

# Phenotypic confirmatory disc diffusion test (PCDDT), double disc synergy test (DDST), E-test OS diagnostic tool for detection of extended spectrum beta lactamase (ESBL) producing Uropathogens

## Abstract

**Background:** The aim of this study was to probe the best diagnostic tool for the detection of Extended Spectrum  $\beta$ -lactamase (ESBL) producing uropathogen and their antimicrobial susceptibility profile to treat the infections properly.

**Methods:** Clinical samples of urine were cultured on Cysteine Lactose Electrolyte Deficient (CLED) Agar medium. Antimicrobial sensitivity tests were carried out by Kirby-Bauer disc diffusion method. Phenotypic methods were used for further confirmation of  $\beta$ -lactamase production by phenotypic confirmatory disc diffusion test (PCDDT), double disc synergy test (DDST) and by E-test for ESBL production. Aswab on Mueller-Hinton (MH) agar plates was used for further studies and the Optical Density (O.D.) of the cultures was set to 0.1 (at 530nm).

**Results:** Sample size N= 200 was selected from patients suffering from UTI. Out of the 200 samples, n=141 samples yielded Aerobic Gram Negative Bacteria (AGNB). The commonest organism isolated was *E. coli* n=108, best antimicrobial result of 95% was shown by imipenem. Among the AGNB isolates, 20 organisms (12.98%) were ESBL producers. *E. coli* showed highest ESBL production of 85%. The most effective antimicrobial in ESBL producers was Imipenem (84%) Augmentin was least sensitive (05%).

**Conclusion:** ESBL production is a common phenomenon in UTI patients and screening by DDST for these enzymes is a good epidemiological tool to assess the overall situation in a certain setup. It was also seen that Imipenem (Carbapenem) is the drug of choice.

**Keywords:** ESBL, CLED, DDST, AGNB isolates, imipenem

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**Abbreviations:** ESBL, extended spectrum  $\beta$ -lactamase; CLED, cysteine lactose electrolyte deficient; PCDDT, phenotypic confirmatory disc diffusion test; MH, mueller-hinton; OD, optical density; AGNB, aerobic gram negative bacteria; UTI, urinary tract infection; OPD, outpatient door; COD, casualty outdoor; DDST, double disc synergy test

## Introduction

Urinary tract infection (UTI) is one of the most common problems for people to get hospitalized or get medical advice and is one of the most common nosocomial infections. Examination of bacterial spectrum and antibiotics sensitivity is necessary for the empirical treatment of nosocomial urinary tract infections.<sup>1</sup> Different aerobic Gram-negative bacteria (AGNB) produce beta lactamases which cause resistance to beta-lactam agents.<sup>2</sup> ESBLs were discovered in 1980, nursing homes and hospitals are found as main reservoirs of extended spectrum beta- lactamase (ESBL) producing bacteria. Hospital patients serve as a reservoir for these resistant organisms.<sup>3-5</sup> Enterobacteriaceae produce ESBL and are globally serious nosocomial pathogens.<sup>6,7</sup> ESBLs producing organisms are clinically important and remain a major cause of treatment failure with Cephalosporin's and other classes of antibiotics in the world.<sup>8</sup>

Third generation of Cephalosporin's was introduced in 1980, which brought a relief in the fight against beta-lactamase resistance to antibiotics. Cephalosporin's were introduced due to increased resistance by beta-lactamases produced by different Gram-negative bacteria such *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, *Proteus spp.* and may disseminate new host, for example, produced by *Neisseria gonorrhoea* and *Hemophilus influenzae*.<sup>9</sup> ESBLs are plasmid or chromosomal mediated  $\beta$ -lactamases ( $\beta$ -lactamases are enzymes that cleave the  $\beta$ -lactam ring) of already existing mutant  $\beta$ -lactamases (SHV-1, TEM-1 and TEM-2) as a result of frequent use of third generation cephalosporin's and aztreonam.<sup>10,11</sup> Clavulanic acid or sulbactam are used as inhibitors of ESBLs.<sup>5</sup> However Amp-c lactamase overproduction and porin loss result in the production of inhibitor resistant ESBLs.<sup>12</sup> The severity and location of infection, liver function, kidney function, geographical resistance and the presence of implants will determine the efficacy of antibiotics. It is also observed that the effectiveness of antibiotics also depend upon the lactation and age of pregnancy.<sup>2</sup>

