Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

MOLECULAR DETECTION OF *BLACTX-M* AND *BLASHV* GENES IN *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* IN A TERTIARY CARE HOSPITAL IN PUDUCHERRY,INDIA

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ABSTRACT

Extended-spectrum β-lactamases producing Enterobacteria represent a rapidly emerging resistance problem in many countries. The present study was undertaken to find out the presence of *blaCTX-M3* and *blaSHV* genes by PCR technique among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae.* A total of 186 faecal specimens were collected in our hospital, were subjected to isolation, identification of *E.coli* and *K. pneumoniae*, antimicrobial susceptibility testing was performed by disc diffusion method as per Clinical Laboratory Standard Institute.The PCR detection of *E.coli* revealed 40/50 (80%) for *CTX-M-3* and (24/50) 48% for *blaSHV* genes were positive. Moreover, antimicrobial resistance for *E. coli* showed 74% to cefotaxime, 62% to ceftriaxone, *K. pneumoniae* showed 72% of *CTX-M-3* and 80% of *blaSHV* positive genes and resistance to cefotaxime (84%), ceftriaxone (56%), ceftazidime (66%), cefuraxime (68%), cefoperazone (58%), *blaCTX-M3* and *blaSHV* genes transfers are possible mechanisms for wide prevalence of cephalosporin resistant among *E.coli* and *K. pneumoniae* strains.

KEY WORDS: ***E.coli*, *K. pneumoniae*, ESBL genes and PCR.**

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INTRODUCTION

The health care acquired infections among patients have become a serious threat due to wide spread of extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* and *E.coli*, and they have enormously spread across the world[1]. The ESBLs are β-lactamases which can hydrolyze third generation cephalosporins and aztreonam[2]. The β-lactamase enzyme producing gram-negative and gram-positive bacteria can hydrolyse the β-lactam ring of penicillin and cephalosporin related compounds, which in turn helps the bacteria to develop β-lactam antibiotic resistance producing serious infections[3]. Generally, the bacterial β-lactamases is divided into12 leading groups 1, 2, 2a, 2b, 2be, 2br, 2c, 2d, 2e, 2f, 3 and 4 according to their functional characteristics. But the most widely used Ambler classification of β-lactamases divides them into class A, B, C and D based on their amino acid sequence[4, 5]. Furthermore, the revised Ambler classification of β-lactamases was proposed by Hall and Barlow in 2005, suggesting further division of class B metallo β-lactamases into Class B1, B2 and B3 based on their differences in their amino acid sequences[6]. Classic evolutions of ESBLs originated by point mutations in the sequences of TEM-1 and SHV-1 β-lactamase plasmid genes[7, 8]. In 1960s, plasmid mediated TEM-1 β-lactamase was first reported in gram-negative bacteria[7]. Similarly in 1972, Pitton has reported first *blaSHV*-1 β-lactamase[9]. Moreover, both TEM and SHV derived β-lactamases were found to be plasmid encoded enzymes belonging to class A and group 2b β-lactamases[5]. In addition to TEM and SHV ESBLs, the CTX-M family was first reported in 1990s and it was found to be the rapidly spreading dominant ESBL among non-TEM and non-SHV ESBLs producing bacteria. Then, it was later also followed by the report of another ESBL type named *AmpC,* which are more prevalent in the world among Enterobacteriaceae[10, 11].

