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RESEARCH ARTICLE

DISTRIBUTION OF CLASS D OXACILLINASES AMONGST THIRD GENERATION CEPHALOSPORIN RESISTANT NOSOCOMIAL UROPATHOGENIC *Escherichia coli* ISOLATES, THEIR PHYLOGENETIC BACKGROUND AND CLONAL ANALYSIS

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ABSTRACT

Wide diversity of β-lactamases causes resistance to third-generation cephalosporins (3GCs) in uropathogenic *E. coli* (UPEC) the frequently encountered nosocomial pathogen creating treatment complications. In the present study incidence of OXA type ß-lactamase in 3GC resistant UPEC was explored. Phylogenic background and genetic relatedness between the isolates was also analyzed. 40.5% *E. coli* resistant to 3GCs were isolated from 190 urine culture positive samples from hospitalized patients clinically diagnosed for urinary tract infection (UTI). Highest resistance was observed against non-β-lactams; ciprofloxacin(100%), levofloxacin(90%), cotrimoxazole(95%) and intermediate resistance against amikacin(55%). ESBL(44.15%) production and β-lactamase inhibitor resistance (BLIR;55.84%) was observed with varied imepenem(100%, 88.37%) susceptibility respectively. Multiple β-lactamases (OXA group I, II, III, TEM, CTX-M) were prevalent. Occurrence of OXA group I, III and TEM was highest amongst ESBL(44.1%) compared to BLIR(32.6%) isolates. OXA group I, II and III β-lactamases either alone or in combinations with other β-lactamases were carried on IncF type plasmid (IncFrepB, IncFIB). Majority of the UPEC belonged to B2(75.32%) and D(22.07%) and their ERIC-PCR profiles indicated genetic homogeneity. This study for the first time demonstrated high distribution of OXA group I and III in 3GC resistant nosocomial UPEC isolates of homogeneous genetic origin from the eastern region of India.

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INTRODUCTION

Plasmid acquired β-lactamases (SHV, TEM and CTX-M, OXA, AMPC) resistant to 3GCs were commonly detected either alone or in combination in clinically challenging *E.coli* (Doi *et al*., 2013) and *Klebsiella pneumonia* (Coque *et al*., 2002). OXA-type β-lactamases are classified into five groups (Sorour *et al*., 2008). Group I extend their substrate profile to produce class D-OXA ESBLs imparting resistance to expanded-spectrum cephalosporins, by accumulating one to several amino acid substitutions (Antunes *et al*., 2014). Exploring treatment options against 3GC resistance indicated carbapenem as an ultimate drug of choice (Meyer *et al*., 2010). Increased carbapenem consumption was associated with carbapenem resistance due to production of carbapenem-hydrolyzing class D β-lactamase (CHDLs) in *K. pneumoniae* and imipenem-resistant *A. baumannii* (Antunes *et al*., 2014; Meyer *et al*., 2010). Moreover OXA-ESBLs were frequently detected in clinical isolates of *E.coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and were poorly inhibited by clavulanic acid a commonly used β lactamase inhibitor (Sahuquillo-Arce *et al*., 2015; Sorour *et al*., 2008). Furthermore expansion of the substrate profile of OXA β-lactamases (group I,II) to include carbapenem antibiotics was also reported in *Pseudomonas aeruginosa* and *Acinetobacter baumanni* (Sahuquillo-Arce *et al*., 2015). There are few studies on the epidemiology and geographical spread of OXA β-lactamases in *E.coli* from different parts of Indian subcontinent (Bhattacharjee *et al*., 2007; Chaudhary and Payasi, 2014) High incidence of resistance against extended-spectrum cephalosporins was reported in multidrug-resistant uropathogenic *E. coli* (UPEC) from eastern region of India (Mukherjee *et al*., 2013) but there is no knowledge on distribution of OXA β-lactamases amongst these pathogens. The OXA enzymes also impart resistance to inactivation by

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medically important β-lactam–β-lactamase inhibitor combinations (Bethel *et al*., 2008) and poses serious challenges in treatment. However no specific phenotypic test can identify OXA enzymes amongst other β-lactamases and molecular detection was considered as the gold standard. In this study distribution of OXA β-lactamase groups was investigated amongst the β-lactamase resistant genes in 3GC resistant UPEC. The isolates were further characterized at molecular level with respect to their plasmid replicon types, phylogenetic background and genetic relatedness by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR analysis to provide an insight into appropriate therapeutics.