## Materials and methods

### Collection of Samples

Urine samples of symptomatic UTI were identified by conventional

techniques. Samples of urine were collected from midstream clean catch, transurethral catheterization, plastic bag, or supra-pubic aspiration according to clinical status and age of patient. N=200 was selected for this study.

- i. Inclusion Criteria: All indoor and Outpatient Door (OPD) samples from UTI patients were included irrespective of age and sex.
- ii. Exclusion Criteria: All clinical urine samples for routine examination and other than UTI patients were excluded.

### Sample processing

Clinical samples of urine were collected from indoor, Casualty outdoor (COD) and OPD patients at Microbiology Laboratory Department and were cultured on CLED Agar and incubated for 24hours at 37°C. Cultures of bacteria were processed for sub-culturing, Gram staining and Biochemical tests for identification. Antimicrobial sensitivity testing was carried out by Kirby-Bauer disc diffusion method and Phenotypic Confirmatory Disc Diffusion Test (PCDDT), Double Disc Synergy Test (DDST) and E-Test was For ESBL detection.

### Identification of isolates

Identification of all aerobic gram negative bacteria (*E.coli*, *Klebsiellaspp*, *Pseudomonas aeruginosa*, *Proteusspp.*) was performed by morphological characteristics, Gram staining, biochemical tests and specific disc pattern for ESBL production. Different biochemical tests were performed on the isolates for confirmation of all aerobic gram negative bacterial colonies. Known controls were also put up

**Table 1** Interpretation of biochemical tests on API 20E strip

Test	Active ingredients	Reactions/enzymes	Result	
			Negative	Positive
ONPG	2-nitrophenyl-βD-galactopyranoside	beta-galactosidase	Colorless	Yellow
ADH	L-arginine	Arginine dihydrolase	Yellow	Red/orange
LDC	L-lysine	Lysine decarboxylase	Yellow	Red/orange
ODC	L-ornithine	Ornithine decarboxylase	Yellow	Red/orange
CIT	Trisodium citrate	Citrate utilization	Pale green/yellow	Blue-green/blue
H2S	Sodium thiosulfate	H2S production	Colorless	Blackdeposit/thin line
URE	Urea	Urease	Yellow	Red/orange
TDA	L-tryptophane	Tryptophane deaminase	Yellow	TDA/immediate.1 Reddishbrown
IND	L-tryptophane	Indole production	Colorless	Jammes/immediate.2Pink
VP	Sodium pyruvate	Acetoin production	Colorless	Vp1+Vp/10 min.3Pink/red
GEL	Gelatin	Gelatinase	No diffusion	Diffusion of black pigment
GLU	D-glucose	F/O4, glucose	Blue-green/blue	Yellow
MAN	D-mannitol	F/O, mannitol	Blue-green/blue	Yellow
INO	Inositol	F/O, inositol	Blue-green/blue	Yellow
SOR	D-sorbitol	F/O, sorbitol	Blue-green/blue	Yellow
RHA	L-rhamnose	F/O, rhamnose	Blue-green/blue	Yellow
SAC	D-sucrose	F/O, saccharose	Blue-green/blue	Yellow
MEL	D-melibiose	F/O, melibiose	Blue-green/blue	Yellow
AMY	Amygdalin	F/O, amygdalin	Blue-green/blue	Yellow
ARA	L-arabinose	F/O, arabinose	Blue-green/blue	Yellow