After the initial reports, the ESBL prevalence rate has increasing continuously and became a global health problem[12]. The prevalence rate of ESBL producing *E.coli* reported in different countries are 31% in China, 17% in Hong Kong, 15% in Singapore, 12% in the Philippines, 6% in Japan, 4% in Korea, 2.5% in South Africa, 14.5% in Taiwan and 2% in Australia in 2007. Similarly, the reported prevalence rate for *K. pneumoniae* were 37% in China, 36.5% in Singapore , 30.5% in South Africa, 27% in Korea, 26% in Philippines, 21% in Taiwan, 17.5% in Hong Kong, 10% in Japan and 4.5% in Australia[13]. During 2009, a study from India has reported the prevalence rate of ESBL producing *E.coli* as 63.6% and *K. pneumoniae* as 66.7%[14]. Evolutions in the bacterial plasmids have facilitated antimicrobial resistant genes to exchange between bacteria of different origins through conjugation mechanism[15]. This plasmid-mediated type of antimicrobial resistant gene exchange is found to be one of the predominant factors for the spread of multi-drug resistance (MDR) bacteria. The use of antimicrobial substances in both human and veterinary medicines were also one of the reasons for the appearance and dissemination of drug resistant clones due to plasmids transfer between bacteria. The treatment of diseases caused by ESBL producers has encountered serious problems of management. To overcome these problems and initiate control measures one should monitor the spread of ESBL producing strains by strain typing[16]. Due to advancements made in the molecular biology, the identification of ESBL producing bacteria become feasible and rapid[17]. By these effective control measures, it can be identified well in advance. The Present study represents phenotypic and molecular characterization of cephalosporins resistant genes in *E.coli* and *K. pneumoniae* isolated from clinical specimens.

MATERIALS AND METHODS

Bacterial isolates and detection of ESBL

A total of 186 faecal specimens collected during July-2012 to June 2013 in our hospital and were subjected to isolation and identification of *E.coli* and *Klebsiella pneumoniae* as per the standard microbiological techniques. All the isolates were stored for further studies in brain heart infusion broth with 15% glycerol at -20º C.

Antimicrobial susceptibility assay

Antimicrobial susceptibility patterns of *E.coli* and *K. pneumoniae* isolates were tested against various routinely used antibiotics. Besides the ESBL producing strains were identified using discs containing ceftazidime (30 µg) and cefotaxime (30 µg) with and without clavulanate (10 µg) by disc diffusion technique as per Clinical Laboratory Standard Institute[18]. The reference strain used was *E.coli* ATCC 25922.

I. Extraction of Plasmid DNA[19]

A single bacterial colony from the isolates was inoculated in 2 ml of LB medium and incubated overnight. 1.5 ml of culture was taken in a microfuge tube and centrifuged at12000 g for 2 mins at 4ºC. The pellet was suspended in 100 µL of ice-cold Solution-A and vortexed vigorously. Then, 200 µL of Solution-B was added and mixed well by inverting the tube rapidly. Later, 150 µL of ice cold Solution-C was added and mixed by inverting the tube. Finally, the tubes were kept in ice bath for 5 mins and centrifuged at 12000 g for 5 mins at 4º C. The supernatant solution was transferred in to a fresh tube. DNA was precipitated by adding two volumes of 100% ethanol at room temperature and mixed well for 2 mins. Again, it was centrifuged at 12000 g for 5 mins at 4º C. The pellet was resuspended in 50 µL of TE buffer (pH 8.0)[19] until use.

II. PCR Amplification of CTX-M and SHV genes

DNA template was extracted from bacterial strains as described above and amplified by PCR. The *CTX-M-3* Forward primer 5'AAT CAC TGC GCC AGT TCA CGC T 3' and Reverse primer 5' GAA CGT TTC GTC TCC CAG CTG T 3' and *SHV* Forward primer 5'CCG GGT TAT TCT TAT TTG TCG CT 3' and Reverse primer 5' TAG CGT TGC CAG TGC TCG 3' obtained from Sigma-USA was employed for amplification. The PCR was performed by using Lab net instrument (Lab net International, Inc USA) according to manufacturer's instructions in final volume reaction of 10 µL containing 1µL of 10x PCR buffer with $MgCl_2$, 250 µm of dNTPs, 80 nm of each primers, 5 units of 0.25 µL Taq DNA polymerase enzymes and 1 µL of template DNA. A Lab net (Lab net International Inc USA) thermo cycler was used for amplification. The program for both *CTX-M-3* and *SHV* genes amplification included a step of Initial denaturation at 94º C for 5 mins followed by 35 cycles of 94º C for 45 sec,50º C for 45sec for annealing and 72º C for 1min for extension and final extension step at 72º C for 7 mins. The PCR product was loaded in 1% wt/vol agarose gel (Sigma -USA) prepared in Tris Acetic acid EDTA buffer and detected by UV-transilluminator (Cleaver Scientific Ltd-UK).

