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ARTICLE

Occurrence of virulence genes, 16S rRNA methylases, and plasmid-mediated quinolone resistance genes in CTX-M-producing *Escherichia coli* from Pakistan

M. A. Habeeb · A. Haque · A. Iversen · C. G. Giske

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Abstract The aim of the study was to conduct a comprehensive molecular characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* collected from Pakistan. Genetic relatedness among 98 ESBL-producing *E. coli* was measured by pulsed-field gel electrophoresis (PFGE). The presence of genes encoding ESBLs, virulence factors (VFs), 16S rRNA methylases, plasmid-mediated quinolone resistance (PMQR) encoding elements, plasmid replicon types, phylogenetic groups of *E. coli*, prevalence of the worldwide disseminated clone *E. coli* ST131, and phylogrouping of CTX-M enzymes was investigated by polymerase chain reaction (PCR). All isolates carried $bla_{\text{CTX-M}}$ genes and, except for one isolate from CTX-M phylogroup 9, they all belonged to CTX-M phylogroup 1. The isolates were genetically diverse with PFGE. Phylogenetic group D (36 %) was most abundant in this collection of *E. coli*, whereas isolates belonging to B2 (22 %) had the highest content of virulence genes. PMQR genes were found in 84.6 % of the isolates; among them, 93 % isolates were positive for variants of acetyltransferases (*aac(6')-Ib-cr*), whereas *qnrB*, *qepA*, and *qnrS* were present in 11 %, 5 %, and 4 % of the isolates, respectively. Only 3 % of the isolates contained genes encoding 16S rRNA methylases. The most abundant replicon type was IncF (96 %), and 18 % of the isolates belonged to the ST131 clone. Out of 34 investigated VFs, 24 genes encoding different types of adhesins, protectins, toxins, siderophores, and other VFs were found. Although the isolates in this collection were highly resistant to many antimicrobials, susceptibility to amikacin and meropenem was retained.

M. A. Habeeb · A. Haque
Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan

M. A. Habeeb · A. Haque
Pakistan Institute of Engineering and Applied Sciences (PIEAS), Nilore, Islamabad, Pakistan

M. A. Habeeb · A. Iversen · C. G. Giske (✉)
Clinical Microbiology L2:02, Karolinska Institutet—MTC, Karolinska University Hospital Solna, 17176 Stockholm, Sweden
e-mail: christian.giske@karolinska.se

M. A. Habeeb
Institute of Molecular Biology and Biotechnology, The University of Lahore, Defense Road, Lahore, Pakistan

Introduction

Plasmid-mediated extended-spectrum β-lactamases (ESBLs) usually confer resistance to penicillins, cephalosporins, and aztreonam. At present, CTX-M ESBLs in Enterobacteriaceae, particularly in *Escherichia coli*, have increased significantly in most regions of the world [1]. *E. coli* is one of the most significant etiological agents for both hospital- and community-acquired infections by causing systemic and urinary tract infections in humans across the world. Therapeutic possibilities are scarce as a result of CTX-M-producing Enterobacteriaceae frequently being resistant also to non-β-lactams [1–3]. ST131, a frequently CTX-M-producing *E. coli* clone, has been found to be largely responsible for the worldwide dissemination of CTX-M [4]. This clone is also considered virulent and belongs to phylogroup B2. Commensal pathogens belong mostly to A and B1 phylogenetic groups, while B2 and D groups are more frequently also detected extraintestinally. A link between *E. coli* phylogeny and virulence has been described previously [5].

CTX-M-containing plasmids are also frequently encoding genes responsible for resistance to quinolones, aminoglycosides, macrolides, tetracyclines, sulphonamides, trimethoprim, and chloramphenicol [6]. ESBL-producing Enterobacteriaceae are commonly resistant to fluoroquinolones and the discovery of the plasmid-mediated quinolone resistance (PMQR), *qnr*, and *aac(6')-Ib-cr* genes has contributed to the observed

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fluoroquinolone resistance in such isolates. The aminoglycoside (6) N-acetyltransferase (*aac(6')-Ib-cr*) gene reduces susceptibility to two unrelated classes of antibiotics; aminoglycoside and fluoroquinolones. Methylation of the 16S rRNA confers pan-resistance to aminoglycosides, which is commonly associated with genes of other well-dispersed resistances in human clinical isolates [7, 8].

