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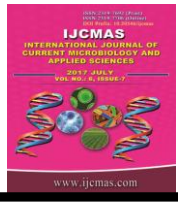

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The CTX-M Type ESBL Gene Production by *Klebsiella* Species in Urinary Tract Infection

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A B S T R A C T

Urinary Tract Infection (UTI) is one among the most common type of community and hospital acquired infection. Antibiotic resistance is a deep scientific concern both in hospital and community settings. The prevalence of ESBL range from 30% to 50% and the resistance due to CTX-M is increasing drastically from 30% to 90% around the world. Aim of the study is to detect ESBL CTX-M gene in *Klebsiella* isolates from patients with UTI in SRM medical college, hospital and research center a tertiary care hospital for a period of 1 year. Identification of organisms was done by routine urine analysis by semi quantitative urine culture. Antimicrobial susceptibility testing with first line and second line drugs as per CLSI guidelines 2016. Phenotypic confirmation of ESBL production in *Klebsiella* is done by double disk diffusion method as per CLSI guidelines 2016. PCR Detection of CTX-M Gene (HELINA KIT BASED) by gel electrophoresis. Totally we have examined 150 patients mid-stream samples of which 70 samples (46.6%) were positive for bacterial culture positive. One of the frequent pathogen associated with UTI in our hospital was *Klebsiella* species (25.71%), most frequent *Klebsiella* species isolated was *Klebsiella pneumoniae* 17(94.4%), ESBL production was determined by phenotypic disk diffusion method (58.82%) and we have demonstrated CTX-M gene in 70.5% by conventional PCR method.

Introduction

Urinary tract infections (UTIs) have been reported to affect up to 150 million individuals worldwide every year [1] and caused mainly by *Escherichia coli (*68%); *Klebsiella (*16.9%); *Proteus (*5.5%); *Enterobacter* (5.3%); *Staphylococcus saprophyticus* (2.8%); and others (1.5%) of the isolates in India [18]. The World Health Organization and the European Commission have recognized the importance of studying the emergence and determinants of acquired anti-microbial resistance and the need to devise appropriate strategies for their control [2-4]. Extended-spectrum β-lactamases (ESBLs) have increased dramatically among clinical *Enterobacteriaceae* during last 3 decades. CTX-M enzymes are a group of class A extended-spectrum β-lactamases (ESBLs) that are more rapidly spreading among *Enterobacteriaceae* family worldwide. Since the initial isolation of CTX-M-1 from a European in the late 1980s, more than 130 CTX-M variants have been described so far. At present CTX-M family comprises 40 enzymes. The most closely related β-lactamases with identities, 62 to 75% are the

chromosomal class A enzymes of *Klebsiella oxytoca*, *Serratia fonticola*, *Proteus vulgaris*, and *Citrobacter koseri.* These CTX-M variants are divided into 5 major phylogenetic groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 or CTX-M-25 on the basis of their amino acid sequence [5, 6, and 8]. During past decade CTX-M enzymes have become the most prevalent ESBL enzymes in clinical *Enterobacteriaceae* family [5, 7]. Most preferred drug for the treatment of UTI are Narrow- and extended-spectrum cephalosporins. However, management of UTIs has become increasingly problematic due to the increasing production of extended-spectrum β-lactamases (ESBLs) (Y. Huang *et al.,*). Aim of our study is to detect Extended Spectrum β-Lactamases (ESBL) producing *Klebsiella* species with CTX-M gene in patients attending urology department with UTI in SRM medical college, hospital and research center a tertiary care hospital in Tamil Nadu, Kancheepuram district for a period of1 year.

Materials and Methods

We have taken 150 inpatients of urology ward with the clinical diagnosis of UTI, we collected mid-stream urine from each patient with the appropriate instruction and consent. Identification of organisms by routine urine analysis by semi quantitative urine culture with Cystine Lactose Electrolyte Deficient (CLED) agar for differentiation of lactose fermenting and non-lactose fermenting organisms and Blood agar for identification of fastidious organisms and appropriate biochemical reactions. Species differentiation of *Klebsiella* was done by biochemical (e.g. indole) and sugar fermentation test (e.g. lactose) [21, 22]. Antimicrobial susceptibility testing by disk diffusion method with first line and second line drugs as per The Clinical Laboratory Standards Institute Guidelines (CLSI) 2016.

