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To Evaluate different Phenotypic Diffusion Methods in the identification of ESBL producing Uropathogenic Escherichia coli

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Abstract

Introduction: A urinary tract infection (UTI) is an infection in any part of urinary system namely kidneys, ureters, bladder and urethra. Most infections involve the lower urinary tract the bladder and the urethra. For complicated UTIs, the order of prevalence for causative agents, following UPEC as most common, is Enterococcus spp., K. pneumoniae, Candida spp., S. aureus, P. mirabilis, P. aeruginosa and GBS. Extended spectrum β lactamases (ESBLs) producing pathogens exhibit resistance not only to newer β-lactams, including third generation cephalosporins and monobactams.

Materials and Methods: This prospective study was conducted in the Department of Microbiology, Index Medical College, India. A total of 300 consecutive urine samples were screened from patients with symptomatic UTI. Clean-catch midstream urine samples were collected in sterile disposable container and processed within one hour. Semi quantitative loop measuring 2.2 mm diameter with a holding capacity of 0.005 ml was employed to culture urine on CLED agar and MacConkey's agar. The inoculated plates were incubated over night at 37^{o}C. Isolates in significant number (colony count $\geq 10^5$ CFU/ml) was identified by standard procedures.

Results: In our study maximum number of patients were in the age gathering of 21-30 years age gathering which were 34% (n =68) of complete followed by age group 31–40 years having 26.5% (n = 200). The present study shows the pathogens causing UTIs and their antibiotic susceptibility pattern. Escherichia. coli 45.6% was the predominant pathogen followed by Klebsiella pneumoniae 28.6%, Proteus spp. 9.6%, Staphylococcus aureus 8.6%, Citrobacter spp. 3.3%, Pseudomonas aeruginosa 2.6%, Enterococcus faecalis 0.6%, Staphylococcus saprophyticus 0.3% and Acinetobacter spp. 0.3%. ESBL positives with augmentation zone of inhibition diameter is ≥ 10 mm. The mean zone augmentation (95% CI) was 17.4 (12.9, 22.1) mm for ceftazidime, 14.3 (12.1, 19.3) mm for cefotaxime and 17.1 (12.0, 17.3) mm for cefpodoxime.

Conclusion: UTIs are the leading cause of outpatient visits, so understanding the epidemiology of Uropathogenic Escherichia coli can contribute to better treatment

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and reduced morbidity of UTIs. This study has determined that ESBL-producing Uropathogenic Escherichia coli isolates are present in India, hospitals, with 24.2% of the isolates being resistant to the commonly prescribed cephalosporin drugs for UTIs.
Keywords: Uropathogenic, Escherichia coli, Phenotypic diffusion methods.

Introduction

A urinary tract infection (UTI) is an infection in any part of urinary system namely kidneys, ureters, bladder and urethra. Most infections involve the lower urinary tract the bladder and the urethra. [1] Women are at greater risk of developing a UTI than are men. Infection limited to your bladder can be painful and annoying. However, serious consequences can occur if a UTI spreads to kidneys. [2] Clinically, UTIs are categorized as uncomplicated or complicated. Uncomplicated UTIs typically affect individuals who are otherwise healthy and have no structural or neurological urinary tract abnormalities; these infections are differentiated into lower UTIs (cystitis) and upper UTIs (pyelonephritis). Several risk factors are associated with cystitis, including female gender, a prior UTI, sexual activity, vaginal infection, diabetes, obesity and genetic susceptibility. [3]

Complicated UTIs are defined as UTIs associated with factors that compromise the urinary tract or host defence, including urinary obstruction, urinary retention caused by neurological disease, immunosuppression, renal failure, renal transplantation, pregnancy and presence of foreign bodies such as calculi, indwelling catheters. [4] UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. The most common causative agent for both uncomplicated and complicated UTIs is uropathogenic Escherichia coli (UPEC). [5] For the agents involved in uncomplicated UTIs, UPEC is followed in prevalence by Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcusfaecalis, group B Streptococcus (GBS), Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus and Candida spp. [6]

For complicated UTIs, the order of prevalence for causative agents, following UPEC as most common, is Enterococcus spp., K. pneumoniae, Candida spp., S. aureus, P. mirabilis, P. aeruginosa and GBS. [7] Extended spectrum β lactamases (ESBLs) producing pathogens exhibit resistance not only to newer β-lactams, including third generation cephalosporins and monobactams, but also to other classes of antibiotics. [8] ESBL resistance genes are located on plasmids which are transferrable to other strains, thus posing considerable infection control issues. UTIs caused by ESBL-producing E. coli and K. pneumoniae are the most common ESBL infections in childhood. [9]

The CLSI has proposed disk diffusion methods for screening for ESBL production by klebsiellae, Escherichia coli, and Proteus mirabilis. Laboratories using disk diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production.