MATERIALS AND METHODS

Bacterial Isolates

250 urine samples were collected from Carmichael Hospital for Tropical Diseases, Kolkata from patients clinically suspected for urinary tract infection. 190 samples yielded significant growth (>10^5 cfu/ml). *E.coli* was detected in 77 isolates by colony morphology on MacConkey agar plates and speciated by standard biochemical test (Mukherjee *et al*., 2013) The study protocol was approved by the institutional ethical committee.

Antibiotic Susceptibility

Antimicrobial susceptibility testing of UPEC isolates was performed by Kirby-Bauer method (CLSI document M100-S21.2013). Antimicrobial disks of ciprofloxacin (CIP; 10 µg), levofloxacin (LE; 5 µg), ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), cefepime(CPM; 30 µg), cotrimoxazole(COT; 25 µg), amikacin(AK;10 µg), imepenem(IPM; 10 µg) tested were obtained from Hi-Media labs, Mumbai, India. *E.coli* ATCC 25922 was used as a negative control strain.

ESBL confirmatory test

ESBL confirmatory test was performed using CAZ and ceftazidime-clavulanic acid (CAC;30+10 µg), CTX and cefotaxime-clavulanic acid (CEC;30+10 µg) and CPM and cefepime-tazobactam (CPT;30/10 µg) combination disks on 3GC resistant isolates (Mukherjee et.al.,2013). Interpretation of results was as per CLSI 2011 guideline (CLSI document M100-S21.2013).

Plasmid DNA isolation, identification of β-lactamase genes and plasmid replicon typing

Plasmid DNA was prepared from the clinical isolates that were lysed by lysis buffer (50mM Tris-HCl, 3%SDS, 2(N) NaOH) incubated at 55˚C for 60 minutes (Kado.1981). Phenol-chloroform extraction followed by CH_3COONa/95% C_2H_5OH precipitation was performed. The plasmid pellet was washed with 70% ice cold C_2H_5OH air dried and resuspended in 20µl of 1x TE (10mM tris, 1mM EDTA) buffer and used as template to identify β-lactamase genes *TEM*, *CTX-M* and *OXA* (Sorour *et al*., 2008; Mukherjee *et al*., 2011) The *OXA* positive isolates were further screened by PCR for identification of OXA groups (Sorour *et al*., 2008). Plasmid replicon types were assigned to incompatibility groups by PCR based replicon typing (PBRT) using IncFrep, F1B, N, I1, A/C, H1, X, Y, L/M, W primers as described (Carattoli *et al*., 2005).

Cluster analysis

Cluster analysis was performed using heatmap.2 function in gplots library (version 2.17.0) in R software package (version 3.2.0). Euclidean distance was used for both row and column dissimilarity. Clustering used complete linkage and was used for data visualization, so no formal statistics were run (Ibrahim *et al*., 2016)

Bacterial total DNA isolation, detection of phylogenetic groups and ERIC typing

Bacterial total DNA was extracted from whole cells by boiling method (Farshad and Emamghorashi, 2009) and stored at –20 ^{0}C until use. PCR was performed on extracted DNA to detect the phylogenetic group of the isolates using specific primers against the three DNA markers; *chuA*, *yjaA* and the DNA fragment *TSPE4.C2* (Clermont *et al*., 2000) and ERIC-PCR typing using primersERIC-1; 5'-AGCTCCTGGGGATTCA-3'and ERIC-2;5'-AAGTAAGTGACTGGGGTGAGCG-3' (Dhanashree and Mallya 2012). A dendrogram was constructed from comparison of ERIC PCR profiles, using the Dice coefficient, and clustered by unweighted pair group method with arithmetic averages (UPGMA) (Durmaz *et al*., 2015).

RESULTS

Antibiotic resistance

The antibiogram analysis on 77UPEC isolates indicated highest resistance against 3GCs, CAZ (95%) and CTX(97.4%), fourth generation cephalosporin, CPM(91%), followed by resistance against non-β-lactam drugs CIP(100%), LE(90.00%) and COT(95.00%). Resistance to AK was intermediate (55%). ESBL production was detected in 44.15% (34/77) and remaining isolates 55.85% (43/77) were β-lactam-β-lactamase inhibitor resistant (BLIR). Moreover all ESBL producers were sensitive to IPM with a high level (88.37%) of sensitivity against BLIR isolates. The antibiotic resistance profile of each *E coli* isolate against the β-lactam and non-β-lactam drugs was clustered using heatmap.2 function of R statistical package based on their sensitivity to β-lactam-β-lactamase inhibitor combination. Clustered resistance against non-β-lactam drugs (CIP, LE) was observed in both ESBL and BLIR isolates. Moreover isolates sensitive to IPM formed a discrete cluster with respect to other antibiotics irrespective of β-lactamase inhibitor sensitivity (Fig. 1).