The prevalence of CTX-M ESBL production is increasing faster than SHV and TEM ESBLs in Asia, and particularly in Pakistan. The prevalence of CTX-M in this region is lower than in Spain and Brazil, but higher than in Portugal and USA [9].

In this study, we aimed to investigate the genotypic characterization of ESBLs and other associated resistances among clinical isolates from two Pakistani cities. Moreover, we studied the prevalence of the ST131 clone, the prevalence of plasmid-mediated quinolone resistance genes, and of 16S rRNA methylases. Lastly, we characterized CTX-M phylogroups, plasmid replicon types, and virulence factors in the collection.

Materials and methods

Bacterial isolates

A total of 98 non-replicate ESBL-producing *E. coli* were collected in 2005 ($n=32$) and during the period November 2009–March 2010 ($n=66$) from hospitals and communities in two geographically different regions in Pakistan. During this period, hospitals and clinical laboratories in Islamabad and Faisalabad routinely sent *E. coli* isolates non-susceptible to extended-spectrum cephalosporins for further characterization at the Human Enteric Pathogens Laboratory of the National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan. The isolates were mainly derived from patients with complicated infections or from cultures taken in patients with infections that were non-responsive to empirically given antibiotics. All *E. coli* collected from different clinical sources were identified by colony morphology on MacConkey agar (Merck, Darmstadt, Germany) and assessed biochemically by commercially available kits (API 20E bioMérieux, Marcy I'Etoile, France and RapID Systems, Remel, Lenexa, KS, USA).

Phenotypic detection of ESBLs

Initial screening and confirmatory tests were carried out on Mueller–Hinton agar (Merck, Darmstadt, Germany) by culturing isolates for 16 to 18 h at 37 °C according to Clinical and Laboratory Standards Institute (CLSI) guidelines for disk diffusion tests [10]. The antibiotics tested were cefotaxime and ceftazidime with and without clavulanic acid.

Antimicrobial susceptibility testing

All ESBL-producing isolates were examined for co-resistance to other classes of antimicrobial agents on Mueller–Hinton agar by the disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [11]. The following antibiotics were tested: amikacin (AK), cefotaxime (CTX), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (CN), meropenem (MEM), piperacillin/tazobactam (TZP), and trimethoprim–sulphamethoxazole (SXT).

Molecular detection of ESBLs

DNA templates were obtained from boiled suspension of bacterial cells. For the amplification of a 585-bp fragment of the $bla_{\text{CTX-M}}$ gene, primers CTX-M F SCS ATG TGC AGY ACC AGT AA and CTX-M R ACC AGA AYV AGC GGB GC were used [12]. All assays were performed in Rotor Gene 3000 apparatus (Corbett Research) using a 25-μl polymerase chain reaction (PCR) reaction mixture containing 12.5 μl QuantiTect SYBR Green PCR Kit (Qiagen, Germany), 1.25 μl of 10 μM primers, 7.5 μl RNase-free water, and 2.5 μl DNA template. The thermal conditions were as follows: denaturation at 95 °C for 15 mins, then 30 repeats of 30 s at 95 °C, 1 min at 52 °C, and 1 min at 72 °C, and a final extension of 65 °C for 30 s. A PCR-positive control was used to test for any amplification failure for the CTX-M gene. Analyses were carried out using Rotor Gene Real Time Analysis Software (versions 6.0 and 6.1).