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[10] Cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone is used. However, the use of more than one of these agents for screening improves the sensitivity of detection. If any of the zone diameters indicate suspicion for ESBL production, phenotypic confirmatory tests should be used to ascertain the diagnosis. [11]

Materials and Methods

This prospective study was conducted in the Department of Microbiology, Index Medical College, India. A total of 300 consecutive urine samples were screened from patients with symptomatic UTI. Clean-catch midstream urine samples were collected in sterile disposable container and processed within one hour. Semi quantitative loop measuring 2.2 mm diameter with a holding capacity of 0.005 ml was employed to culture urine on CLED agar and MacConkey's agar. The inoculated plates were incubated over night at 37^{o}C. Isolates in significant number (colony count $\geq 10^5$ CFU/ml) was identified by standard procedures. [12] Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method [34] using antibiotic discs: ampicillin (10µg), amoxicillin/clavulanic acid (20/10µg), co-trimoxazole (1.25/23.75 µg), amikacin (30µg), imipenem (10µg), gatifloxacin (5µg) and tobramycin (10 µg).

Disc susceptibility test to screen ESBLs

All the isolates were screened for ESBL production by using three indicator cephalosporins, namely ceftazidime (30 µg), cefotaxime (30 µg) and cefpodoxime (30 µg). The isolates were considered to be resistant, if the inhibition zone diameter of ceftazidime, cefotaxime and cefpodoxime was < 22mm, <27mm and <17mm respectively.

The strains which showed resistance to at least one of the three cephalosporins were further included for phenotypic confirmation method. [13]

Double disc synergy test (DDST)

The *Escherichia coli* showing decreased susceptibility to any of the three cephalosporins used was further tested for ESBL production by DDST method. Ceftazidime, cefotaxime, cefpodoxime and amoxy-clav was used in this method. [14] Over the lawn cultured Muller-Hinton agar plates, amoxy-clav and third generation cephalosporin discs were placed at a distance of 20mm from the center. The Plates were incubated at 37ºC for 8 hours. The augmentation in the zone of inhibition of cephalosporins towards the amoxy-clav disc was considered to be positive for ESBL. The standard strains of Klebsiella pneumoniae ATCC 700603 and *E. coli* ATCC 25922 was used as controls.

Inhibitor potentiated disc diffusion test (IPDD)

The turbidity of *E.coli* in a broth was matched with 0.5 McFarland turbidity standards and inoculated onto two Muller-Hinton agar plates by streak method. Of the two plates, one was supplemented with 0.004 mg/L Potassium clavulanate and another without clavulanate. The ceftazidime, cefotaxime and cefpodoxime disks were placed

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on both of these plates. The inoculated agar plates were then incubated at 37ºC for 8 hours. The inhibition zones of disks were compared between the plates with and without potassium clavulanate. The difference in the zone size of ≥ 10 mm diameter was taken as positive for the production of ESBL. [15]

ESBL Epsilometer-strip test (E-strip test)

The commercially available ESBL E-strip (make: AB Biomerieux) contains two gradients of antibiotic drugs. At one end, the strip is impregnated with ceftazidime (0.5 to 32 mg/ml) and on the other end is with ceftazidime (0.125 to 8 mg/ml) with clavulanate (4 mg/ml). The overnight growth of *E.coli* isolate was suspended in saline to match the turbidity with 0.5 McFarland standards and was then inoculated on Muller Hinton agar plate by lawn culture technique. After drying, the E -test strip was placed on the plate and incubated overnight at 37ºC. The MICs on both ends of the E-strip was interpreted as the point of intersection of the inhibition eclipse with the E-test strip edge. The ratio of ceftazidime/ ceftazidime with clavulanate MIC ≥ 8 indicates the presence of ESBL enzymes. [16]

Statistical analysis

The results of the study was statistically analyzed using SPSS v 25.0 software wherever suitable. The Chi- square test was done to analyze statistical significance. The p-value of less than 0.05 was considered statistically significant.

Results

Table 1: Distribution of the Percentage of patients according to age group

Age group in Years	Frequency	Percentage
<20	41	13.6
21-30	83	27.6
31-40	69	23.0
41-50	53	17.6
51-60	35	11.6
>60	19	6.3
Total	300	100

In table 1, maximum number of patients were in the age gathering of 21-30 years age gathering which were 34% (n =68) of complete followed by age group 31–40 years having 26.5% (n = 200).

