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RESEARCH ARTICLE

POTENTIAL PLASMIDIC CO-TRANSFER, EXTENDED SPECTRUM β--LACTAMASE LINEAGE AND INTEGRON ASSOCIATION IN UROPATHOGENIC *E. coli* FROM AN ENDEMIC REGION

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ABSTRACT

Resistance to extended-spectrum β-lactams was mediated by plasmidic extended-spectrum β-lactamases (ESBLs) and/or AmpC β–lactamases in uropathogenic *E. coli* (UPEC) a primary etiologic agent of urinary tract infections (UTI). Often the isolates were multidrug resistant harboring integrons and cause great hindrance in treatment. This study aims to identify genetic relatedness between plasmids and their correlation with β-lactamase resistant genes and integrons in nosocomial UPEC isolates. 29 drug resistant patterns were observed in 82 *E. coli* isolates after urine culture and biochemical detection. 33(45.8%) isolates were ESBL producers and 39 (54.2%) resistant to ESBL production. The former showed lowest resistance (3%, 24.2%) than the latter (43.6%, 35.9%) group to meropenem and nitofurantoin respectively. Conjugal transfer of plasmids was successful for 52 isolates. *bla*TEM was present in all transconjugants, alone or in combination with other β-lactamases and integrons. Mantel test revealed significant correlation (Rxy=0.997, p <0.0001) between plasmids, β-lactamase genes and integrons. Therefore this study demonstrated that flow of genetic information was due to mobile genetic elements instead of genetic recombination between plasmid pairs. Furthermore awareness, detection and distribution of resistant determinants were an absolute necessary for designing therapeutics for optimal patient care.

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INTRODUCTION

The clinical management of urinary tract infection is complicated by the increasing incidence of infections caused by strains of *Escherichia coli* that are multidrug resistant (MDR) (Vijayakanthi *et al*., 2013; Bajpai *et al*., 2014). There are several factors responsible for dissemination of MDR genes among the different bacterial strains, and plasmid-mediated transfer has been considered one of the most important mechanisms for the horizontal MDR (Brolund *et al*., 2013). High incidence of extended-spectrum *β*-lactamases (ESBLs) produced by the MDR uropathogenic *E. coli* was reported in isolates circulated in eastern region of India (Mukherjee *et al*., 2013) as well as at different other geographical locations (Khadgi *et al*., 2013, Matsumura *et al*., 2013). Most ESBLs were mutants of TEM and SHV enzymes along with CTX-M type beta lactamases that originated from the environmental species of genus *Kluyvera* that were often carried on plasmids (Carattoli, 2009).

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Incidence of hyper-production of TEM-1 penicillinase, production of ESBL and AmpC β-lactamase simultaneously, production of inhibitor- resistant TEM (IRT), complex mutant TEM (CMT)-like ß-lactamase, OXA-type β-lactamase and carbapenemase production respectively can hinder the phenotypic detection of ESBL producers as they revealed resistance against β-lactam–β-lactamase inhibitor combinations (Matsumura *et al*., 2013, Park *et al*., 2012). Therefore phenotypic confirmatory test for detection of the ESBL-producers have limitations and must be addressed by appropriate genotypic investigations to implement appropriate infection control measures. Plasmids conferring resistance to important antibiotics such as the extended-spectrum cephalosporins along with fluoroquinolones and aminoglycosides may be sustained in the host conferring a selective advantage in the presence of the respective antibiotics (Carattoli, 2013). Therefore co-resistance to ß -lactam and non-ß lactam antibiotics may be explained by co transfer of resistance determinants in the same genetic elements leading to the dissemination of multiple drug resistance in these microbes (Sharma *et al*., 2010). The fact that ESBL genes could be acquired by strains harboring particular integrons may enlarge the possibilities of selection of these isolates by a variety of

different antimicrobials. Moreover, ESBL genes can be located on integrons, which may facilitate the spread of such genetic elements (Machado *et al*., 2005). The study was designed to characterize extended spectrum β-lactam resistant uropathogenic *E. coli* isolated from hospitalized patients from Kolkata, an eastern region of India with respect to ESBL production and transfer of resistance. Further attempts were made to explore a correlation if any between the transmissible plasmids and their respective genes to understand the mechanism of spread of drug resistance.

