Community onset of CTX-M extended spectrum β-lactamases among uropathogenic *E. coli* and *K. pneumoniae* from Karachi, Pakistan

Sakina Fatima[1], Iyad Naeem Muhammad[1]*, Muhammad Naseem Khan[2], Subia Jamil[3], Tuba Siddiqui[4] and Humera Khatoon[3]

[1]Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Karachi
[2]Microbiology Section, FMRRC, PCSIR Laboratories. Complex Karachi
[3]Department of Pharmacology, Faculty of Pharmacy, Jinnah University for Women, Karachi
[4]Department of Pharmaceutics, Faculty of Pharmacy, Federal Urdu University of Arts, Science and Technology, Karachi

Abstract: Urinary tract infections (UTIs) are major health issue in developing countries like Pakistan, become more complicated with extended spectrum β-lactamase (ESBL) expression in *Escherichia coli* and *Klebsiella pneumoniae*. The ground of this present study was to evaluate the incidence of cefotaxime (CTX-M) gene in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. The clinical isolates from various specimens were collected for one-year duration from January till December 2015. After initial screening (n=352) isolates were examined for phenotypic expression of ESBLs by double disc synergy test. Furthermore, eight-four isolates were analyzed by polymerase chain reaction for identification of Cefotaxime (CTX-M), Temoneira (TEM) and Sulfhdryl variable (SHV) genes. Among eighty-four clinical isolates CTX-M was dominant and found positive in 50 isolates (59.5%) followed by TEM in 35 (41.6%) and SHV in 11 (13%). In uropathogenic *E. coli* and *K. pneumoniae,* ESBLs gene was found in 50 and 6 isolates out of 57 and 7 respectively. Among uropathogens CTX-M was most prevalent 78% (39/50) in *E. coli* followed by *K. pneumoniae*. In uropathogenic *E. coli*, CTX-M was found dominant in females. The study concluded that ESBL related uropathogenic *E. coli* were CTX-M dominant, showed community onsets of UTIs that can be preventive and controlled with modified hygienic practices.

Keywords: ESBLs, Urinary tract infections (UTIs), CTX-M, *E. coli*, *K. pneumonia*

INTRODUCTION

Extended spectrum β-lactamases (ESBLs) related community acquired infections has increased because of Cefotaxime (CTX-M) variants, a beta-lactamase of molecular class A (Bush *et al.*, 1995) originated from environmental bacteria with highly transferrable plasmid, this link is related in circulation of ESBLs in the community (Pitout *et al.*, 2005). In 1983, first report of CTX-M was published, CTX-M was suggested due to enzymatic activity against cefotaxime by hydrolysis (Knothe*et al.*, 1983). The β-lactamase inhibitors are ineffective against hydrolytic activity of these enzymes (Bradford, 2001). The prevalence of CTX-M enzymes reported from all over the world in both nosocomial and community settings (Cantón and Coque, 2006).

E. coli with CTX-M has previously reported from various household animals and different food related products, several reports indicated that stool samples from healthy human subjects and sewage also contained CTX-M producing *E. coli*. In most regions *E. coli* has already replaced as the predominant species of ESBLs-*Enterobacteriaceae* other than *Klebsiella spp* (Carattoli *et al.*, 2005; Kojima *et al.*, 2005). In Pakistan, recent studies indicated increased prevalence of ESBLs in *E. coli* (Ali *et al.*, 2016; Rahman *et al.*, 2016)

**Corresponding author:* e-mail: iyadnaeem@uok.edu.pk

Previously there were several published reports of true community-onset infection or colonization of *E. coli* with ESBLs (Colodner *et al.*, 2004; Woodford *et al.*, 2004; Rodríguez-Bano *et al.*, 2004; Akram *et al.*, 2007; Laupland *et al.*, 2008; Rodríguez-Baño *et al.*, 2008; Meier *et al.*, 2011; Søraas *et al.*, 2013;). These reports revealed urinary tract infections related with CTX-M positive *E. coli* from Spain, Turkey, India, Switzerland, Norway, the United Kingdom and Canada. In previous years various reports from Pakistan were published on prevalence of CTXM-ESBLs among *E. coli* and *K. pneumoniae* from hospital setups (Habeeb *et al.*, 2014;Rahman *et al.*, 2016; Abrar *et al.*, 2017).

This study was designed to determine CTX-M dominance among various *Enterobacteriaceae* isolates such as *E. coli*, *K. pneumoniae* and *P. mirabilis* reported from non-hospitalized patients.