with each biochemical test.<sup>13</sup>

### Indole production test

*Pseudomonas* was indole negative and *E.coli* was indole positive.<sup>13</sup>

- i. MR-VP Test: *E.coli* was MR positive and VP negative while *Klebsiella spp* was MR negative and VP positive.<sup>13</sup>
- ii. Citrate Utilization Test: Positive control was *Klebsiella pneumoniae* and negative control was *E.coli* ATCC 25922.<sup>13</sup>
- iii. Oxidase Test: Positive control was *P. aeruginosa* ATCC 27853 and negative control *E. coli* ATCC 25922.<sup>13</sup>

### Analytical profile index (API) 20E

Non-conclusive samples were run on API and were interpreted according to API chart. API 20E is known as a standardized identification system for gram negative rods and *Enterobacteriaceae*. It has 20 biochemical parameters. Bacterial suspension was prepared equal to 0.5 McFarland index. A homogeneous bacterial suspension was achieved by careful emulsification. Strips wells were charged with bacterial suspension. Tubes were filled according to the test requirement. H<sub>2</sub>S, ADH, LDC, Urea and ODC tests were incubated with overlaying mineral oil. To check the quality of suspension, a purity plate was prepared by inoculation from the suspension. After 18 to 24 hours at 35±2°C incubation, add reagents in respective wells. Seven digits numerical profile was determined after positive results and which was looked up in API code comparable to organism identification<sup>13</sup> (Table 1).

## ESBL screening using PCDDT, DDST and e test as diagnostic tool

Phenotypic methods were used for further confirmation of  $\beta$ -lactamase production for all the isolates showing resistance to 3<sup>rd</sup> generation cephalosporin, namely Ceftazidime, Cefotaxime and Ceftriaxone. Swab on Mueller-Hinton (MH) agar plates was used for further studies and the Optical Density (O.D.) of the cultures was set to 0.1 (at 530nm). The screening was done according to CLSI instructions.<sup>14</sup>

### Phenotypic Confirmatory Disc Diffusion Test (PCDDT) For ESBL Detection

A combination of clavulanic acid (10 mcg) and ceftazidime (30 mcg) was used. Both discs were placed on Muller Hinton agar plates which were earlier swabbed by respective culture and incubated for 24hrs. At 37°C. More than 5mm increase in the zone diameter for Ceftazidime-Clavulanic acid was considered positive ESBL production<sup>14</sup> (Figure 1).

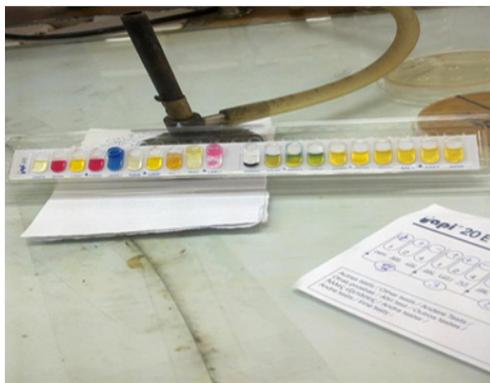


Figure 1 Extended spectrum  $\beta$ -lactamase.

### Double Disc Synergy Test (DDST)

Five antibiotics were used for DDST namely Aztreonam (30mcg), Amoxicillin-Clavulanic acid (20/10mcg), Ceftriaxone (30mcg), Ceftazidime (30mcg) and Cefotaxime (30mcg). At center Amoxicillin-Clavulanic acid disc was placed and these discs were placed at a distance of 1.5cm. Development of the zone of inhibition towards the Clavulanate disc at 37°C after 24hrs incubation was indicative of a potential ESBL positive organism<sup>15</sup> (Figure 2).