Real-time TaqMan PCR for CTX-M groups

CTX-M-positive isolates were subjected to further analysis to determine the phylogenetic group by assigning them to one of the following groups: CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M 8/25. Primers and designed standard probes for each group were used as previously described [13]. Each PCR reaction contained 25 μl of reaction mixture, having 12.5 μl of qPCR MasterMix No ROX (Eurogentec, USA), 0.5 μM of primers, 0.05 μM of each probe, 5.25 μl RNase-free water, and 5 μl DNA template. Using the following thermal conditions: incubation at 50 °C for 2 mins, hold for 10 mins at 95 °C, and 40 repeats of 10 s at 95 °C and 60 s at 58 °C, the PCR assay was carried out using Rotor Gene 3000 apparatus. PCR-positive controls were included to confirm the amplification of all CTX-M groups. A single fluorescence reading for all four channels was done at the end of the extension stage and analyzed by Rotor Gene Real Time Analysis Software.

Molecular typing by PFGE

All isolates were further investigated for molecular epidemiological relationships by *XbaI* pulsed-field gel electrophoresis

(PFGE) analysis, as previously described [14]. Profiles obtained after 22 h of electrophoresis in CHEF-Mapper (Bio-Rad, Hercules, CA, USA) gels stained with GelRed were photographed in Gel Doc EQ (Bio-Rad, Hercules, CA, USA) and further analyzed with the GelCompar II software version 5.10 (Applied Maths, Belgium). A dendrogram exhibiting the diversity among PFGE profiles was generated using cluster analysis of the dice similarity based on the unweighted pair-group method using arithmetic averages (UPGMA).

Screening for acquired 16S rRNA methylase genes

Real-time PCR was performed to study the distribution of acquired 16S rRNA methylase genes which confer pan-resistance to aminoglycosides. Primers amplifying the genes *armA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD* were previously described elsewhere [8, 15]. A 25-μl PCR reaction mixture having the same reagents as described above in the methodology for the molecular detection of ESBLs was amplified in Rotor Gene apparatus under thermal conditions as follows: denaturation at 95 °C for 15 mins, then 35 repeats of 30 s at 95 °C, 1 min at 58 °C, and 1 min at 72 °C, and ending at 65 °C for 30 s. Positive controls for *armA*, *rmtB*, *rmtD* were included in the PCR reaction. The final results were analyzed using Rotor Gene Real Time Analysis Software.

Detection of plasmid-mediated quinolone resistance genes

The prevalence of plasmid-mediated quinolone resistance genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and plasmid-mediated active efflux pumps conferring low-level fluoroquinolones resistance (*qepA*) was determined with previously published protocols [8, 16]. The PCR mix, thermal conditions, and analysis were the same as those mentioned above in the methodology for the molecular detection of ESBLs. The plasmid-mediated fluoroquinolone resistance gene *aac(6')-lb* was amplified using PCR primers *aac(6)*F TGA CCA ACA GCA ACG ATT CC and *aac(6)*R TTA GGC ATC ACT GCG TGT TC, as used previously [8]. All isolates were tested for the prevalence of *aac(6')-lb* genes in a 25-μl PCR reaction mixture containing similar reagents as mentioned above for the molecular detection of ESBLs in Rotor Gene apparatus with the following thermal conditions: incubation at 95 °C for 15 mins, then 45 repeats of 20 s at 95 °C, 40 s at 59 °C, and 20 s at 70 °C, and, finally, at 65 °C for 30 s. Isolates both positive and negative for *aac(6')-lb-cr* were included in the reactions. All isolates positive after analysis by software were purified by using the PCR Product Purification Spin Kit (Genomed, Germany). These purified products were digested with BstF5I (New England Biolabs, Ipswich, MA, USA) [17], separated on 2 % agarose E-Gel (Invitrogen, USA), and were photographed in Gel Doc EQ (Bio-Rad Laboratories, Hercules, CA, USA) for the prevalence of *aac(6')-lb-cr*.