RESULTS

Antimicrobial susceptibility pattern of ESBL-producing E. coli and K. pneumoniae

During one year period (July 2012-June 2013), a total of 186 faecal specimens were collected from our hospital and isolated 50 *E. coli* and 50 *K.pneumoniae* strains. The results of antimicrobial susceptibility pattern of *E. coli* and *K.pneumoniae* against various antibiotics is shown in Table-1. Higher percentage of resistance with both organisms was shown against cephalosporin group of antibiotics. The resistance pattern were analysed by paired two sample t-Test for both *E. coli* and *K. pneumoniae. p-value* of 0.04006 (which is <0.05) was obtained for *E.coli* and *K. pneumoniae* antibiotic resistance. Resistance pattern of E.coli were cefotaxime (74%), ceftriaxone (62%), ceftazidime (64%), cefurxime (58%), cefoperazone (48%), nalidixic acid (48%), ciprofloxacin (32%).. Resistance pattern of K. *pneumonia* were cefotaxime (84%), cefuroxime (68%), ceftazidime (66%), cefoperazone (58%), ceftriaxone (56%), nalidixic acid (52%), ciprofloxacin (48%). Meropenem (82%) and imepenem (90%) showed better sensitivity in *E.coli*, whereas in *K. pneumonia,* meropenem (60%) and imepenem (74%) showed moderate sensitivity.

Table 1
Antimicrobial susceptibility pattern of E.coli and K.pneumoniae

S. No	Antibiotics	Discs Potency µg	*E.coli*			*K. pneumoniae*		
			S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
1	Ciprofloxacin	5	25 (50)	09 (18)	16 (32)	16 (32)	10 (20)	24 (48)
2	Nalidixic acid	30	08 (16)	18 (36)	24 (48)	16 (32)	08 (16)	26 (52)
3	Cefotaxime	30	06 (12)	07 (14)	37 (74)	03 (06)	05 (10)	42 (84)
4	Ceftriaxone	30	18 (36)	01 (02)	31 (62)	17 (34)	05 (10)	28 (56)
5	Ceftazidime	30	12 (24)	06 (12)	32 (64)	07 (14)	10 (20)	33 (66)
6	Cefuroxime	30	15 (30)	06 (12)	29 (58)	09 (18)	07 (14)	34 (68)
7	Cefoperazone	75	16 (32)	10 (20)	24 (48)	12 (24)	09 (18)	29 (58)
8	Cefepime	30	24 (48)	04 (08)	22 (44)	28 (56)	07 (14)	15 (30)
9	Piperacillin/Tazo	100/10	25 (50)	23 (46)	02 (04)	21 (42)	22 (44)	07 (14)
10	Imepenem	10	45 (90)	05 (10)	00 (00)	37 (74)	11 (22)	02 (04)
11	Meropenem	10	41(82)	04 (08)	05 (10)	30 (60)	10 (20)	10 (20)
12	Gentamicin	10	38 (76)	07(14)	05 (10)	33 (66)	06 (12)	11 (22)

Table 2
Paired two sample t-Test for E.coli and K.pnuemoniae antibiotics resistance

Antibiotics	*E.coli* Resistance %	*K.pneumoniae* Resistance %	t-Test: Paired Two Sample for Means			Significant difference
Ciprofloxacin	16	24		*K.pneumoniae*	*E.coli*	
Nalidixic acid	24	26	Mean	21.75	18.91667	
Cefotaxime	37	42	Variance	155.2955	167.9015	YES
Ceftriaxone	31	28	Observations	12	12	
Ceftazidime	32	33	Pearson Correlation	0.945682353		
Cefuroxime	29	34	Hypothesized Mean Difference	0		
Cefoperazone	24	29	df	11		
Cefepime	22	15	t Stat	2.327159838		
Piperacillin/Tazo	2	7	P(T<=t) one-tail	0.020034571		
Imepenem	0	2	t Critical one-tail	1.795884814		
Meropenem	5	10	P(T<=t) two-tail	>0.040069143		
Gentamicin	5	11	t Critical two-tail	2.200985159		