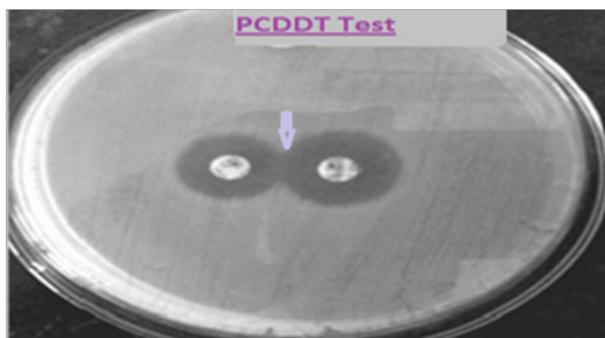


Figure 2 Phenotypic confirmatory disc diffusion test.

### Confirmation of ESBL Production by E-Test

Multi-Enzyme (Ezy) Strips were used for confirmation of ESBL production. These strips differ from the conventional E-strips in that

they contain a gradient of 3 antibiotics with and without Clavulanic acid on either sides of the strip respectively instead of one antibiotic and so these strips are different from conventional strips. The Multi-Ezy Strips have Cefotaxime, Cefipime and Ceftazidime (Noted as MIX side) on one side in a two-fold gradient and the same antibiotics with Clavulanic acid (Noted as MIX+side) on the other side. A ratio of inhibition zones of MIX and MIX+for  $\geq 8$ mm was found as a positive E-test<sup>14</sup> (Figure 3).

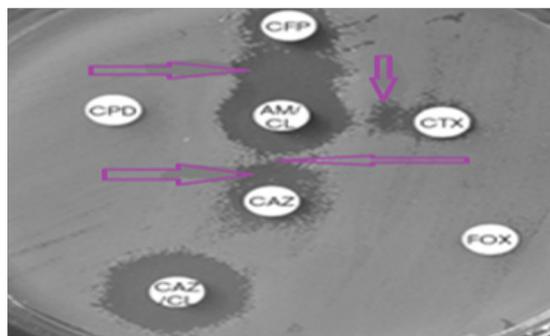


Figure 3 Double disc synergy test.

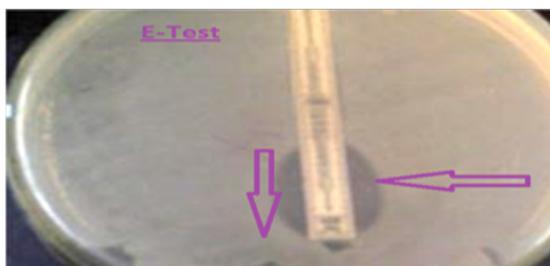


Figure 4 Confirmation of ESBL by E-test.

## Results

Two hundred samples N=200 were selected for current study, samples were collected from patients suffering from urinary tract infection (UTI). N=141 were AGNB. We got 154 different pathogenic strains (some samples yielded double and triple growths). The most common organism isolated was *E.coli* i.e. n=108, followed by *Coliform spp*, *Klebsiellaspp*, *Pseudomonas aeruginosa* and *Proteus spp* in an order n=22, n=12 n=04 and n=03 out of 154 pathogenic strains yielded from 141 positive samples respectively. Organism's breakup and percentages are given in Table 2. Of the N=154, best results of 95% shown by imipenem, 84% and 80% sensitivity were seen in case of Tazocin and Amikacin respectively. Augmentin showed the poorest sensitivity of 6%. Individual details of the susceptibility pattern of the applied panel of antibiotics are shown in Table 3.