PCR-based replicon typing

Plasmid replicons were examined among ESBL-producing *E. coli* according to Carattoli et al. [18]. Eighteen replicon types were searched for using five multiplex PCRs and three simplex PCRs in Rotor Gene apparatus. The reaction mixture for each multiplex PCR recognizing three different replicons contained 12.5 μl of QuantiTect SYBR Green PCR Kit, 1 μl of 10 μM primer each of three pairs of primers, 2.5 μl DNA template, and 4 μl RNase-free water to produce a total volume of reaction of 25 μl. Simplex PCR contained reaction mixture as mentioned above in the methodology for the molecular detection of ESBLs. Thermal conditions for multiplex and simplex assays were the same as those described by Carattoli et al. [18]. Positive isolates for all replicon types were included in the assay to confirm amplification. The products obtained from multiplex PCRs were separated on 2 % agarose E-Gel (Invitrogen, USA) and photographed in Gel Foc EQ, while simplex PCR results were analyzed using Rotor Gene Real Time Analysis Software.

PCR detection of the *pabB* gene

Real-time PCR using Rotor Gene apparatus was conducted to determine the prevalence of the worldwide distributed clone ST131. With primers already described [5] and PCR reagents the same as those mentioned above for the molecular detection of ESBLs, the thermal conditions in the assay were: denaturation for 15 mins at 95 °C, 40 repeats of 20 s at 94 °C, 20 s at 65 °C, and 20 s at 72 °C, ending in a final extension of 30 s at 65 °C. PCR-positive control was also included. All results were analyzed by the Rotor Gene software.

Phylogenetic groups

All ESBL-producing isolates were subjected to the investigation of major phylogenetic groups for *E. coli* (A, B1, B2, and D) using a triplex PCR previously established [19]. In each 25-μl reaction tube, the reagents were 12.5 μl QuantiTect SYBR Green PCR Kit, 2.5 μl DNA template, 1.25 μl of each 10 μM primer, and 2.5 μl RNase-free water was used to make up the volume. The cycling conditions in the Rotor Gene apparatus were the same as those described above for CTX-M detection. Gel electrophoresis was performed using 2 % agarose E-Gels and photographed in Gel Doc EQ. The resulting photographs were analyzed according to grouping patterns established previously [19].

PCR-based O typing

A multiplex reverse primer PCR was performed using primers for O types which have been reported as frequently involved in bloodstream infections (BSI) and the assay followed a previously described method [20]. The allele-specific PCR

assay was conducted in Rotor Gene apparatus for the following BSI O types: O1, O2a, O18, O16, O6a, O7 and O4, O12, O25, O75, O15, and O157. The PCR reagents in a 25-μl reaction were 12.5 μl QuantiTect SYBR Green PCR Kit, 4 μl of 10 uM forward primer, 1 μl of each 10 μM reverse primers in a set, and 2.5 μl DNA template. The thermal conditions were the same as for CTX-M detection. The PCR products were separated by gel electrophoresis using 2 % agarose E-Gels and photographed in Gel Doc EQ.