β-lactamase genotypes and plasmid replicon types

All 77isolates harbored β-lactamase genes including TEM alone in 13(16.9%) isolates, 1 (1.3%) each for CTX-M and OXA group III alone and rest 62(80.5%) for multiple β-lactamase genes. Combination of OXA group I, III and TEM β-lactamase was present in 29(37.7%) isolates. Highest occurrence was observed amongst ESBL (44.1%) producers compared to BLIR (32.6%) isolates. Moreover combination of only OXA group I and III present amongst ESBL (11.8%) and BLIR (11.4%) isolates were comparable. Similarly CTX-M in combination with multiple β-lactamases in both ESBL (20.6%) and BLIR (20.5%) isolates were also comparable. OXA group II was present in 1 BLIR isolates along with OXA group I and in another in combination with OXA group I, TEM, CTX-M respectively (Fig 1).

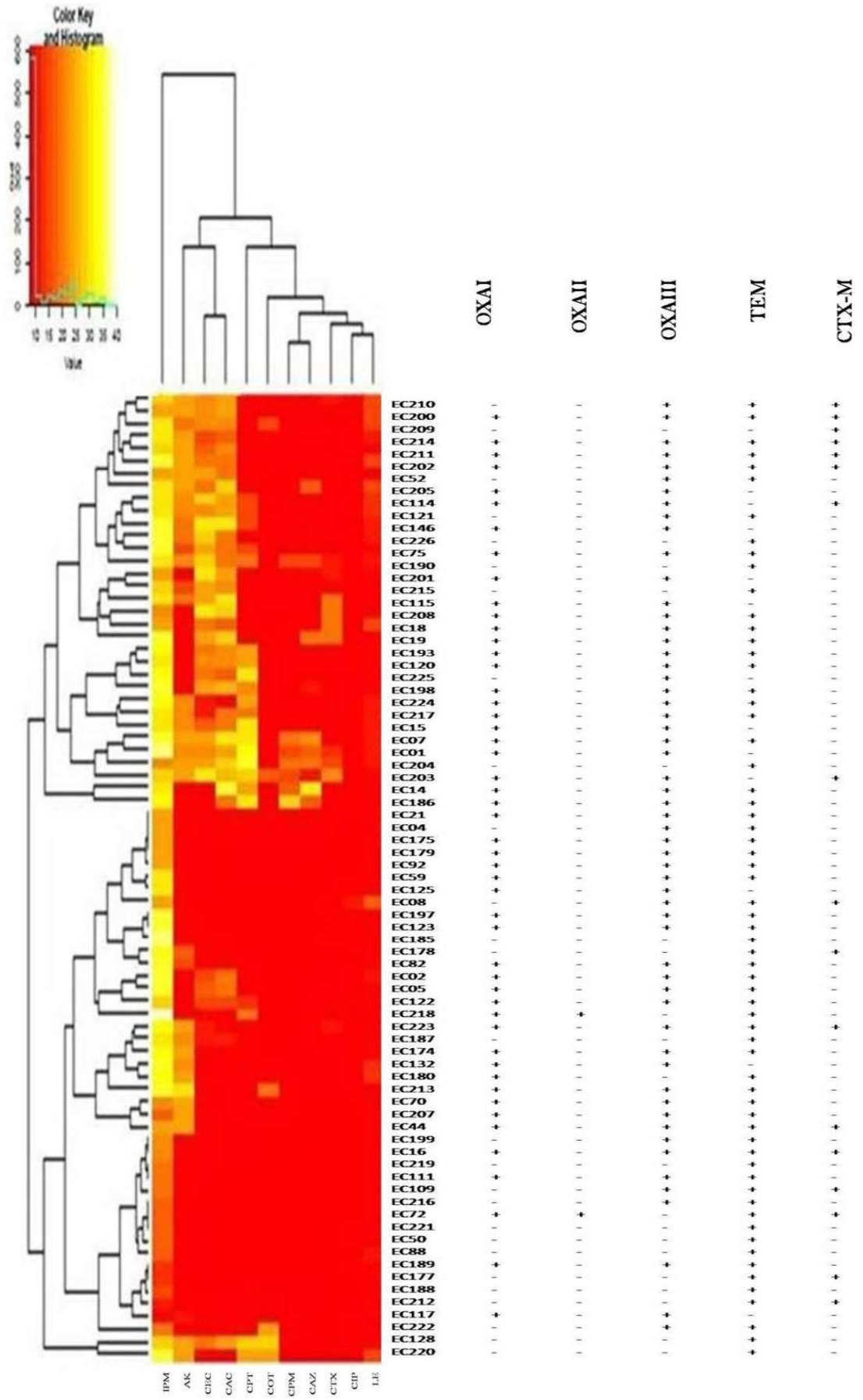

Fig. 1. Heatmap representation of zone of growth inhibition surrounding standard antibiotic disks to 11 antibiotics for 77 *E. coli* isolates. The size of the zone of inhibition was represented by the colour spectrum in the diagram, red; no zone of inhibition (highly resistant), red to orange; zone of inhibition 10–22 mm depending on antibiotic (resistant), orange to yellow; zone of inhibition 17–27 mm depending on antibiotic (sensitive) and yellow to white; zone of inhibition of 30–37 mm (highly sensitive)

None of the isolates harbored SHV β-lactamase. When the distribution of the β-lactamases in each *E. coli* isolate was clustered using heatmap.2 function of R statistical package, isolates that harbored OXA group I, III and TEM β-lactamases formed a discrete cluster followed by related sub-clusters of isolates that harbored β-lactamase genes either alone or in combination respectively (Fig. 2).

Plasmids that belong to IncF type replicons (IncFrepB, IncFIB ; 64/77) and IncF and IncI1 types (13/77) was distributed in isolates that harbor OXA group I, II and III in combinations with other β-lactamases. However IncFrepB, IncFIB (6/77) and IncFIB (3/77) plasmid replicon types were distributed in isolates that harbored only OXA group I and III respectively (Fig 3).