Table 2 Overall organisms identified

Organisms	No of Isolates N=154
<i>Enterococcus</i>	05(3.5%)
<i>E.coli</i>	108(70%)
<i>Klebsiellaepneumoniae</i>	12(08%)
<i>Pseudomonas aeruginosa</i>	04(2.5%)
<i>Coliform</i>	22(14.28%)
<i>Proteus spp</i>	03(02%)
Total	154(100%)

**Table 3** Overall sensitivity pattern

Antibiotics	Sensitive	Intermediate	Resistant
Amikacin(AK)(applied in 93cultures)	(74) 80%	(05) 05 %	(14) 15%
Augmentin(AUG)(154)	(09) 06%	(01) 0.6%	(144) 93.4%
Tazocin(TZP)(86)	(72) 84 %	(08) 09%	(06) 07%
Cefepime (FEP)(07)	(02) 28%	(01) 14%	(04) 58%
Ceftazidime(CAZ)(96)	(23) 24%	(02) 02%	(71) 74%
Ceftriaxone(CRO)(98)	(20) 20%	(02) 02%	(78) 78%
Ciprofloxacin(CIP)(76)	(15) 20%	(06) 08%	(55) 72%
Imepenem(IMI) 115	(109) 95%	(03) 2.5%	(03) 2.5%
Gentamycin(CN)(30)	(13) 43%	(01) 03%	(16) 54%
Nitrofurantoin (F)(110)	(82) 75%	(05) 04%	(23) 21%
Tobramycin(TOB)(05)	(01) 20%	(01) 20%	(03) 60%
Suxzone(CES)(29)	(23) 79%	(01) 3.5 %	(05) 15%
Chloramphenicol(C)(5)	(02) 40%	(01) 20%	(02) 40%
Fosfomycin(FOS)(18)	(04) 22 %	(01) 06%	(13) 72%
Cefotaxime(CTX)(37)	(13) 35%	(01) 2.7%	(23) 62.3%
Moxyfloxacin(MXF)(51)	(19) 37%	(01) 02%	(31) 61%
Nalidixic Acid(NA)(18)	(01) 5.5%	(01) 5.5%	(16) 89%
Ampicillin(AMP)(06)	(01 ) 17%	(01) 17%	(04) 66%
Sulphmethazole((SXT)(29)	(03) 10%	(01) 4%	(25) 86%
Teicoplanin(TEC)(07)	(05) 72%	(01) 14%	(01) 14%
Vancomycin(VAN)(05)	(03) 60%	(01) 20%	(01) 20%
Pipedinic Acid(PIP)(37)	(07) 19%	(01) 03%	(29) 78%
Meropenem(MRP)(09)	(07) 78 %	(01) 11%	(01) 20%
Polymixin B(PB)(05)	(03) 60%	(01) 20%	(01) 20%

Among the AGNBs isolates, n=20 (12.98%) were ESBL producers. These organisms included *E.coli*, *Enterococcus*, *Coliform* and *Proteus spp*. *E.coli* showed highest ESBL production of 17(85%). Lesser degree of ESBL production was also seen in case of *Proteus spp* (05%), *Coliform spp* (05%) and *Enterococcus* (05%) (Table 4). The most effective antimicrobial in ESBL producers was Imipenem (84%) followed by Amikacin and Tazocin respectively. Augmentin was least sensitive (05%) as shown in Table 5. Non conclusive isolates, which were not identified by cultural characteristics and Gram staining, were run on API20E Figure 1. PCDDT was used as one of the confirmatory tests for ESBL production as recommended by CLSI. The Ceftazidime discs alone or in combination with clavulanic acid were applied on Muller Hinton (MH) agar plates having the inoculation of test strain. A 5mm increase in the zone of inhibition of the combination discs or ceftazidime alone were considered as positive ESBL production test. The test stain is inoculated on Muller Hinton agar plates. Amoxiclave/Clavulanic acid disc was applied in the center of agar plate while Ceftazidime, Ceftriaxone, Cefotaxime and Cefixime were applied at a distance of 20mm from center to center from each other. The plates were incubated at. An enhancement of the zone of inhibition greater than 5mm on the amoxiclave side of the disc were considered as ESBL producers. The test organism was inoculated on MH agar on which

E-test strips were applied. The plates were incubated at 37°C for 16-18hours. A ratio of inhibition zones of TZ and TZL for ≥8mm was found as a positive E test *Escherichia coli* ATCC 25922 was used as the negative control and ESBL producing *Klebsiella pneumoniae* ATCC 700603 was used as the positive control throughout the study.