Detection of virulence factors

The presence of extraintestinal virulence factors was investigated among ESBL-producing *E. coli* by PCR. Primers for genes *papAH*, *papC*, *papEF*, *papG*, *papG* allele I, allele II, allele III, *sfa*/*focDE*, *sfaS*, *focG*, *afa*/*draBC*, *bmaE*, *gafD*, *nfaE*, *hylA*, *cnf*1, *cdtB*, *fyuA*, *iutA*, *kpsMT*II, *kpsMT*III, *ibeA*, *cvaC*, *traT*, PAI, *fimA*, *fimH*, *neuC*, *iss*, *tsh*, *sat*, *iroN*, *fliCh*7, and *usp* were included as described previously [21–27]. Six multiplex PCRs with 28 sets of primers were designed according to amplicon sizes and primers validation. In each PCR, the virulence factors were as follows: PCR 1: *papAH*, *kpsMT*III, *ibeA*, *papEF*, and *fimA*; PCR 2: *papG* allele III, *sfa*/*focDE*, *bmaE*, *iutA*, and *iroN*; PCR 3: *papC*, *papG* allele I, *nfaE*, *kpsMT*II, and *tsh*; PCR 4: *papG* allele II, *focG*, *cvaC*, *traT*, and *cdtB*; PCR 5: *sfaS*, *afa*/*draBC*, *cnf*1, *neuC*, and *fimH*; PCR 6: *usp*, *iss*, and *fliCh*7. Amplification for PCRs 1–5 was done in a 25-μl reaction mixture containing 2.5 μl DNA template, 12.5 μl QuantiTect SYBR Green PCR Kit, and 1 μl of each 10 μM primer, and PCR 6 was also conducted in a 25-μl reaction mixture with 12.5 μl QuantiTect SYBR Green PCR Kit, 1.25 μl of each 10 μM primer, 2.5 μl DNA template, and 2.5 μl RNase-free water to make up the volume. The cycling conditions for all multiplex PCRs in the Rotor Gene apparatus were the same as those described above for the molecular detection of ESBLs. The products of assays were separated by electrophoresis using 2 % agarose E-Gels (Invitrogen, USA). Conventional singleplex PCR reaction for virulence genes *hylA*, *fyuA*, *papG*, *gafD*, *sat*, and PAI was conducted using a Peltier Thermal Cycler PTC-200 (MJ Research, USA). PCR amplification was performed using 12.5 μl GoTaq Green Master Mix (Promega, USA), 1.25 μl of 10 μM primers, and 7.5 μl RNase-free water to make up the volume of 25 μl in each tube, and the thermal conditions were as described previously [21]. The PCR results were visualized by electrophoresis on 2 % agarose E-Gels and products were visualized in Gel Doc EQ (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Proportional comparisons between different groups were conducted using Fisher's exact test. A two-tailed p-value of <0.05 was considered to be statistically significant.

Results

Characterization of ESBL-producing isolates

All 98 *E. coli* displayed a positive phenotypic test in the combined disk test conducted with cefotaxime and ceftazidime ± clavulanic acid. Genes encoding CTX-M were detected in all isolates by qPCR amplification. Probe-based PCR showed that all isolates except one had CTX-M enzymes belonging to phylogroup 1, whereas CTX-M phylogroup 9 was detected in one isolate.

PFGE

Figure 1 shows the genetic diversity among *XbaI*-restricted CTX-M-producing isolates. In the collection, 92 distinct genotypes could be observed with ≥65 % similarity. Eight clusters were observed with ≥85 % similarity (i–viii) and each cluster comprised a minimum of four isolates. With a few exceptions, all isolates in the respective clusters revealed close relationships by presenting similar features with respect to phylogenetic groups, contents of virulence factors, serotypes, antimicrobial resistance profiles, and replicon types.