Calculation: Performed in excel 2007 using QI Macros 2010

Table 3
Paired two sample t-Test for E.coli and K.pnuemoniae antibiotics resistant genes

Genes	*E.coli* resistance		*K.pneumoniae* resistance		t-Test: Paired Two Sample for Means			Significant difference
	No of isolates	Percentage	No of isolates	Percentage		*K.pneumoniae*	*E.coli*	
CTX-M3	40	80	36	72	Mean	76	32	NO
SHV	24	48	40	80	Variance	8	512	
					Observations	2	2	
					Pearson Correlation	-1		
					Hypothesized Mean Difference	0		
					df	1		
					t Stat	0.6		
					P(T<=t) one-tail	0.32797913		
					t Critical one-tail	6.313751514		
					P(T<=t) two-tail	0.655958261		
					t Critical two-tail	12.70620473		

Calculation: Performed in excel 2007 using QI Macros 2010

I. Molecular detection of blaCTX-M3 and blaSHV genes

All ESBL-producing isolates were detected by single disc diffusion method and subjected to confirmation by PCR technique for *bla*CTX-*M-3* and *blaSHV* in both *E.coli* and *K. pneumoniae.* An interesting result was found that 80% of *blaCTX-M-3* and 48% of *blaSHV* positivity were found in *E.coli* isolates Fig-1and Fig-2.

Figure-1
Ethidium bromide stained UV document of Agarose gel representing the blaCTX-M3 PCR product in E.coli and K. pneumoniae (479bp)

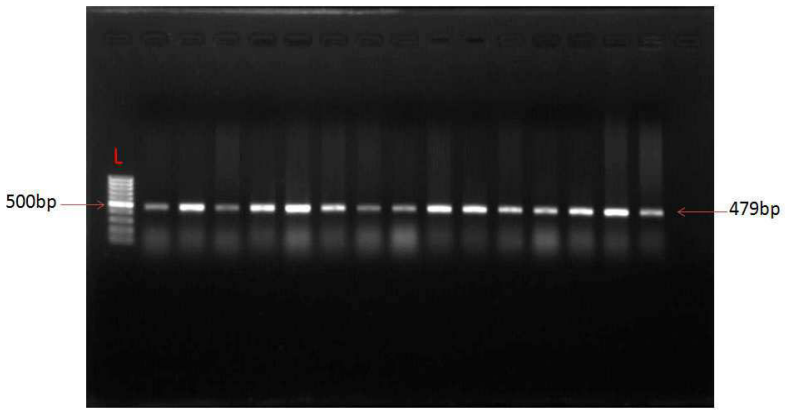
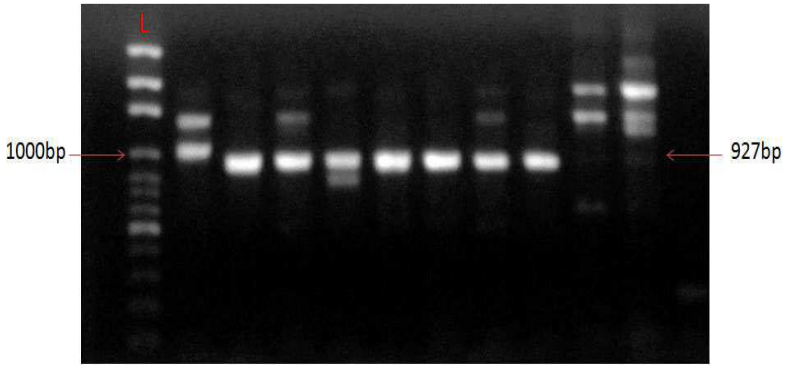


Figure-2
Ethidium bromide stained UV document of Agarose gel representing the blaSHV PCR product in E.coli and K. pneumoniae (927bp)