MATERIALS AND METHODS

Bacterial culture

A total of 150 urine samples were collected from Carmichael Hospital for Tropical Diseases, Kolkata from patients with UTI. Among these, 100 urine samples yielded significant growth. A significant monomicrobic bacteriuria was defined as the culture of a single bacterial species from the urine sample at a concentration of $>10^5$ cfu/ml. Only a single positive culture per patient was included in the analysis. *E. coli* were detected in 82 isolates out of 140 by standard biochemical tests. The media which were used in the study included, MacConkey's Agar, Muller Hinton agar and Luria Bertani Broth (Hi-Media, Mumbai). The cultures were incubated at 37ºC for 24 hrs respectively. These *E. coli* isolates were sub-cultured on Luria Bertani broth and were maintained on Luria bertani agar plates at 4ºC for further studies. The study protocol was approved by the institutional ethical committee.

Antibiotic Sensitivity and ESBL confirmation

The antibiotic sensitivity of the isolates against amikacin (AK; 10µg), gentamicin (GEN; 10µg), tobramycin (TOB; 10 µg), ceftazidime (CAZ;30 µg), cefotaxime (CTX; 30 µg), cefoxitin(CX; 30 µg), ciprofloxacin (CIP; 5 µg), levofloxacin (LE; 5µg), cotrimoxazole (COT; 25 µg), nitrofurantoin (NIT; 300 µg), and meropenem (MRP;200 µg) (Hi-Media, Mumbai) was tested on Muller Hinton agar using Kirby Bauer disc diffusion method. *E. coli* (ATCC 25922) was used as quality control strain. The zone of inhibition was interpreted following CLSI 2010 guidelines. An isolate was considered as multidrug resistant if it was resistant to ≥3 groups of antibiotics. ESBL testing was done using CLSI recommended phenotypic confirmation test (CLSI 2010) on all isolates that were resistant to any cephalosporin disks. The sensitivity testing against cefepime- tazobactam (CPT; 30/10 µg Hi media, Mumbai India) was performed on isolates that were negative in the ESBL phenotypic confirmation test. The cefepime zone sizes as per CLSI 2010 were used to interpret the susceptibility of cefepime-tazobactam as no criteria for this combination were given by CLSI 2010 and the susceptible isolates were interpreted as ESBL producers (Sharma *et al*., 2012).

Molecular analysis

Transfer of resistance plasmids was performed by broth mating assays using a sodium azide-resistant *E. coli* J53 recipient. Transconjugants were selected on Mac Conkey agar plates containing sodium azide (100 µg /ml) and cefotaxime (30 µg /ml) (Loncaric *et al*., 2013). Plasmids were isolated from the transconjugants by alkali lysis method. The samples were run on 0.8% agarose gels and visualized by Gel documentation system (BioRad). Plasmid bands of varied size (approximate) were detected using molecular weight marker, lambda/HindIII double digest. Plasmids obtained by conjugation were designated as pUEC. Numbers were chosen according to the isolate number respectively. PCR analysis for detection of ESBL genes; bla_{TEM} and $bla_{CTX\text{-}M}$ (Mukherjee *et al*., 2011), pAmpC beta-lactamase gene; $bla_{CMY\text{-}2}$ (Winokur *et al.,* 2001) and Class 1 (*intI1*) and 2 (*intI2*) integron genes (Machado *et al.,* 2005) using established gene specific primers and conditions was performed on plasmid DNA isolated from the transconjugants and whole genomic DNA of the recipient strain as a negative control. Nucleotide sequencing reactions were carried out on all $bla_{CTX\text{-}M}$ and all bla_{TEM} amplified products in which $bla_{CTX\text{-}M}$ or $bla_{CMY\text{-}2}$ or integron genes were absent to validate the identity of both genes. A dendogram was created on bootstrap test (500 replicates) and distances were computed using Maximum Composite Likelihood method with MEGA v 4 software (Johnson *et al*., 2009).