Antibiotic resistance profile

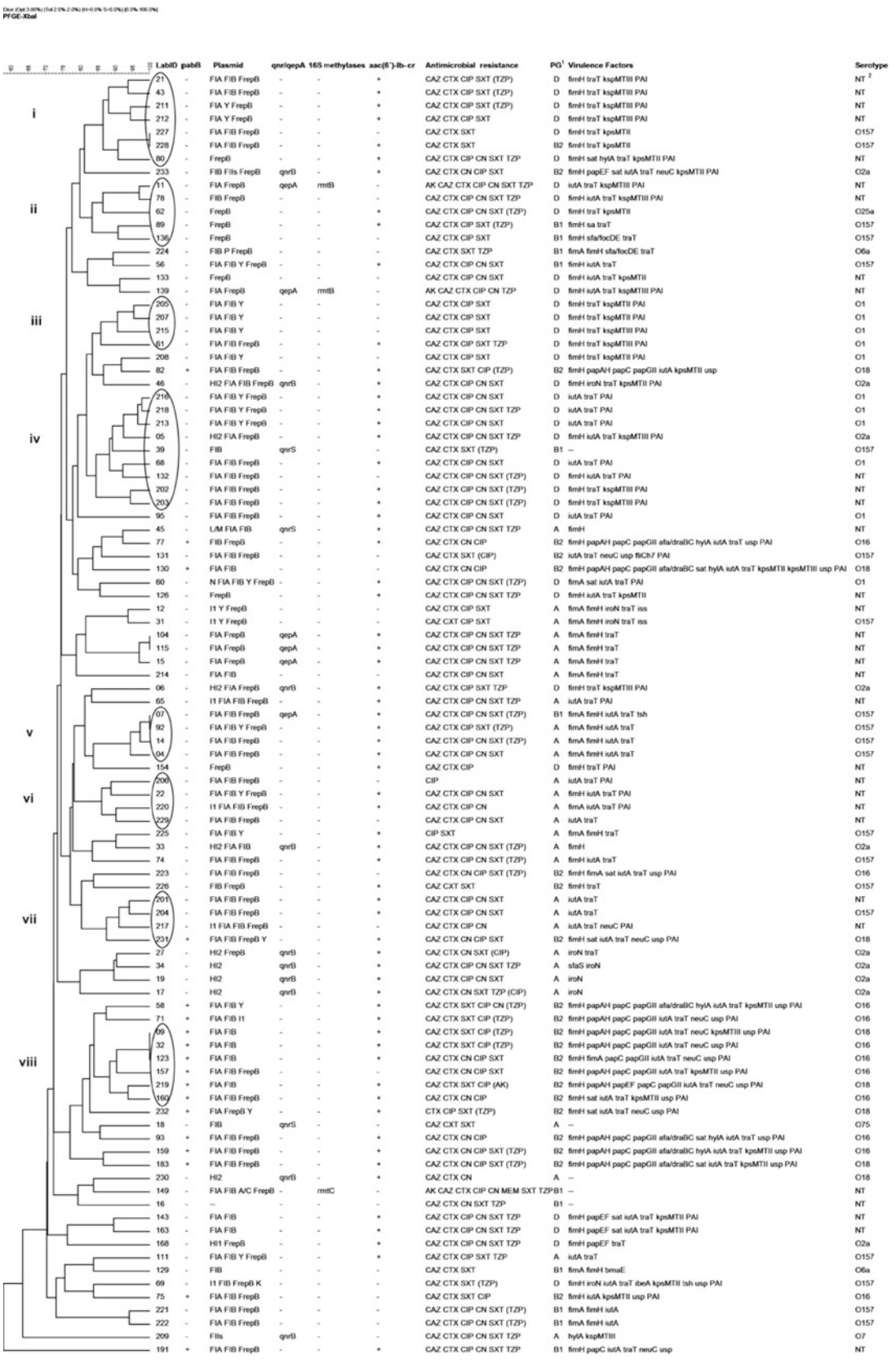
CTX-M-producing *E. coli* isolates showed high antimicrobial resistance rates versus the tested antimicrobials. A high proportion of isolates were resistant to cefotaxime (98 %), ceftazidime (97 %), trimethoprim–sulfamethoxazole (91 %), ciprofloxacin (89 %), gentamicin (62 %), and piperacillin–tazobactam (27 %). Only one isolate was resistant to meropenem and three were resistant to amikacin.

Distribution of phylogenetic groups and O types

By PCR-based phylogenetic grouping, isolates were assigned four major groups: 30 % belonged to A, 12 % to B1, 22 % to B2, and 36 % to group D. O types often causing BSI were found in all B2 *E. coli*; among 22 isolates, 12 were O16 (55 %), six were identified as O18 (27 %), three isolates were O157 (14 %), and one isolate belonged to O2a (Table 1). O157 types were present in 17 non-B2 *E. coli* (22 %), 11 isolates among other phylogenetic groups were O1 (14 %), and nine and two isolates were O2a (12 %) and O6a (3 %), respectively. The serotypes O18, O25a, O7, and O75 occurred only once each. Among the non-B2 *E. coli*, 33 isolates (44 %) were non-typable with PCR (Tables 2, 3, and 4).