MATERIALS AND METHODS

Study layout

Over a one-year period (January-December 2015), isolates were collected in cross-sectional manner from various specimens of non-hospitalized patients in Karachi, Pakistan. Total n=352 non-repeated clinical specimens (*E. coli*, *K. pneumoniae* and *P. mirabilis*) were selected for phenotypic investigation of ESBLs. The

criteria of investigation for initial screening of ESBLs were determination of inhibitory zones i.e., ceftazidime≤22 mm and cefotaxime≤27mm, additionally ceftriaxone≤25mm (Wayne, 2014) .

Phenotypic detection of ESBLs by double disc synergy test (DDST)

After initial screening, phenotypic identification of ESBLs was determined by synergy between cephalosporins and amoxicillin/clavulanic acid on muller-hinton agar. Both cephalosporins i.e., cefotaxime and ceftazidime were placed 30 mm apart from middle antibiotic disc of amoxicillin/clavulanic acid and incubated for 24 hours at 37ºC.The presence of ESBLs was confirmed with enhanced inhibited zone of cephalosporins towards clavulanic acid (Jarlier *et al.*, 1988).

Genotype identification

The CTX-M, TEM and SHV genes were analyzed using PCR. ESBLs positive clinical isolates were screened for the identification of all three genes, gene amplification was performed with forward and reverse primers.. The forward and reverse sequences of above-mentioned genes are listed in table 1.

Sub culture of preserved isolates

For sub-culturing, 20µl of previously preserved culture was transferred in to 2.5ml of BHI broth (Oxoid, UK), under controlled sterilized conditions and further incubated for 18-24 hours at 37°C.

DNA extraction

The extraction of DNA was performed using bacterial DNA Extraction kit (MOLEQULE-ON, New Zealand). Freshly cultured broth (100µl) was transferred in sterilized Eppendorf tubes (Nest, China) and centrifuged at speed of 8000 rpm for 5min. After centrifugation, discard the supernatant layer and keep the pallet in Eppendorf tubes. Further, 400µl of digestion solution and 3µl of and proteinase k solution were added and incubated for 5min at 55°C.After incubation, the Eppendorf tubes were centrifuged for 2min at speed of 10000 rpm and 260 µl of ethanol were added. Transferred the solution from Eppendorf tubes in to the collection tubes and again centrifuged for 2min at speed of 10000 rpm. Discard the fluid from collection tubes and 500µl wash solution was added and centrifuged at speed of 10000 rpm for 2min. Again repeatthe process with wash solution and a spin column was placed in clean Eppendorf tube. 30 to 50µl elution buffer was added and centrifuged for 2 min at speed of 10000 rpm. The eluted aliquot of 50µl (extracted DNA) was kept in freezer at -20°C.

Polymerase chain reaction (PCR)

The composition of reaction mixture was 25µl of master mix (Absolute Master Mix MOLEQULE-ON, New Zealand), 2.5µl of both forward and reverse primers (previously diluted individually with PCR water, 1:10 dilution) and 18µl PCR water (Merck, Germany). 2µl of extracted DNA was transferred in 0.2ml PCR tubes along with reaction mixture. Gene amplification was carried out in thermocycler (Bio-Rad Icycler, USA) according to the temperature cycles listed in table 2. All genes were amplified with hot start at 95°C and in the end 4°C was adjusted to stop all reactions.

Gel electrophoresis

The amplified products were analyzed by gel electrophoresis. 1% agarose gel (MOLEQULE-ON, New Zealand) was prepared in Tris/borate/EDTA (TBE) buffer (Sigma-Aldrich, Germany), 3µl of ethidium bromide dye was added in the solution and solidified in gel casting assembly. The amplified products and 100bp DNA ladder were loaded on gel; electrodes were attached for 25-30 minutes (80-100 volts). The amplified genes were compared with DNA ladder under UV transilluminator.

RESULTS

Phenotype identification of ESBLs

After phenotypic identification of ESBLs, out of 352 clinical isolates 96 (27.27%) were ESBL positive., Among ESBL positive isolates 72 were *E. coli* (75%), 16 were *K. pneumoniae* (16.6%) and 1 was *P. mirabilis* (1.04%). The specimen distributions among ESBL-producing isolates is depicted in table 3 .The urine specimens were found to be 75% and out of 72 urine specimens *E. coli* and *K. pneumoniae* were frequently recovered pathogens from *i.e.,* 63 and 9 respectively.

Genotype identification

Out of 96 ESBL-producing clinical isolates, 84 were tested for ESBLs gene; fig.1 showed presence of gene in tested isolates. The TEM gene was amplified in 35 isolates, whereas, SHV was found in 11 and CTX-M in 50 isolates. None of the above ESBLs genes were detected in 15 clinical isolates and all three genes were amplified in 2 clinical isolates.