Cluster analyses

Cluster analysis of plasmid pattern obtained from transconjugants was performed according to the genetic distance method of Jacard and graphically represented by UPGMA method. The relationship between the distribution of individual gene and their plasmid profile data amongst all the transconjugants was correlated with respect to their Jacard distance matrices using a Mantel test (Sherley *et al*., 2004).

RESULTS

ESBL detection and Antibiotic Sensitivity

E. coli was detected in 82 out of 140 urine culture positive isolates. All 82 isolates except 10 were presumptive ESBL producers showing resistance against third generation cephalosporins. The combined-disk method characterized by synergy between clavulanate and at least one of the standard antibiotic disks (ceftazidime and cefotaxime) and against cefepime-tazobactam confirmed 21 and 12 out of the 72 cephalosporin resistant *E. coli* isolates to be ESBL producers respectively.

The antibiotic sensitivity pattern of confirmed ESBL producers (33/72) and the β–lactamase inhibitor resistant (39/72) presumptive ESBL producers against potential antibiotics indicated that the latter group had similar resistance compared to the former against ciprofloxacin (93.9%, 92.3%), levofloxacin (78.8%, 76.9%), gentamicin (87.9%, 82.1%) and tobramycin (78.8%, 76.9%). Increased resistance was observed in the inhibitor resistant isolates against amikacin (48.5%, 66.7%) and cotrimoxazole (72.7%, 84.6%) respectively. Moreover 97% and 75.8% of the ESBL producers were susceptible against meropenem and nitrofurantoin but the inhibitor resistant isolates showed 43.6% and 35.9% resistance against the two drugs respectively (Fig. 1). Overall 29 different antibiotic resistant profiles were observed. Each of the patterns was common in 1 or upto 9 isolates. 7 isolates were found to be resistant against all antibiotics used in this study (Table 1).

Resistance transfer: plasmids and antibiotic resistance genes

Transfer of resistant plasmids by conjugation to *E. coli* J53 azide resistant was successful for 18 (52.9%) and 34 (87.2%) out of 33 and 39 β-lactam-β- lactamase inhibitor sensitive and resistant uropathogenic *E. coli* isolates respectively.

Table 1. Resistance pattern of the cephalosporin resistant clinical uropathogenic *E. coli* isolates (n=72)

Antibiotic resistance pattern	No. of isolates
AK GEN TOB CIP LE COT	9
AK GEN TOB CIP LE COT NIT MRP	7
AK GEN TOB CIP LE NIT	1
AK GEN TOB CIP COT	3
AK GEN TOB CIP LE COT MRP	6
GEN CIP LE COT NIT	2
GEN CIP COT NIT	1
AK GEN TOB CIP LE COT NIT	7
AK GEN TOB NIT	1
GEN CIP LE COT	3
GEN TOB CIP LE	3
GEN CIP LE	1
AK GEN TOB CIP LE	2
AK GEN TOB CIP	1
CIP LE COT	3
GEN TOB CIP COT	3
GEN TOB CIP COT NIF	1
GEN TOB CIP LE COT	5
CIP LE	2
CIP COT	1
AK GEN TOB CIP COT NIT	1
AK GEN TOB CIP COT MRP	1
AK GEN TOB COT	1
AK GEN TOB CIP	1
GEN TOB CIP LE COT MRP	1
AK GEN TOB CIP LE NIT MRP	1
TOB CIP LE	1
CIP LE COT NIT MRP	2
AK GEN TOB COT NIT	1