Amoxycillin/Clavulanic acid (20/10µg), Piperacillin/Tazobactam (100/10µg), Cefazoline (30µg), Cefepime (30µg), Cefotaxime (30µg) Ceftriaxone (30µg), Ceftazidime (30µg), Cefaperazone/sulbactam Ertapenem (10µg), Imipenem (10µg),Meropenem (10µg), Gentamicine (10µg), Amikacin (10µg), Netilmicine (30µg), Ciprofloxacin (5µg), Nalidixic acid (30µg), Norfloxacin (10µg), Nitrofurantoin (300µg), Co-trimoxazole (1.25µg/23.75µg).

Screening for ESBL producing isolates

All isolates showing ≤ 22 mm inhibition zone size for ceftazidime (30 µg) and ≤ 25 mm inhibition zone for ceftriaxone(30 µg) in the antimicrobial sensitivity test is identified as potential ESBL producers according to CLSI guidelines and subjected to double disc synergy test for phenotypic confirmation. Phenotypic confirmation of ESBL production in *Klebsiella* spices by double disk diffusion method.

Double Disc Synergy test

Detecting ESBL producing organism employs a beta-lactamase inhibitor clavulanate, in combination with the 3rd Generation Cephalosporins (3GC) such as ceftriaxone and cefotaxime. A bacterial suspension of potential ESBL with 0.5 Mcfarland turbidity standard is inoculated on the MHA plate, ceftazidime (30µ g) disc, ceftriaxone (30µ g) discs are placed at a distance of 20 mm from ceftazidime + clavulanic acid (30µ g/10 µg), ceftriaxone + clavulanic acid (30 µg/10µ g) respective and incubated under 37°c for 16-18 hours.

Positive result- difference of ≥ 5 mm in the zone diameter for ceftazidime +clavulanic acid against ceftazidime or ceftriaxone +clavulanic acid against ceftriaxone.

Negative result- difference of ≤ 4 mm in the zone diameter for ceftazidime + clavulanic acid against ceftazidime or ceftriaxone + clavulanic acid against ceftriaxone.

Conventional Polymerase Chain Reaction (PCR) detection of CTX-M

DNA extraction and the PCR procedure was done in King Institution of Preventive Medicine, Gundy (HELINA KIT BASED): Cells are lysed during a short incubation with proteinase K in the presence of chaotropic salt, which immediately inactivates all the nucleases. Cellular nucleic acids bind selectively to special glass fibers prepacked in the pure fast purification filter tube. Bound nucleic acids are purified in series of rapid "wash and spin" steps to remove contaminating cellular components. Finally low salt elution releases the nucleic acid for the glass fiber. The primer sequencing of CTX-M gene is given in table 1.

PCR product size is almost 276 base pair

The product of amplified DNA from PCR is added to the wells prepared (2% agarose gel) and subjected to electrophoresis using TAE buffer at 50v. Gel viewed in UV transiluminator and documented (Table 2 and Fig. 1).

Results and Discussion

Out of 150 patient's urine samples 70 samples (46. 6%) were bacterial cultures were positive. Among the culture positive *E. coli* was the most isolated organism accounting for 23 (33%). The second commonest isolate was *Klebsiella* species accounted for 18 (26 %). Followed *Pseudomonas aeruginosa* 5(7%), Gram positive cocci accounted for 24(34%). In the isolated *Klebsiella* species was *Klebsiella pneumoniae* 17 (94.4%) and one species was *Klebsiella oxytoca*. Table 3 explains the gender distribution of culture and *Klebsiella* positive isolates.

The following drugs were increasingly used by the department of urology in hospital due to high degree of sensitivity (Chart 1).

By double disk diffusion test 10 isolates were positive for ESBL production among *Klebsiella* species isolated. When it was subjected to molecular detection by conventional PCR Detection of CTX-M Gene in gel electrophoresis, the finding shows CTX-M (7) among ESBL producing *Klebsiella* species is 70%.

The present study, done on molecular aspect is aimed at screening ESBL producing *Klebsiella* species with CTX-M genes. This study found out resistance due to ESBL producing *Klebsiella* species is increasing and the most prevalent gene in circulation is CTX-M in this geographical our area. In the present study, majority of the cases were from males compared to females. Study by Eshwarappa *et al.*, in south India also showed similar results [19] (Table 4).

E. coli was the most isolated organism accounting for 23 (33%). The second commonest isolate was *Enterococci* 19(27%) followed by *Klebsiella pneumoniae* which accounted for 18 (26 %).