Prevalence of CTX-M in ESBL-producing uropathogens

Among 84 clinical isolates, 64 were uropathogens (table 4), *E. coli* was dominant uropathogen in females, *i.e.*, 70.1% (40/57) as compared to male gender 29.8% (17/57). CTX-M gene was prevalent in ESBL-producing uropathogenic *E. coli* and *K. pneumoniae*. table 4 indicated TEM, SHV and CTX-M gene among uropathogenic clinical isolates and gender. The isolates with none of the ESBLs gene were indicated in the results as No gene.

DISCUSSION

The present study investigated the prevalence of broad-spectrum cephalosporin's resistant gene of TEM, SHV and CTX-M among ESBL positive isolates. Total

Table 1: Primers sequences used for ESBLs genes (Forward and reverse primer sequences previously defined with product size)

Primer	Primer sequence 5´_3´	Size (bp)	Reference
TEM F	TCG GGG AAA TGT GCG CG	971	(Saladin *et al.*, 2002)
TEM R	TGC TTA ATC AGT GAG GCA CC		
SHV F	GATGAACGCTTTCCCATGATG	214	(Kim *et al.*, 2009)
SHV R	CGCTGTTATCGCTCATGGTAA		
CTX-M F	SCS ATG TGC AGY ACC AGT AA	554	(Saladin *et al.*, 2002)
CTX-M R	CCG CRA TAT GRT TGG TGG TG		

F: Primer forward sequence, R: Primer reverse sequence, bp: base pair

Table 2: PCR-thermocycler requirements for ESBLs Genes

Genes	Denaturation	Cycles	Final elongation
TEM	94 °C for 5 min	30 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min	72 °C for 5 min
SHV	95 °C for 5 min	30 cycles of, 95 °C for 1 min, 61 °C for 1 min,72 °C for 1 min	72 °C for 10 min
CTX-M	95 °C for 5 min	30 cycles of 95 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min	72 °C for 10 min

Table 3: Percentage distribution of ESBLs positive clinical isolates from various specimens

Specimen	No. of Isolates	%age	*E. coli*	*K. pneumoniae*	*P. mirabilis*
Urine	72	75	63	9	-
Pus	9	9.37	8	1	-
Sputum	3	3.57	-	3	-
Stool	4	4.16	4	-	-
HVS	5	5.20	3	2	-
Throat Swab	1	1.04	-	-	1
Cystic Fluid	1	1.04	1	-	-
Semen	1	1.04	-	1	-
Total	96	100%	72	16	1

*HVS: high vaginal swab

Table 4: Distribution of ESBLs gene in uropathogenic clinical isolates

Organism	ESBL-producing isolates	Uropathogens	Male	Female	No Gene	TEM	SHV	CTX-M
E. coli	72	57	17	40	7	25	3	39
K. pneumoniae	11	7	2	5	1	3	4	2
P. mirabilis	1	-	-	-	-	-	-	-
Total	84	64	19	45	8	28	7	41

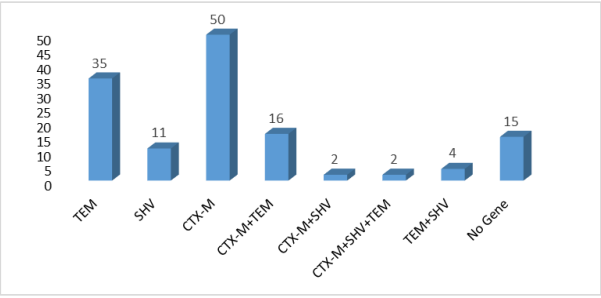

Fig. 1: Distribution of CTX-M, TEM and SHV among ESBL producing clinical isolates (out of eight-four clinical isolates the number of gene identified alone or in combination with other gene)

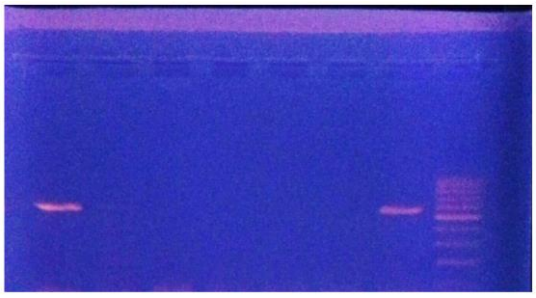

Fig. 2: Amplification of CTX-M gene on 1% agarose gel electrophoresis using 100 bp DNA ladder: From right in Column 1; Column 2 and Column 8 were amplified with product of 554 bp indicated positive results of CTX-M gene.

n=84 ESBL isolates were screened for gene amplification and 69 were positive with ESBLs gene *i.e.,* 82.14% (69/84) with TEM, SHV and CTX-M genes, none of the gene was found in 17.85% (15/84) isolates (fig. 1).