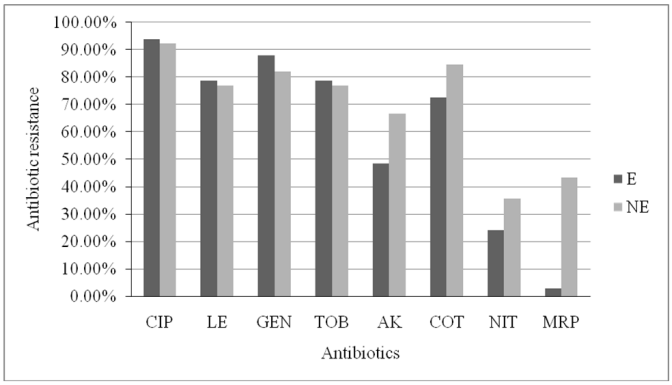

Fig. 1. Antimicrobial resistance amongst the cephalosporin resistant clinical uropathogenic *E. coli* isolates (E; ESBL producers; n=33, NE; resistant to ESBL production; n=39). CIP; ciprofloxacin (5 µg), LE; levofloxacin (5µg), AK; amikacin (10µg), GEN; gentamicin (10µg), TOB; tobramycin (10 µg), COT; cotrimoxazole (25 µg), NIT; nitrofurantoin (300 µg), MRP; merpenem (10 µg). All assays were performed in triplicate with each pathogenic isolate

All 18 and 1 out of 34 transconjugants obtained from β-lactam—β-lactamase inhibitor sensitive and resistant clinical isolates respectively were ESBL producers. Plasmid profiles from all the 52 transconjugants indicated one to six plasmids of approximate sizes ranging from 17 kb to 1 kb. Prevalence of a plasmid of an approximate size in the range of 12 kb was common amongst all the transconjugants.