**Table 4** Overall distribution of ESBL positive isolates (N=20).

Isolates	ESBL positive
<i>Enterococcus</i>	01(05%)
<i>E.coli</i>	17(85%)
<i>Coliform</i>	01(05%)
<i>Proteus spp</i>	01(05%)
Total	20(100%)

**Table 5** Susceptibility pattern of ESBL producers: (N=20)

Antibiotics	Sensitive	Intermediate	Resistant
Amikacin(AK)(used in 20cultures)	(16) 80%	(02) 10 %	(02) 10%
Augmentin(AUG)(20)	(01) 5%	(01) 05%	(18) 90%
Tazocin(TZP)(20)	(15) 75 %	(03) 15%	(02) 10%
Cefepime(FEP)(06)	(02) 33.34%	(01) 16.66%	(03) 50%
Ceftazidime(CAZ)(20)	(0) 0%	(02) 10%	(18) 90%
Ceftriaxone(CRO)(20)	(0) 0%	(03) 15%	(17) 85%
Ciprofloxacin(CIP)(20)	(06) 30%	(02) 10%	(14) 70%
Imepenem(IMI)(19)	(16) 84.21%	(02) 10.52%	(01) 5.26%
Polymixin B(PB)(20)	(11) 55%	(04) 20%	(05) 25%

## Discussion

The development and discovery of antibiotics was no doubt one of the greatest achievements of modern medicine. Unfortunately the appearance of bacterial resistance to antibiotics is frightening for the efficacy of many antimicrobial agents due to which hospital stay of the patients has increased and as a result great increase in the economic burden found.<sup>16</sup> In this study, maximal numbers of ESBL producing organisms were derived from indoor patients. This could be due to the fact that infections caused by nosocomial organisms in admitted patients are more common than in OPD & COD. The continuous exposure to the hospital environment plus the fact that the patients are on antibiotics or have prosthetic devices makes them more susceptible to infection.<sup>17</sup> A similar finding was demonstrated by Supriya et al.<sup>17</sup>

In this study the commonest organism isolate was *E.coli* i.e. 70%, followed by *coliform* 14%, *Klebsiella spp* (8%), *Enterococcus* 3.5%, *Pseudomonas aeruginosa* 2.5%, and *Proteus spp* (2%). Although predominant organisms vary from one institution to another. This difference could be attributed by differences in geographical locations and standards of hygiene.<sup>2</sup> In this study the high level of resistance to Augmentin may be as a result of inadvisable use of these drugs in the study population which lead to high selection pressure of resistant bacteria. Prescription of these drugs is common; therefore the use of these prescribed drugs in our settings is questionable.<sup>18</sup> These are widely used because they are more affordable than other antibiotics. Fluor quinolones are effective agents for treatment of gram negative bacterial infection and resistance to ciprofloxacin is an early warning sign.<sup>8</sup> In our country irrational and indiscriminate antibiotic usages as well as lack of effective antibiotic policies at all levels of treatment are the main contributory factors towards growing antimicrobial resistance. Mortality, morbidity and cost of treatment have considerably risen because of these resistant bacteria.<sup>19</sup>

Some Microbiology Laboratories may fail to detect ESBL positive organisms and can sometimes erroneously detect isolates to be sensitive to any of the third generation cephalosporin's (3GC) leading to therapeutic failures. Therefore, two more drugs are added to the routine panel of antimicrobial drugs for the regular detection of ESBL by PCDDT method. Every laboratory where molecular methods cannot be performed, should perform this technically simple PCDDT method, as this method is highly sensitive and specific compared to genotypic methods.<sup>20</sup> To detect ESBL isolates many other methods like E Tests and Double Disc Synergy Test (DDST) commercially available, besides PCDDT. But these cannot be used routinely are

expensive. Whereas, PCDDT method is useful in busy diagnostic Microbiology laboratories, technically easy, reproducible, sensitive and cost effective.<sup>20</sup>