Similar to the study by Varghese *et al.*, showed *E. coli* (48%), *Klebsiella pneumoniae* (17%) followed by *Candida* species (10%) and other *Klebsiella* species (2%) [8].

Klebsiella species which are harboring ESBL may usably acquire additional resistance to some group of antibiotics like trimethoprim, aminoglycosides tetracylines, co-trimoxazole, and quinolones [9].

Table.1 CTX-M type ESBL gene primer

Gene Detected	Primer
bla CTX-M	F-ACGTGGCGATGAATAAGCTG R-AACCCAGGAAGCAGGCAGT

Table.2 PCR amplification protocol

PCR amplification protocol	Time	Temperature
Initial Denaturation	5 min	95°C
Denaturation Annealing	30 sec	95°C
Extension	30sec	58°C
Final extension	30 sec	72°C
	5 min	72°C

Table.3 Gender distribution of the study population

(n=150)

Number (%) of patients with clinical diagnosis with UTI	Number (%) of bacterial culture positive	Number (%) of *Klebsiella* species in bacterial culture positive
MEN(104)	50 (71.5)	12 (66.7)
WOMEN (46)	20 (28.5)	6 (33.3)
TOTAL (150)	70	18

Table.4 Phenotypic and genotypic detection of ESBL

Klebsiella species isolated in bacterial culture positive	Among *Klebsiella* species isolated ESBL detection by double disk diffusion test	Among ESBL *Klebsiella* species CTX-M Gene detection by PCR
18 (24.4%)	10 (58.82%)	7(70%)

Fig.1 DNA ladder

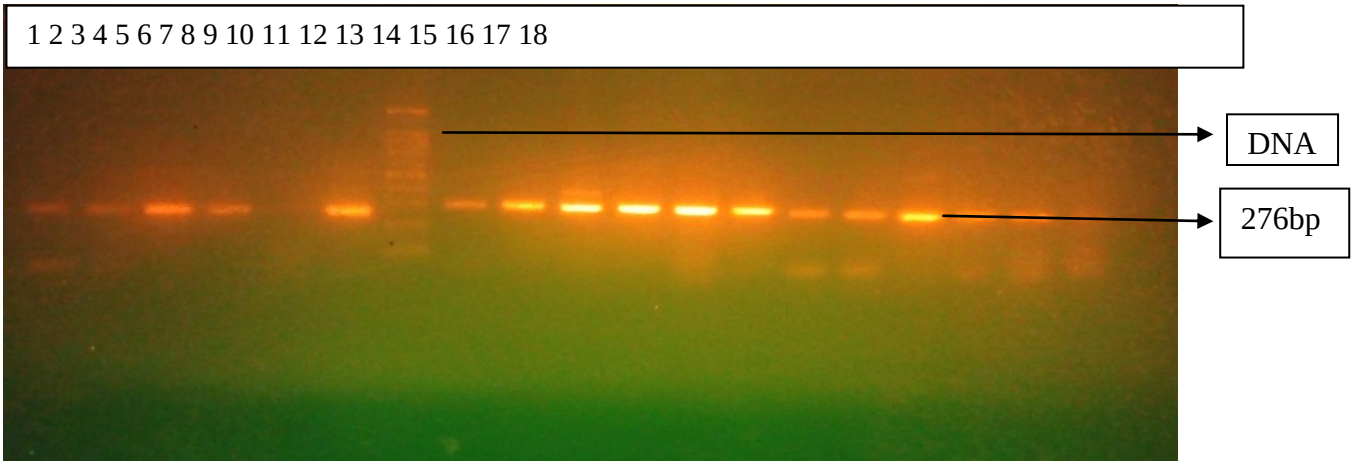
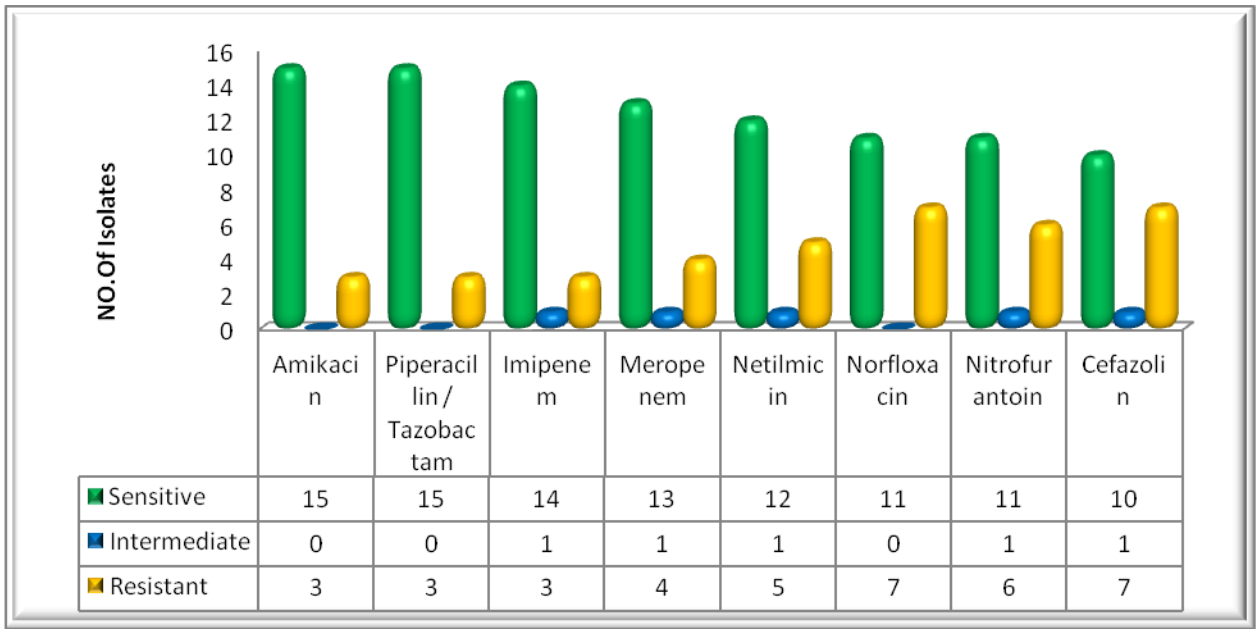