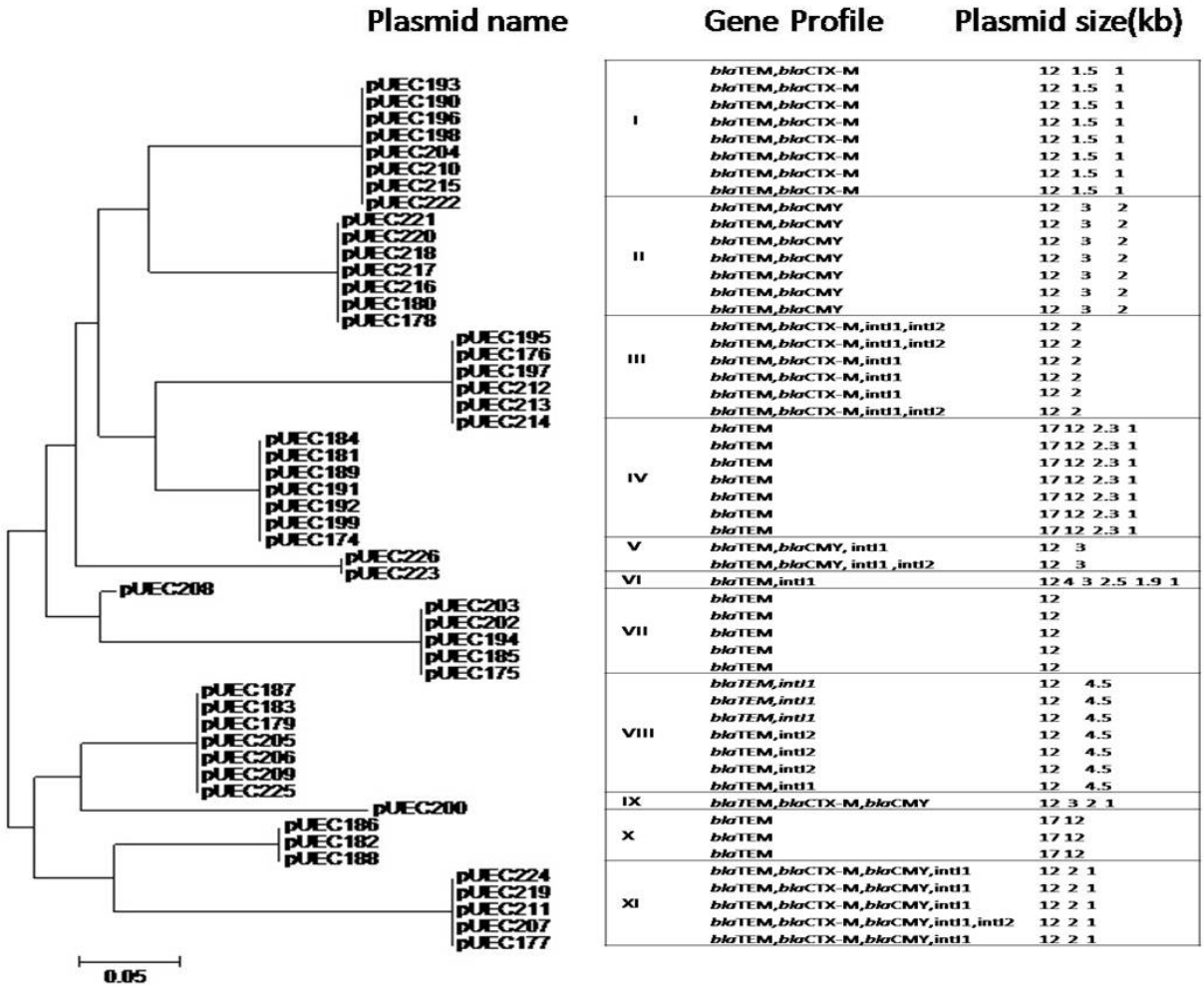

Fig. 2. Genetic relatedness among 52 transconjugants obtained from clinical uropathogenic *E. coli* isolates based on plasmid pattern and gene acquisition

The plasmids with the sizes of ~1-2 kb were the second most frequent and were observed in about 65.3% of the transconjugants while ~2-3kb, ~4-5 kb and ~17 kb were detected in only 32.6%, 15.4%, 19.2% of the isolates respectively. A total of three plasmid associated ß-lactamase genes (bla_{TEM}, bla_{CTX-M} and bla_{CMY-2}) were identified. bla_{TEM} was observed in all the 52 tranconjugants either alone (15/52) or in combination with bla_{CTX-M} (8/52), bla_{CMY-2} (7/52) or both (1/52). Incidence of class I and class II integrase genes that were often associated with multiple drug resistance was observed in combination with bla_{TEM} (5/52, 3/52) only, with both bla_{TEM} and bla_{CTX-M} (3/52, 0/52), bla_{TEM} and bla_{CMY-2} (1/52, 0/52) and bla_{TEM}, bla_{CTX-M} and bla_{CMY-2} (0/52, 1/52) respectively. Moreover 3, 1 and 1 out of 52 isolates harbored both class I and II integrase genes in combination to bla_{TEM}, bla_{CMY-2}, and bla_{TEM}, bla_{CTX-M}, bla_{CMY-2} respectively (Table 2). CTX-M-15 was identified by nucleotide sequence analysis from all bla_{CTX-M} positive isolates.

Table 2. Distribution of β-lactamase and integron encoding genes amongst the transconjugants obtained from cephalosporin resistant uropathogenic *E. coli* isolates (n=52)

Genotypes	*E. coli* isolates n (%)
bla_{TEM}	15 (28.8)
bla_{TEM} + bla_{CTX-M}	8 (15.4)
bla_{TEM} + $bla_{CMY\,2}$	7 (13.5)
bla_{TEM} + *intI1*	5 (9.6)
bla_{TEM} + *intI2*	3(5.8)
bla_{TEM} + bla_{CTX-M} + *intI1*	3 (5.8)
bla_{TEM} + bla_{CTX-M} + *intI1* + *intI2*	3(5.8)
bla_{TEM} + bla_{CTX-M} + $bla_{CMY\,2}$ + *intI1*	3 (5.8)
bla_{TEM} + bla_{CTX-M} + $bla_{CMY\,2}$ + *intI2*	1(1.9)
bla_{TEM} + bla_{CTX-M} + $bla_{CMY\,2}$ + *intI*+ *intI2*	1(1.9)
bla_{TEM} + bla_{CTX-M} + $bla_{CMY\,2}$	1 (1.9)
bla_{TEM} + $bla_{CMY\,2}$ + *intI1*	1 (1.9)
bla_{TEM} + $bla_{CMY\,2}$ + *intI1*+ *intI2*	1(1.9)

Genetic relatedness between transmissible plasmids and resistance gene

The genetic relationship among the 52 *E. coli* transconjugants indicated 11 well defined clusters by UPGMA method (Fig.2). Furthermore plasmids with similar size and number were found to harbor similar combinations of resistant genes.

