general population. An individual is at a significantly higher risk of being infected by ESBL-producing uropathogens if he/she is exposed to antibiotics for a long period of time, undergoes instrumentation or catheterization procedures, suffers from any severe illness and is admitted in the Intensive care units (ICUs) for a prolonged period, or is a resident in nursing homes or institutes which frequently use 3rd generation cephalosporins [3,9].

Table-3 Antibiotic susceptibility pattern among urinary isolates of gram negative bacilli for the commonly used antibiotics (N = 156)

Antibiotics tested	Percentage of susceptible isolates among ESBL producers (N = 69)	Percentage of susceptible isolates among Non-ESBL producers (N = 87)	Chi-Square (χ^2) & *p value
Ceftazidime	8.7%	29.9%	$\chi^2 = 10.596$, $p = 0.001$
Cefotaxime†	11.9%	47.1%	$\chi^2 = 20.230$, $p < 0.001$
Ceftriaxone†	5.9%	28.6%	$\chi^2 = 12.102$, $p = 0.001$
Cefepime	29.0%	49.3%	$\chi^2 = 6.678$, $p = 0.010$
Aztreonam‡	4.3%	26.7%	$\chi^2 = 13.755$, $p < 0.001$
Ciprofloxacin	15.9%	59.8%	$\chi^2 = 30.703$, $p < 0.001$
Norfloxacin‡	78.3%	82.6%	$\chi^2 = 0.453$, $p = 0.501$
Imipenem	100%	95.4%	$\chi^2 = 3.256$, $p = 0.071$
Meropenem	100%	97.7%	$\chi^2 = 1.607$, $p = 0.205$
Piperacillin-tazobactam	81.2%	87.4%	$\chi^2 = 1.135$, $p = 0.287$
Nitrofurantoin§	86.6%	91.3%	$\chi^2 = 0.777$, $p = 0.378$
Cotrimoxazole†	13.4%	44.3%	$\chi^2 = 15.764$, $p< 0.001$

*N = Number of isolates. *p value < 0.05 was considered as statistically significant. † These antibiotics were not tested against Pseudomonas aeruginosa, hence, N = 67 and 70 for ESBL and Non-ESBL producers respectively.‡ These antibiotics were not tested against Acinetobacter spp., hence, N = 86 for Non-ESBL producers. § This antibiotic was not tested against both Pseudomonas aeruginosa and Acinetobacter spp., hence, N = 67 & 69 for ESBL and Non-ESBL producers respectively*

Most of the nosocomial UTIs are caused by Gram-negative bacteria, particularly *Escherichia coli, Klebsiella spp., and Pseudomonas spp*. Collectively, they account for more than 80% of the culture positive cases of UTIs and the rest are caused by Gram positive cocci and fungi [23]. ESBL producing strains of Gram negative bacilli have emerged due to selective pressure imposed by extensive use of antibiotics, especially in ICUs [24]. Recently, the incidence of ESBL producing isolates has considerably increased resulting in the limitation of therapeutic options, thereby making the treatment of UTIs a challenging issue for clinicians [4,25]. As ESBL producing organisms which were initially confined to hospitalized patients only are now a day found in outpatients also, therefore, continuous monitoring systems and effective infection control measures are absolutely necessary to prevent the rapid and worldwide spread of ESBL producing organisms [23]. In the present study, amongst 156 urinary isolates, *Escherichia coli* constituted the most frequent isolate (44.2%) followed by *Klebsiella pneumoniae* (26.9%). This finding corresponds well with those of previously done studies which also reported *Escherichia coli* and *Klebsiella spp*. as the most common organisms causing UTI [2,26]. The detection of ESBLs in strains that produce inducible chromosomal AmpC beta-lactamase is nearly impossible with confirmatory tests using clavulanate as the ESBL inhibitor, since AmpC beta-lactamases do interfere with the inhibition of ESBLs by clavulanate and false-negative results using double disk synergy test (DDST) or phenotypic confirmatory disk diffusion test (PCDDT) have been reported in some earlier studies [12,15]. Hence, to overcome this problem, we have used modified double disk synergy test (MDDST) using cefepime as indicator agent and piperacillin-tazobactam as ESBL inhibitor. Some previous done studies have reported good results for detecting ESBL producers in the presence of AmpC co-production [12,27].