The high incidence of CTX-M gene was reported from all over the world among ESBLs (Blanc *et al.*, 2014;Chen *et al.,* 2014; Zhang *et al.,* 2014; Graham *et al.,* 2016; Djuikoue *et al.,* 2017). Similar trend was found in present study and CTX-M was found to be most prevalent gene (59.5%) followed by TEM and SHV i.e., 41.6% and 13% respectively. Previously from Pakistan, a study by Habeeb*et al.* (2013) revealed rapid emergence of CTX-M gene within five years i.e., in year 2005 it was 3.5% then 42.5% in the year 2009-10, the present work indicated that the dissemination of CTX-M gene has increased up-to 17%as compared to the previously published report in the year 2009-10 *i.e.,* 42.5%

In Recent years, community related ESBLs infections were reported frequently from different regions, dominantly *E. coli* related urinary tract infection (UTIs) (Alyamani *et al.*, 2017; Belmont-Monroy *et al.*, 2017; Djuikoue *et al.,* 2017; Jean *et al.,* 2017). Globally, this community dissemination of UTIs associated with ESBL producing *E. coli* (Pitout*et al.,* 2005) have two major relations, first one is due to *E. coli*, found in humans as normal intestinal flora as a major pathogen (Tenaillon *et al.,* 2010) and other reason is production of CTX-M (Bauernfeind *et al.,* 1996). In present study, CTX-M gene was dominant in uropathogens *i.e.,* 64%.The CTX-M was found positive in 41 uropathogens out of 64 urine specimens. The current finding has an increased rate as compared to previous report from Pakistan that indicated CTX-M was positive in 47% ESBL related UTIs (Habeeb*et al.,* 2013). Of 64 uropathogens, 57 were *E. coli* and preponderance of CTX-M was found in 78% uropathogenic *E. coli* (table 4), whereas, 7 uropathogenic-*E. coli* were not detected with any gene. The present findings has similar trends as previously published studies, indicated high prevalence rate of CTX-M in uropathogenic *E. coli* 71.4% from Sudan (Ahmed *et al.*, 2013), 42.5% from China (Zhao *et al.*, 2015),95.2% from Korea (Kim *et al.*, 2016) and 59.54% (Rahman *et al.*, 2016) from Pakistan.

Previously, prevalence of TEM and SHV genes were studied in several reports revealed that TEM was more prevalent gene *i.e.,* 29.6% (Habeeb *et al.,* 2013) and 40.5% (Rahman *et al.,* 2016) in *E. coli*, whereas SHV were positive in 11.76% (Kargar *et al.,* 2014). In present study similar prevalence trend was observed *i.e.,* 50% and 6% of uropathogenic *E. coli* were TEM and SHV positive respectively. Comparison with previous studies, the prevalence of TEM has increased in present findings from Pakistan but less frequent than CTX-M.

In uropathogenic *K. pneumonia*, ESBL genes were positive in 6 isolates (table 4). CTX-M was not dominant and found in 33% (2/6). Several published studies indicated that community onset of *K. pneumoniae* related UTIs has increased with dominance of CTX-M gene (Doi *et al.,* 2008;Lee *et al.,* 2011; Peirano *et al.,* 2012). In the present findings, low incidence was revealed in community related UTIs associated with *K. pneumoniae* because of low prevalence of CTX-M in Karachi, Pakistan. The SHV gene preponderance was high in uropathogenic *K. pneumonia* i.e., 66% followed by TEM 50% (Table 4), similarly studies published from Sudan (Ahmed *et al.,* 2013) and Sri Lanka (Tillekeratne *et al.,* 2016).

CTX-M and TEM was dominant in female gender *i.e.,* 62.5% (35/56) and 46.4% (26/56) respectively, whereas SHV gene was dominant in male gender 14.3% (4/28). In females, 82.8% (29/35) were positive with CTX-M gene recovered from uropathogenic *E. coli* followed by TEM and SHV i.e., 73% (19/26) and 28% (2/7) respectively. Previously, a study indicated 100% CTX-M gene in women (Djuikoue*et al.,* 2017). In present study, uropathogens from both genders were positive with ESBLs genes either uropathogenic-*E. coli* or *K. pneumoniae*. A previously published report revealed that more often cases of *E. coli* associated UTIs in females and *K. pneumoniae* related respiratory tract infections in males (Bialvaei *et al.,* 2016). Although, present findings showed SHV gene dominance in females with *K. pneumoniae* associated UTIs *i.e.,* 42.8% (3/7).

CONCLUSION

The study concluded that CTX-M gene was highly prevalent in uropathogenic *E. coli*, indicating community-onset of urinary tract infections (UTIs). The incidence of community related UTIs can be reduced and controlled with better hygienic practices. Prescribing of third generation cephalosporins should be adequate with detailed culture reports. However, rationale practices must be employed for utilization of other broad-spectrum antibiotics.

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