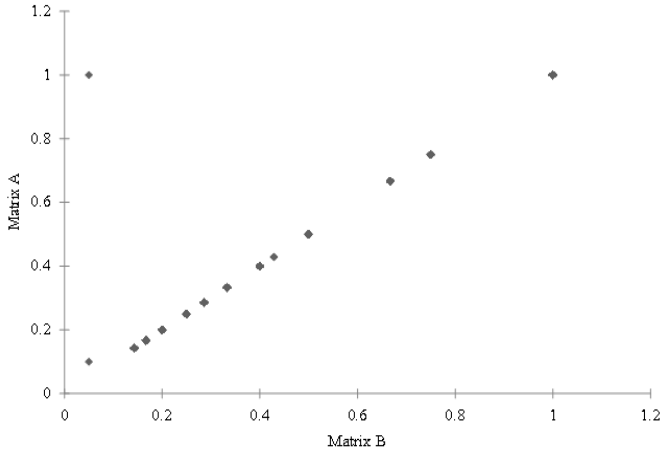

Fig. 3. The correlation between the plasmid mediated β-lactamase encoding genes, integrons and the plasmid pattern. Distance matrices were created to describe the distribution of β-lactamase genes either alone or in combination with the integron genes (Matrix A) for each plasmid pattern (Matrix B). These matrices were compared using Mantel test

Mantel test analysis confirmed that there exist a statistically significant (Mantel correlation coefficient; Rxy=0.997, p <0.0001) correlation amongst the plasmid clusters and acquisition of ß-lactamase and integron encoding genes amongst the 52 transconjugants obtained from the cephalosporin resistant clinical uropathogenic *E. coli* isolates analyzed (Fig. 3).

DISCUSSION

This study describes plasmid dissemination along with group I class A and class C ß-lactamse encoding genes and class I and class II integrons and their genetic relatedness obtained from cephalosporin resistant uropathogenic *E. coli* isolated from hospitalized patients from Kolkata an eastern region of India. Antibiotic resistance profile for clinical *E. coli* isolates have well been documented by various workers (Jan *et al*., 2009, Baral *et al*., 2012). It was found from this study that samples collected throughout the year 2013 reports 87.8% resistance against third generation of cephalosporins; ceftazidime and cefotaxime and 77-94% resistance was observed against ciprofloxacin, levofloxacin, gentamycin and cotrimoxazole amongst the presumptive ESBL producers respectively (Fig.1). Replacing cephalosporins with antibiotic containing β lactamase inhibitor combinations (cepipime-tazobactam) were reported to help to reduce the occurrence of ESBL producing organism (Biswas, 2013). In this study resistance against β-lactamase inhibitor combinations using clavulanate and tazobactam was observed in the 39 cephalosporin resistant clinical isolates indicating their restricted use in this region of our country. Moreover our results suggest that fluoroquinolones and cotrimoxazole that were once used as potent drugs along with cephalosporins in urinary tract infection have completely lost their efficacy, however meropenem and nitrofurantoin may be a better choice. Outbreaks of the infections which were caused by ESBL producing multiple drug resistant organisms had been earlier reported from virtually every European country (Luzzaro *et al*., 2006) as well as from the Indian sub-continent (Sarma *et al*., 2011). Therefore detailed investigation on the drug resistant isolates poses an absolute necessity to administer appropriate treatment measures as well as to prevent the spread of resistance. ESBL producers can often transfer resistance to multiple bacterial species through plasmid-mediated conjugation increasing the rate of antimicrobial resistance transmission. The widespread use of antimicrobials, coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and integrons, further contribute to increase the prevalence of antimicrobial resistance in these pathogenic microbes.