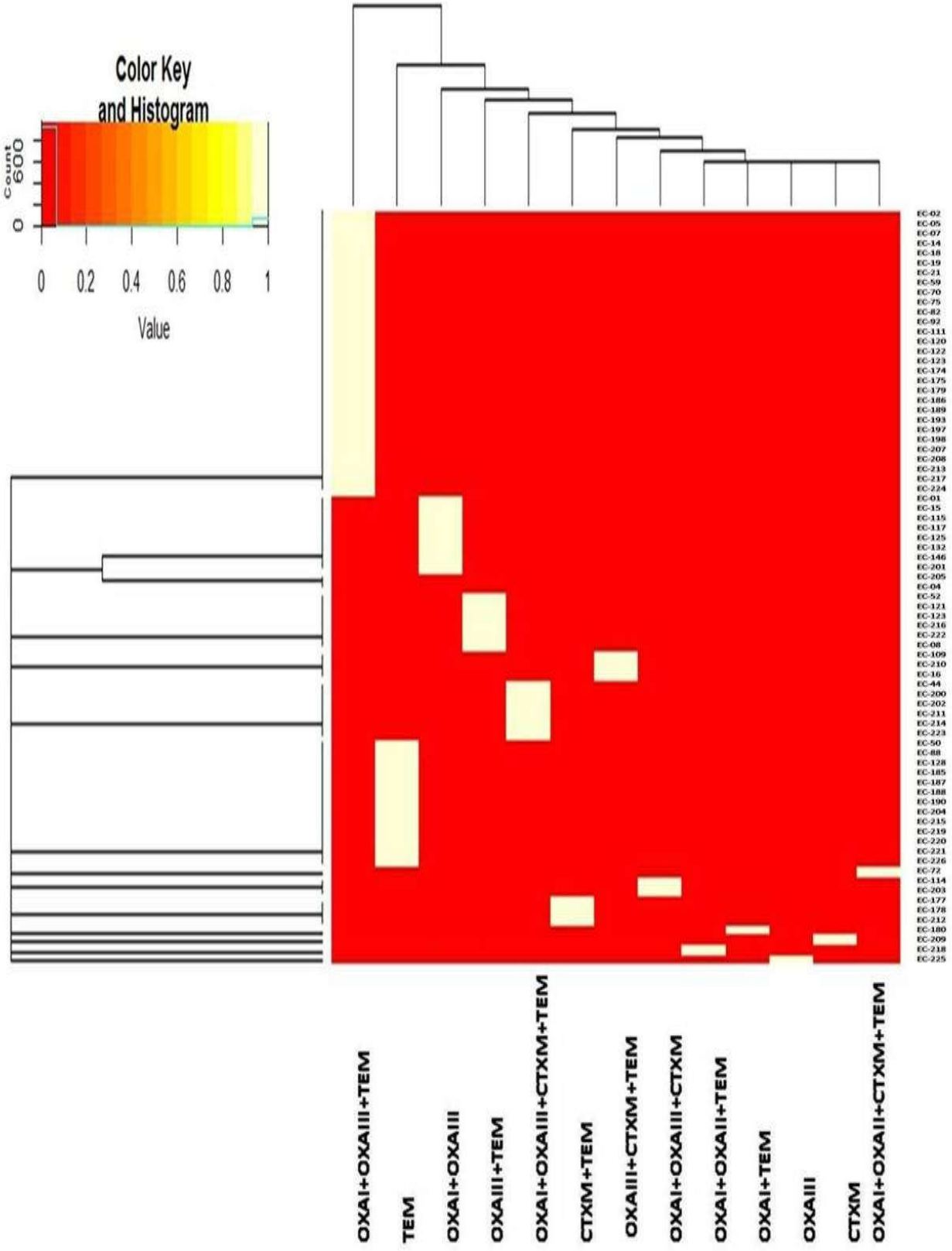

Fig. 2. Heatmap representation of distribution of β-lactamase genes *blaOXA*, *blaTEM*, *blaCTX-M* either alone or in combination in 77 *E. coli* isolates resistant to 3GCs. The presence and absence was represented by the colour spectrum in the diagram, red; gene absent, white; gene present. Presence of *blaOXAI*, *blaOXAII*, *blaOXAIII*,*blaTEM*, *blaCTX-M* was indicated as (+) and absence as (-) respectively