Table 2: Gender wise distribution

Gender	Frequency	Percentage
Male	89	29.6
Female	211	70.4
Total	300	100

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In table 2, of the 300 samples 211 were female and 89 males, which correspond to 70.4% of female and the 29.6% male.

Table 3: Distribution of various uropathogens in culture positive samples

Name of the organism	Frequency	Percentage
Escherichia coli	137	45.6
Klebsiella pneumonia	86	28.6
Pseudomonas aeruginosa	8	2.6
Acinetobacter spp.	1	0.3
Enterococcus faecalis	2	0.6
Proteus spp.	29	9.6
Staphylococcus aureus	26	8.6
Staphylococcus saprophyticus	1	0.3
Citrobacter spp.	10	3.3
Total	200	100

In table 3, the present study shows the pathogens causing UTIs and their antibiotic susceptibility pattern. Escherichia. coli 45.6% was the predominant pathogen followed by Klebsiella pneumoniae 28.6%, Proteus spp. 9.6%, Staphylococcus aureus 8.6%, Citrobacter spp. 3.3%, Pseudomonas aeruginosa 2.6%, Enterococcus faecalis 0.6%, Staphylococcus saprophyticus 0.3% and Acinetobacter spp. 0.3%.

Table 4: Susceptibility rates of isolated *Escherichia coli* to various tested antibiotics (n=137)

Antibiotic drugs	Frequency	Percentage
Amikacin	77	56.2
Ampicillin	37	27.0
Amoxy–clav	57	41.6
Aztreonam	71	51.8
Cefotaxime	21	21.6
Ceftrioxane	29	21.1
Cefuroxime	17	12.4
Cefatzidime	57	41.6
Ciprofloxacin	16	11.6
Cotrimoxazole	36	26.2
Gentamicin	46	33.5
Imipenem	86	62.7
Levofloxacin	21	15.3
Meropenem	101	73.7
Nitrofurantoin	82	59.8
Norfloxacin	37	27.0
Ofloxacin	77	41.6

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Piperacillin + tazobactam	51	37.2

In table 4, in our study high susceptibility of meropenem (73.7%) and imipenem (62.7%) was seen and least wereCiprofloxacin 11.6%.

Table 5: Susceptibility rates of isolated *Escherichia coli* to various tested antibiotics (n=137)

Antibiotic drugs	Screening test*	Confirmatory tests** (n=60)		
	N=137	Double disc synergy test	Inhibitory potentiated disc diffusion Test	E-strip test
Cefotaxime	39	3	4	NA
Ceftazidime	52	6	13	23
Cefpodoxime	57	1	13	NA
ESBL positives		7	30	23

In table 5, in the Double disc synergy test and Inhibitory potentiated disc diffusion Test screening test for ESBL production, 70 (43.7%) isolates were resistant to at least one of the three representative cephalosporin drugs. The highest resistance was observed with cefpodoxime (n=57; 40.1%) followed by ceftazidime (n=52; 37.9%) and cefotaxime (n=39; 28.4%). Out of the three cephalosporins tested in the study, ceftazidime was found to be the better antibiotic drug for the identification of ESBL production by both Double disc synergy test and Inhibitory potentiated disc diffusion Test.

Table 6: Confirmation of screening test positive ESBL producers by inhibitory potentiated disc diffusion (IPDD) test

Agents	**Mean Zone diameter± S.D (mm)**		**Mean Zone augmentation (mm) (95% CI)**	**-Value**
ESBL Positive strains(n=23)	**MH Agar**	**MH agar + Clavulanate**		
Ceftazidime	17.3±7.4	37.3±5.5	17.1 (12.9, 22.1)	<0.001
Cefotaxime	20.3±5.1	33.3±7.2	14.3 (12.1, 19.3)	<0.001
Cefpodoxime	17.5±4.3	33.3±5.1	17.4 (12.0, 17.3)	<0.001
ESBL Negative strains(n=37)				
Ceftazidime	35.3±3.1	35.5±3.7	1.7 (0.7, 2.3)	<0.001
Cefotaxime	31.9±3.4	33.3±3.3	1.3 (0.3, 2.7)	<0.001
Cefpodoxime	31.3±3.2	34.3±3.8	1.5 (0.7, 2.0)	<0.001

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In table 6, the ESBL positives with augmentation zone of inhibition diameter is ≥ 10 mm. The mean zone augmentation (95% CI) was 17.4 (12.9, 22.1) mm for ceftazidime, 14.3 (12.1, 19.3) mm for cefotaxime and 17.1 (12.0, 17.3) mm for cefpodoxime.