In order to quantify the synergy between extended-spectrum cephalosporin's and clavulanate, E-tests have been developed. The E-tests called CT/CTL, TZ/TZL and PM/PML are two-sided strips containing gradients of cefotaxime (CT), or ceftazidime (TZ) or cefepime (PM), either alone (at one end of the strip), or combined with clavulanate 4mg/L (on the other end). The MIC value of the tested drug is reduced by more than three doubling dilution steps (MIC ratio $\geq$ 8) in the presence of clavulanate, ESBL test is considered as positive.<sup>21</sup> This test is also considered as positive when (a) a deformation of the CT, TZ or PM inhibition ellipse at the tapering end (b) or when there is either a rounded zone (phantom zone) just below the lowest concentration of CTL, TZL or PML gradients, ESBL production is indicated by the presence of a phantom zone or an ellipse deformation. Interpreting results of the ESBL E-test strips requires training and is delicate. In a recent study, it has been reported that laboratories may fail to interpret correctly the inhibition ellipse in 30% of cases.<sup>22</sup> In addition; ESBL detection by E-test may fail when the MIC values for cephalosporins fall outside the range of MICs available on the test strip.<sup>23</sup> Double-disk synergy test (DDST) was the first test specifically designed to detect ESBL production in Enterobacteriaceae.<sup>24</sup> It was initially designed to differentiate between cefotaxime-resistant strains, i.e., those overproducing cephalosporinase, and those producing ESBLs. The test is performed on agar with a 30- $\mu$ g disk of cefotaxime (and/or ceftriaxone and/or ceftazidime and/or aztreonam) and a disk of amoxicillin-clavulanate (containing 10 $\mu$ g of clavulanate) positioned at a distance of 30mm (center to center), i.e., at the distance provided by several types of disk-dispenser. The test is considered as positive when a decreased susceptibility to cefotaxime is combined with a clear-cut enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk, often resulting in a characteristic shape zone referred to as 'champagne-cork' or 'keyhole'. The DDST was first used in epidemiological studies to assess the spread of ESBL-producing Enterobacteriaceae in French hospitals.<sup>25,26</sup> It has been shown to work well with a wide range of Enterobacteriaceae species and ESBL types, and it is generally regarded as a reliable method for the detection of ESBLs, although it is sometimes necessary to adjust the disk spacing. It is important to note that reducing the distance between the clavulanate-containing disk and the third-generation cephalosporin disk (e.g., to 20mm) significantly improves the test sensitivity.<sup>27,28</sup> Since the antibiotic disks are routinely spaced 30mm apart by several types of

marketed disk dispenser, it is necessary, when the result of the test is equivocal (i.e., clear decrease in susceptibility to third-generation cephalosporins without clear synergy), to perform an additional test by arranging the disks by hand with narrower distances.<sup>28</sup>

## Conclusion

In our set up, 12.9% isolates were ESBL producers. ESBL production is a common phenomenon in UTI patients and screening by DDST for these enzymes is a good epidemiological tool to assess the overall situation in a certain setup. It was also seen that Imipenem (Carbapenem) is the drug of choice. Hand washing still can reduce the spread of ESBL. Avoid prolong hospitalization wherever possible. Remove catheter/needles and other prosthetics as early as possible. Need for formulation of appropriate hospital antibiotics policy to avoid misuse and overuse of antibiotics. Formulation of effective infection control policy. Educate hospital staff against danger of cross infection. The knowledge of the resistance pattern of bacterial strains in a geographical area will help to guide the appropriate antibiotic use and such institutional studies will help to formulate an empirical antibiotic policy to treat Gram negative infections.

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## Conflict of interest

The author declares no conflict of interest.

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