In our study out of 156 uropathogens, 84 (53.8%) were potential ESBL producers and amongst these 69 (44.2%) isolates were found to be confirmed ESBL producers by giving positive results in MDDST. Out of these 69 isolates, 6 were found to co-produce AmpC enzyme also. Hence, in the present study the prevalence of ESBL production among uropathogens was found to be 44.2% and that of AmpC co-producers was 3.8%. A study from Egypt done on uropathogenic *Escherichia coli* detected pure ESBL production among 49% isolates, whereas, 15.5% isolates were co-producers of ESBL and AmpC [28]. Previous studies from India have reported ESBL production varying from 6% to 87%, with much higher prevalence (58%) among urinary isolates of Gram negative bacteria [2,29,30]. Another recent study from India reported even higher prevalence of ESBL production of 66.9% among uropathogens [23]. It was found that prevalence of ESBL producers varies greatly in different geographical areas. This geographical difference may be due to different patterns of antibiotic use in different areas [19].

In our study, maximum ESBL production was seen in *Klebsiella pneumoniae* (61.9%) followed by *Escherichia coli* (53.6%). This finding is in agreement with studies done by other Indian workers who have reported higher prevalence of ESBL production among *Klebsiella spp*. as compared to *Escherichia coli* [2,11,26,29].

In the present study, the prevalence of ESBL production among uropathogens was found to be more (61.3%) from inpatients as compared to those from outpatients (8.0%). This is comparable with findings of another study which also showed higher frequency of ESBL producers isolated from inpatients (64.64%) as compared to outpatients (16.89%) [31]. The reason for predominance of ESBL producers among inpatients may be due to the presence of risk factors among hospitalized patients for colonization and infection by ESBL producing organisms [10].

The antibiotic susceptibility profile of the urinary isolates included in our study showed that 62.2% of ESBL producing isolates were resistant to three or more drugs, whereas, multidrug resistance in non-ESBL producers was seen in only 37.8% isolates. This finding is comparable to another study which also showed higher rates of multidrug resistance (90.5%) among ESBL producers as compared to non-ESBL producers (68.9%) [2].

In the present study all the 69 urinary isolates of ESBL producers were found to be 100% sensitive to imipenem and meropenem. These organisms were found to be more sensitive to nitrofurantoin (86.6%) as compared to norfloxacin (78.3%). Also, ESBL producers were found to be quite sensitive to piperacillin-tazobactam (81.2%). This finding correlate with another study which also showed that ESBL producing *Escherichia coli* from urine samples were highly (>80-95%) resistant to norfloxacin, much less resistant to nitrofurantoin (11.66%) and 100% sensitive to imipenem [11]. Hence, nitrofurantoin and piperacillin-tazobactam may be used to effectively treat UTI caused by such resistant uropathogens and carbapenems may be used as reserve drugs for serious UTI cases.

Conclusion

The knowledge of prevalence of ESBL producers is essential for proper management of patients and also to prevent spread of such resistant strains by avoiding injudicious use of antibiotics particularly third generation cephalosporins. The present study showed that ESBL production was quite high among uropathogens. The situation is even worsened due to multidrug resistance seen in ESBL producers. As phenotypic confirmation of ESBL production could be easily and effectively done, hence, routine ESBL testing for uropathogens along with conventional antibiotic susceptibility testing would be useful for deciding proper treatment of all cases of UTI and the therapy must be started only after the urine culture and sensitivity testing has been done.

Application of Research

By performing MDDST test, we detected ESBL production among 69 (44.2%) isolates. Of these 8.7% (6/69) isolates were found to be AmpC beta-lactamase co-producers. Hence, proper treatment of such patients with UTI could be initiated on time.

Research Category: Drug resistance among uropathogens

Abbreviations:

MDDST – Modified double disk synergy test
UTIs – Urinary tract infections
ESBLs – Extended spectrum beta lactamases

TEM – Temoniera
SHV – Sulphydryl variable
ATCC – American Type Culture Collection

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