Table 7: Distribution of ESBL (n=137)

Isolates	ESBL		
	KB disc Diffusion Method	Disk Approximation Method	Combination DiscMethod
Escherichia coli	101 (73.7%)	66 (66.4%)	84 (61.3%)

In table 7 Presence/absence of SHV, CTX-M and TEM genes in samples resistant/susceptible to the third generation Cephalosporins by PCR

Discussion

In the present investigation, the most frequent pathogen seen in the age group of 21 to 30 years (27.6%). According to Kalal et al observed that 31.7% were affected in the age group of 15- 59 years. [17] Savitha T et al observations are dissimilar to our study and proved that UTI is more common in older age group (41- 50 years). [18] Irene Eriksson et al reported that UTI is common in older age due to associated risk factors such as urinary incontinence. [19]

In our study showed that the prevalence of UTI in females (70.4%) was higher than males (39.6%). It strongly correlates with other findings which revealed that the frequency of UTI is greater in females as compared to males. Manikandan C et al observed a prevalence of 69.8% and 52.10% in females when compared to 30.2% and 47.9% in males respectively. [20] The reason behind this high prevalence of UTI in females is shorter urethra, due to its close proximity to anus, sexual intercourse, incontinence and other comorbid condition.

The present study shows the pathogens causing UTIs and their antibiotic susceptibility pattern. Escherichia. coli 45.6% was the predominant pathogen followed by Klebsiella pneumoniae 28.6%, Proteus spp. 9.6%, Staphylococcus aureus 8.6%, Citrobacter spp. 3.3%, Pseudomonas aeruginosa 2.6%, Enterococcus faecalis 0.6%, Staphylococcus saprophyticus 0.3% and Acinetobacter spp. 0.3%. It is strongly supported by the study done in Pattukkottai area in Tamilnadu by Manikandan C et al. in which the second most common uropathogen was *Klebsiella pneumoniae* (11.2%) followed by *Pseudomonas aeruginosa* (10.5%) and *Proteus* spp., (6.8%). [21]

In the present study, high susceptibility of meropenem (73.7%) and imipenem (62.7%) was seen and least were Ciprofloxacin 11.6%. The resistance pattern varies

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from place to place. It is similar to the study conducted by Tabasi M et al and the results suggested that *E. coli* was extremely resistant to Ampicillin (100%). He also observed a resistance of 68.3% to amoxicillin/clavulanic acid, 33% to Cotrimoxazole. [22] This high level of resistance to norfloxacin and nitrofurantoin could be explained by absence of strict antibiotics use policy in the community, added to the exponential increase in prescription of these antibiotics for empiric treatment of hospitalized patients with positive urine cultures. The antimicrobial profile of ESBLs producing isolates showed significantly higher resistanceo aztreonam, all cephalosporin, and amoxicillin clavulanate compared to non-ESBLs producing isolates.

In our study 46.15% ESBL producers belonged to Enterobacteriaceae family. There have been reports of ESBL's from major hospitals in India and some of them have recorded the incidence to be as high as 60-68%. [23] The high incidence of ESBL is a cause of concern to regulators of hospital antibiotic policy. Over reliance on third generation cephalosporins to treat gram negative bacterial infections is one of the prime causes for increased resistance to this class of antibiotics.

The difference observed in detection of ESBL positive isolates by two different methods may be justified by the lower sensitivity of phenotypic method and the influence of environmental factors on the incidence of resistance. [24] The lack of constant sensitivity of different phenotypic methods has been emphasized by some studies. [25] In contrast, the genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity. The cost of molecular method is particularly reduced for the bacteria belonging to enterobactericeae family as their DNA is easily extractable by boiling method, a quick and cost-effective DNA extraction method. [26]

Conclusion

UTIs are the leading cause of outpatient visits, so understanding the epidemiology of Uropathogenic Escherichia coli can contribute to better treatment and reduced morbidity of UTIs. This study has determined that ESBL-producing Uropathogenic Escherichia coli isolates are present in India, hospitals, with 24.2% of the isolates being resistant to the commonly prescribed cephalosporin drugs for UTIs. This resistance level indicates possible antibiotic selection pressure in Indian hospitals and community settings, driving resistance to these widely prescribed drugs.

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