Plasmid replicon types

The PCR-based replicon typing demonstrated the presence of F replicons to a greater extent and other replicons to a lesser



LabID	pabB	Plasmid	qnr/qepA	16S methylases	aac(6')-Ib-cr	Antimicrobial resistance	PG[1]	Virulence Factors	Serotype
21	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP SXT (TZP)	D	fimH traT kspMTIII PAI	NT [2]
43	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP SXT (TZP)	D	fimH traT kspMTIII PAI	NT
211	-	FIA Y FrepB	-	-	+	CAZ CTX CIP SXT (TZP)	D	fimH traT kspMTIII PAI	NT
212	-	FIA Y FrepB	-	-	+	CAZ CTX CIP SXT	D	fimH traT kspMTIII PAI	NT
227	-	FIA FIB FrepB	-	-	-	CAZ CTX SXT	D	fimH traT kpsMTII	O157
228	-	FIA FIB FrepB	-	-	+	CAZ CTX SXT	B2	fimH traT kpsMTII	O157
80	-	FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	D	fimH sat hylA traT kpsMTII PAI	NT
233	-	FIB FIIs FrepB	qnrB	-	+	CAZ CTX CN CIP SXT	B2	fimH papEF sat iutA traT neuC kpsMTII PAI	O2a
11	-	FIA FrepB	qepA	rmtB	-	AK CAZ CTX CIP CN SXT TZP	D	iutA traT kspMTIII PAI	NT
78	-	FIB FrepB	-	-	-	CAZ CTX CIP CN SXT TZP	D	fimH iutA traT kspMTIII PAI	NT
62	-	FrepB	-	-	+	CAZ CTX CIP CN SXT (TZP)	D	fimH traT kpsMTII	O25a
89	-	FrepB	-	-	+	CAZ CTX CIP SXT (TZP)	B1	fimH sa traT	O157
136	-	FrepB	-	-	-	CAZ CTX CIP SXT	B1	fimH sfa/focDE traT	O157
224	-	FIB P FrepB	-	-	-	CAZ CTX SXT TZP	B1	fimA fimH sfa/focDE traT	O6a
56	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP CN SXT	B1	fimH iutA traT	O157
133	-	FrepB	-	-	-	CAZ CTX CIP CN SXT	D	fimH iutA traT kpsMTII	NT
139	-	FIA FrepB	qepA	rmtB	-	AK CAZ CTX CIP CN TZP	D	fimH iutA traT kspMTIII PAI	NT
200	-	FIA FIB Y	-	-	-	CAZ CTX CIP SXT	D	fimH traT kspMTII PAI	O1
207	-	FIA FIB Y	-	-	-	CAZ CTX CIP SXT	D	fimH traT kspMTII PAI	O1
215	-	FIA FIB Y	-	-	-	CAZ CTX CIP SXT	D	fimH traT kspMTIII PAI	O1
61	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP SXT TZP	D	fimH traT kspMTIII PAI	O1
208	-	FIA FIB Y	-	-	-	CAZ CTX CIP SXT	D	fimH traT kspMTII PAI	O1
82	+	FIA FIB FrepB	-	-	+	CAZ CTX SXT CIP (TZP)	B2	fimH papAH papC papGII afa/draBC hylA iutA kpsMTII usp	O18
46	-	HI2 FIA FIB FrepB	qnrB	-	+	CAZ CTX CIP CN SXT	D	fimH iroN traT kpsMTII PAI	O2a
216	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP CN SXT	D	iutA traT PAI	O1
218	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	D	iutA traT PAI	O1
213	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP CN SXT	D	iutA traT PAI	O1
05	-	HI2 FIA FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	D	fimH iutA traT kspMTIII PAI	O2a
39	-	FIB	qnrS	-	-	CAZ CTX SXT (TZP)	B1	--	O157