Experiments suggest that frequencies of conjugative transmission in nature are probably several orders of magnitude higher than those under laboratory conditions. Various reports suggested acquisition of plasmids of varied size and numbers amongst the multiple drug resistant pathogenic *E. coli* isolates worldwide and nationwide (Khadgi *et al*., 2013, Sharma *et al*., 2010) with incidence of a predominant plasmid at 26 kb or 21 kb respectively (Khadgi *et al*., 2013, Jan, 2009]. In this study, transmissible plasmids, numbers and size range between 1-4 and 1-17 kb, respectively with a predominant band at 12 kb were observed in the 52 transconjugants obtained from the multidrug resistant clinical *E. coli* isolates irrespective of their sensitivity against ß -lactam- ß-lactamase inhibitor combinations. Cephalosporin

resistance derived from TEM type ß-lactamases were reported to be carried on transmissible plasmids (Lim *et al*., 2009) Consistent with the observation, an universal distribution of TEM type ß-lactamases in all the transconjugants either alone or in combination with other resistant determinant genes independent of ß -lactam- ß-lactamase inhibitor sensitivity was observed indicating risk of dissemination of resistance against the most widely prescribed ß -lactams. Prevalence of plasmidic AmpC (pAmpC) type ß –lactamase production was not much explored in Indian *E. coli* isolates however high incidence was reported worldwide (Jacoby, 2009). However our study indicated that bla_{CMY} encoding pAmpC type ß-lactamase gene was present in 29.4% of the transconjugants and could be transmissible *in vivo*. CTX-M-15 was the most predominant CTX-M type ESBL in India (Dureja C *et al* 2014) and worldwide (Carattoli, 2009, Shahid *et al*., 2014). CTX-M-15 encoding gene was detected in 39% of the transconjugants in combination with other genes (Table 2). Moreover co-existence of more than one type of plasmid mediated β-lactamases in the transconjugants obtained from the clinical *E. coli* isolates (Table 2) was consistent with other reports (Matsumura *et al*., 2013, Lim *et al*., 2009; Castillo *et al*., 2013) and strongly suggests risk of transmission of the antibiotic resistance determinants in population. It has been well established that the integron carrying plasmids plays a dominant role in multiple drug resistance (Sun *et al*., 2013). In this study 27.5% and 15.7% of the transconjugants harbored *intI1* and *intI2* genes in varied combination in their plasmids with the ß-lactamase resistance genes. Furthermore isolates irrespective of the presence of ESBL genes were found to be resistant to ß –lactamase-inhibitors. This observation may be attributed to either presence of mutant variants of TEM type ß-lactamase, inhibitor resistant TEM type ß-lactamase or other transmissible ESBL genes that were not included in the study (Lim, 2009, Martín *et al*., 2010). Horizontal gene transfer has played a predominant role in the evolution and transmission of resistance to the β-lactam antibiotics among the *Enterobacteriaceae* in both community and hospital infections. The horizontal movements of resistant plasmids between cells were very frequent, exacerbating the flow of genetic information by increasing the opportunity for recombination between novel plasmid pairs (Sherley *et al*., 2004). Moreover involvement of transposons and integron cassette systems were also reported to mediate resistant plasmid evolution rather than recombination events (Sun *et al*., 2013, Brown *et al*., 2000, Carattoli A *et al* 2002). In our study a genetic relatedness (Fig. 2, Fig. 3) between transmissible plasmids and the resistance genes and prevalence of integron cassettes provided evidence of flow of genetic information by mobile genetic elements instead of genetic recombination between plasmid pairs amongst the extended spectrum β-lactam resistant uropathogenic *E. coli* from the eastern region of India.

Conclusion

TEM β-lactamase was the most predominant ESBL in the uropathogenic *E. coli* isolates from hospitalized patients from the eastern part of India. Transmission of resistance to the extended spectrum β-lactam antibiotics in these isolates was attributed to mobile genetic elements instead of genetic recombination between plasmid pairs.

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