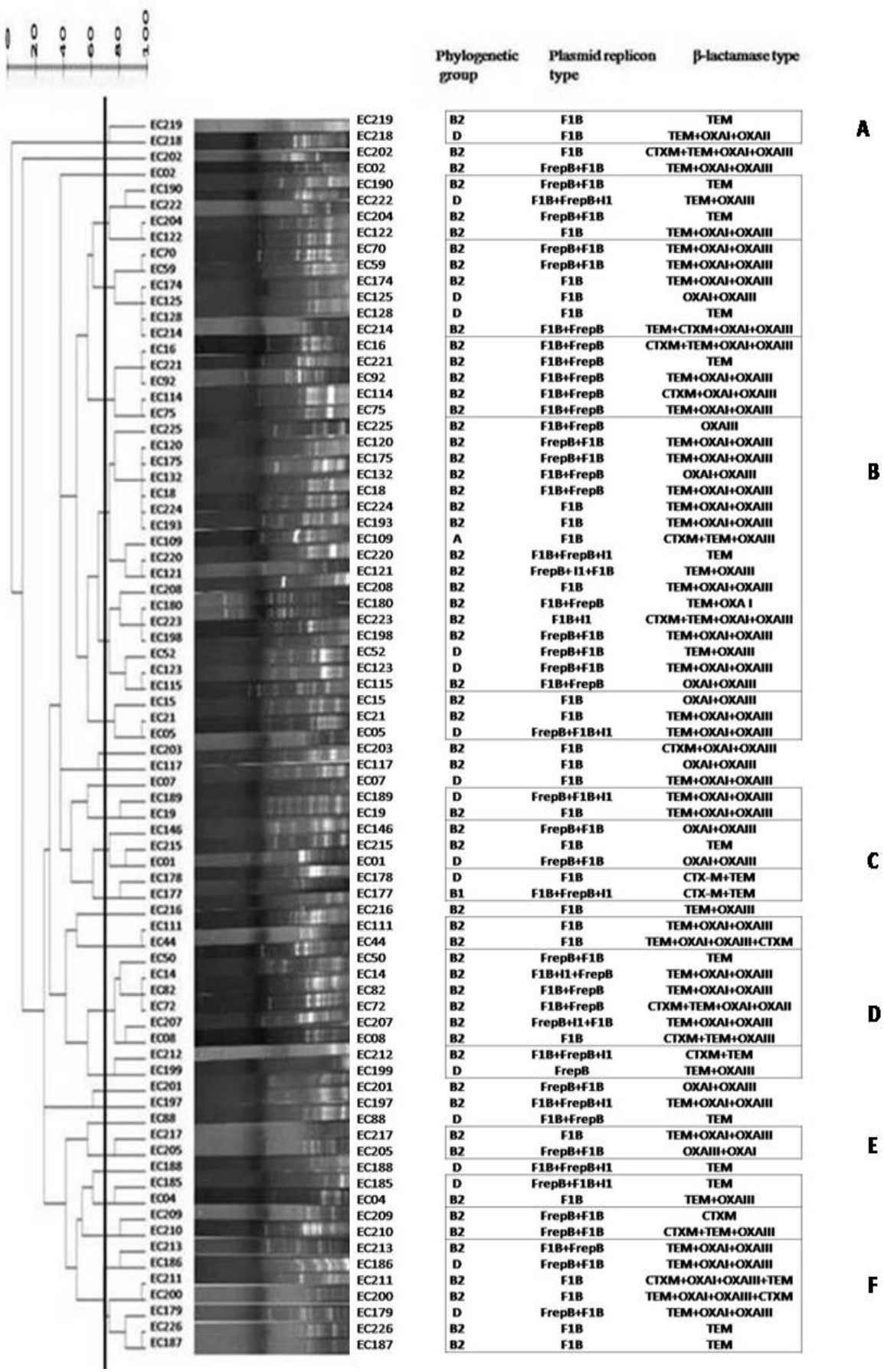

Fig. 3. Genetic relatedness among 77 uropathogenic *E. coli* isolates with 3GC resistance was assessed by cluster analysis of ERIC typing. The column on the right reports the isolate code number, their respective acquired β-lactamase genes, plasmid replicon types and phylogenetic groups. Similarity analysis was performed with Dice's coefficient and clustering by the unweighted pair group method with arithmetic averages. Isolates with a coefficient of similarity value ≥70% (indicated by a solid line) were considered to belong to the same clonal group (groups are in boxes marked as A-F)

Phylogroups and ERIC profiles

Phylogenetic background of 77*E.coli* isolates analyzed indicated prevalence of pathogenic phylotype B2(75.32%, 58/77) followed by D(22.07%, 17/77) with rare incidence of commensal phylogroups B1(1.29%, 1/77) and A(1.29%, 1/77) respectively (Fig. 3). A dendogram was constructed on ERIC-PCR profiles to show the degree of relatedness among the isolates. A genetic relatedness at ≥70 % similarity was observed among 77 *E. coli* isolates represented by 6 related clusters (A-F) with closely related sub-clusters. The largest cluster B consists of 5 sub-clusters followed by the 3 clusters(C, D, F) that is further sub-divided into 3 sub-clusters respectively. Moreover the genetically related isolates were independent with respect to their phylogenetic background, acquisition of multiple replicon type plasmids and β-lactamase resistant genes (Fig. 3).