While with *K. pneumoniae* strains, for 72% *blaCTX-M-3* and 80% for *blaSHV* positivity Fig-1and Fig-2. The data were analysed statistically using paired t-Test. This revealed a *p*-value of 0.6559 (which is >0.05) for *blaCTX-M-3* and *blaSHV* in *E.coli* and *K. pneumoniae*. High resistance rates were observed with cefotaxime in both *E.coli* and *K. pneumoniae* strains, while other cephalosporins drugs were showed reduced percentages of resistance was found. Our study confirms that the *blaCTX-M*-3 ESBL has increasingly spread among our *Enterobacteriaceae* isolates, as this is also the most prevalent among ESBL producing *E. coli* and *K.pneumoniae* isolates in many parts of the world.

DISCUSSION

The spread of ESBL-producing *Enterobacteriaceae* has been rapidly spreading worldwide, indicating the importance of continuous monitoring and initiating effective infection control measures. Therapeutic options for infections due to ESBL producers have also become increasingly problematic

due to their multiple resistant character. Health care workers should have interactions including the rational and restricted uses of antibiotics, particularly oxyiminocephalosporins. Infections and over use of cephalosporins are well-defined risk factors for acquisition of ESBL producing bacterial infections[20-22]. ESBL-producing *K.pneumoniae* appeared to be an important cause of serious infections among patients admitted in intensive-care units. It was also reported that nearly 90% isolates of *K.pneumoniae* are susceptible to carbapenem group of antibiotics. Although β-lactam antimicrobial agents are frequently used for the treatment of *E. coli* and *K. pneumoniae* infections, ESBL-producing isolates are of particular concern because these traits can be horizontally-transmitted to other strains via by plasmid transfer and often cause nosocomial infections[23]. The prevalence and genotype of ESBLs producing isolates vary according to the country and even within hospital. For example, 3% (0-25%) of *Enterobacteriaceae* isolated in USA were reported to be ESBL-producing strains[7, 24] while 0.1% of *E. coli* and 0.3% of *K. pneumoniae* isolates in Japan were ESBL producers[25]. Additionally, there have been several reports on the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in Korea. Previous reports revealed approximately 10 % of *E. coli* and 25-30% of *K. pneumoniae* isolates were ESBL producers[26, 27]. The present study showed an increased percentage of ESBL-producing *E. coli* and *K. pneumoniae* isolated at our hospital.

ESBL-producing isolates carry several *bla* genes, which probably account for high-level of Beta lactam- resistant phenotype. Although CTX-M3 of ESBLs have been known for their rapid spread in Europe and Asia[10, 28], it was remarkable that in this study, 80% of ESBL producing *E. coli* and 72% of ESBL-producing *K. pneumoniae* isolates carried *blaCTX-M*. We therefore report the highest prevalence, to our knowledge, of *blaCTX-M* among ESBL-producing *E. coli* and *K. pneumoniae* and thus indicating *CTX-M*-type ESBL is highly endemic in India and other Asian countries. In conclusion, the present study revealed that increased antimicrobial exposure in particular cephalosporins drugs was the only independent predictor of ESBL-producing *E. coli* or *K. pneumoniae* infection. In addition, molecular study revealed that many of the ESBL-producing E*. coli* and *K. pneumoniae* isolates were closely related. These results suggest that if challenges to control outbreaks of infections due to these organisms are to be successful, such efforts must highlight the rational and judicial use of all antimicrobial agents. Strict barrier nursing should be implemented to prevent the nosocomial spread of ESBL-producing *E. coli* and *K. pneumoniae* infections must also be emphasized. Besides, ESBL-producing *E. coli* and *K. pneumoniae* infections were also associated with a significantly longer duration of hospital stay and increased hospital cost, thereby indicating that these infections have an important impact on clinical outcomes.

ACKNOWLEDGEMENT

The authors thank the Management of Sri Manakula Vinayagar Medical College and Hospital for the financial support of this research and we also thank our laboratory technician for her help in this research.

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