Chart.1 Antibiotic sensitivity pattern of *Klebsiella* species



	Amikacin	Piperacillin / Tazobactam	Imipenem	Meropenem	Netilmicin	Norfloxacin	Nitrofurantoin	Cefazolin
Sensitive	15	15	14	13	12	11	11	10
Intermediate	0	0	1	1	1	0	1	1
Resistant	3	3	3	4	5	7	6	7

The antimicrobial suitability showed that amikacin and piperacilin tazobactem as the most sensitive drugs for *Klebsiella* species.

Antibiotic susceptibility pattern shown by the *Enterobacteriaceae* isolates were variable. Amikacin (83%), piperacillin tazobactam (83%) and Imipenem (78%) was the most effective antibiotic for the isolates similar to the study by Yadav *et al.*, [10] and Xiao *et al.*, [12].

The Multi-drug resistant in *K. pneumoniae* has recently emerged as a troublesome pathogen worldwide. Resistances caused by ESBLs are mainly responsible for the drug resistance to β-lactam antibiotics Liu *et al.*,; Lahlaoui *et al.*, [11].

The proportion of ESBL-producing *K. pneumoniae* (56 %) in this study was higher than that in Italy (32.6%), America (51.8%), Korea (52.9%), and lower than Russia (60.8%) Kim *et al.*, [13]; Edelstein *et al.*,[14]; Marra *et al.*,[15]; Tumbarello *et al.*,[16], and the dominate ESBL enzyme CTX-M (70%) of ESBL producers were similar Edelstein *et al.*,[15,12].

In other studies conducted in Iran and Beijing showed ESBL detection of (59.20%) and (32.21%).The rate of detection of ESBLs in the present study with *K. pneumoniae* was calculated to be 56%; which was lower than the rate observed in Iran (59.20%), and higher than that seen in Beijing (32.21%). However, the rate of detection of CTX-M ESBLs in ESBL producing *K. pneumoniae* (70%) was lower than that seen in Beijing (84.80%), and higher than that in Iran (23.90%) [22- 24].

Antibiotic resistance is a problem of deep scientific concern both in hospital and community settings. Rapid detection in clinical laboratories is essential for the recognition of antimicrobial resistant organisms. Production of extended-spectrum β-lactamases (ESBLs) is a significant resistance-mechanism that impedes the antimicrobial treatment of infections caused by Gram negative bacteria and is a serious threat to the currently available antibiotics,

and resistance by CTX-M is increasing. Proper infection control practices and barriers are essential to prevent spread and outbreaks of ESBL producing bacteria.

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