DISCUSSION

High rate of multidrug resistance among UPEC isolates with varied level of resistance against 3GCs were reported from different parts of India and abroad (Meyer *et al*.,2010; Niranjan, and Malini.,2014). In this study, antibiogram analysis on 3GC resistant UPEC showed high resistance to non-β-lactams with moderate sensitivity to amikacin and highest sensitivity to imepenem. ESBL producing *Enterobacteriaceae* are widely disseminated in India and carbapenems are drug of choice (Taneja *et al*., 2008). TEM, SHV and CTX-M were reported as predominant β-lactamases imparting ESBL genotypes (Doi *et al*.,2013). There is paucity of data on distribution of OXA-ESBL amongst UPEC from India. High incidence of OXA β-lactamase was reported in *E. coli* and *Pseudomonas aeruginosa* showing narrow spectrum and extended-spectrum (ES) β-lactamase resistance respectively (Sahuquillo-Arce *et al*., 2015). ES-OXA was identified as variants of their narrow-spectrum counterparts; OXA-10, OXA-2, OXA-1 (Poirel *et al*., 2010). Alizade *et al*.2015 reported distribution of low frequencies of OXA β-lactamase in UPEC isolates. Similarly another study by Lee *et al*.2005, also showed low prevalence of OXA group I (13.5%), II(2.3%) and III(6.3%) in *P. aeruginosa* isolates. Our study indicated high frequencies of combination of OXA group I and III β-lactamases in the UPEC isolates. CTX-M was considered most prevalent ESBL-worldwide and replaced TEM and SHV as predominant ESBL in many European countries (Livermore *et al*.2007) A recent study from India reported predominance of CTX-M in extra-intestinal pathogenic *E.coli* (Alhetar, and Lakshmidevi, 2015). However our study indicated predominance of co-existence of OXA group I and III with TEM β-lactamases with rare incidence of CTX-M in UPEC isolated from hospital settings irrespective of β-lactam-β-lactamase inhibitor sensitivity. Majority of clinically relevant plasmid mediated OXA β-lactamases were mostly found in gram negative pathogens such as pseudomonads, acinetobacters and members of the *Enterobacteriaceae* (Sorour *et al*., 2008; Nowak P *et al*., 2012). Reports indicated predominance of IncF type plasmid replicons in *E. coli* isolates that harbored different β-lactamase resistant genes. Furthermore IncF type replicons have been previously identified as carriers of CTX-M-15 in different clinical isolates of *E.coli* (Carattoli *et al*., 2008). Our results indicated distribution of IncFrepB and IncFIB plasmid replicon types in isolates that harbored only OXA group I and III respectively. Studies from different geographical regions around the world reported predominance of phylogenetic group B2 amongst the 3GC resistant *E.coli* isolates (Carattoli *et al*., 2008; Lee *et al*., 2015). A recent report indicated prevalence of phylogenetic groups B2 and D in different clinical ESBL producing quinolone resistant *E.coli* isolates (Durmaz *et al*., 2015). In contrast to other reports 3GC resistant strains commonly belonged to phylogroup A and sensitive strains belonged to phylogroups B2 or D respectively (Courpon-Claudinon *et al*., 2011). Genetic relatedness on ERIC-PCR profiles suggests probable clonal spread of 3GC resistance due to transmission of resistant plasmids and other mobile genetic elements possibly from an outbreak of a resistant strain. Therefore the degree of clonality within the resistant *E. coli* population with respect to various phylogenetic groups must be explored to formulate therapeutic options. Our results indicated predominance of phylogenetic group B2 followed by D among the 3GC resistant UPEC isolates regardless of their clonal relatedness at the level of ≥70% similarity. No direct correlation between ERIC-PCR genotypes, phylogenetic groups and resistance gene determinants, suggests multiple subtypes of the species may be involved in the isolates considered in this study.

Therefore this is the first report of its kind stating the distribution of OXA β-lactamase groups in multidrug resistant UPEC isolates in varied combination with other β-lactamases (TEM, CTX-M) that is highly alarming as it may create complications in therapeutics. Interestingly a recent study showed that class D enzymes (OXA-2, OXA-10) that are currently regarded as non- carbapenemases in *E. coli* may in fact be carbapenem-hydrolyzing class D β-lactamases (CHDLs) in nonfermentative *Acinetobacter baumannii***.** However in contrast low level of resistance against carbapenem was observed on expression of CHDLs in *E.coli* (Antunes *et al*., 2014). These studies clearly demonstrated the importance of the bacterial host in determining whether an enzyme can be classified as CHDL. High incidence of OXA group I in our study was alarming. Indiscriminate use of carbapenem may result in possible amino acid variations to accommodate the carbapenem-hydrolyzing property of the extended-spectrum enzymes (Antunes *et al*., 2014; Alizade *et al*., 2015; Carattoli *et al*., 2008) limiting treatment options. Therefore these pathogenic isolates may be considered as potential reservoir of Class D β-lactamases with extended-spectrum activity.

Conclusion

Predominant distribution of class D OXA group I and III and class A TEM β-lactamases was observed amongst the 3GC-resistant nosocomial UPEC from eastern India. ERIC-PCR revealed 6 related clonal groups at the level of ≥70 % genetic similarity with universal distribution of OXA group I and III enzymes either alone or in combination with multiple β-lactamases. Susceptibility towards imepenem indicated suitable treatment option. However its indiscriminate use must be restricted as it may result in dissemination of CHDLs. Therefore implementation of routine molecular analysis of circulated β-lactamases among UPEC must be undertaken to develop appropriate therapeutics to combat 3GC resistance.

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Conflict of Interest: There is no conflict of interest

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