68	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT	D	iutA traT PAI	O1
132	-	FIA FIB FrepB	-	-	-	CAZ CTX CIP CN SXT (TZP)	D	fimH iutA traT PAI	NT
202	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT (TZP)	D	fimH traT kspMTIII PAI	NT
203	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT (TZP)	D	fimH traT kspMTIII PAI	NT
95	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT	D	iutA traT PAI	O1
45	-	L/M FIA FIB	qnrS	-	+	CAZ CTX CIP CN SXT TZP	A	fimH	NT
77	+	FIB FrepB	-	-	+	CAZ CTX CN CIP	B2	fimH papAH papC papGII afa/draBC hylA iutA traT usp PAI	O16
131	-	FIA FIB FrepB	-	-	-	CAZ CTX SXT (CIP)	B2	iutA traT neuC usp fliCh7 PAI	O157
130	+	FIA FIB	-	-	-	CAZ CTX CN CIP	B2	fimH papAH papC papGII afa/draBC sat hylA iutA traT kpsMTII kpsMTIII usp PAI	O18
60	-	N FIA FIB Y FrepB	-	-	+	CAZ CTX CIP CN SXT (TZP)	D	fimA sat iutA traT PAI	O1
126	-	FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	D	fimH iutA traT kpsMTII	NT
12	-	I1 Y FrepB	-	-	-	CAZ CTX CIP SXT	A	fimA fimH iroN traT iss	NT
31	-	I1 Y FrepB	-	-	-	CAZ CXT CIP SXT	A	fimA fimH iroN traT iss	O157
104	-	FIA FrepB	qepA	-	+	CAZ CTX CIP CN SXT TZP	A	fimA fimH traT	NT
115	-	FIA FrepB	qepA	-	+	CAZ CTX CIP CN SXT TZP	A	fimA fimH traT	NT
15	-	FIA FrepB	qepA	-	+	CAZ CTX CIP CN SXT TZP	A	fimA fimH traT	NT
214	-	FIA FIB	-	-	-	CAZ CTX CIP CN SXT	A	fimA fimH traT	NT
06	-	HI2 FIA FrepB	qnrB	-	+	CAZ CTX CIP SXT TZP	D	fimH traT kspMTIII PAI	O2a
65	-	I1 FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	A	iutA traT PAI	NT
07	-	FIA FIB FrepB	qepA	-	+	CAZ CTX CIP CN SXT (TZP)	B1	fimA fimH iutA traT tsh	O157
92	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP SXT (TZP)	A	fimA fimH iutA traT	O157
14	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT (TZP)	A	fimA fimH iutA traT	O157
04	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT	A	fimA fimH iutA traT	O157
154	-	FrepB	-	-	+	CAZ CTX CIP	D	fimH traT PAI	NT
200	-	FIA FIB FrepB	-	-	-	CIP	A	iutA traT PAI	NT
22	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP CN SXT	A	fimH iutA traT PAI	NT
220	-	I1 FIA FIB FrepB	-	-	+	CAZ CTX CIP CN	A	fimA iutA traT PAI	NT
229	-	FIA FIB FrepB	-	-	-	CAZ CTX CIP CN SXT	A	iutA traT	NT
225	-	FIA FIB Y	-	-	+	CIP SXT	A	fimA fimH traT	O157
33	-	HI2 FIA FIB	qnrB	-	+	CAZ CTX CIP CN SXT (TZP)	A	fimH	O2a
74	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT (TZP)	A	fimH iutA traT	O157
223	-	FIA FIB FrepB	-	-	-	CAZ CTX CN CIP SXT (TZP)	B2	fimH fimA sat iutA traT usp PAI	O16
226	-	FIB FrepB	-	-	+	CAZ CXT SXT	B2	fimH traT	O157
201	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT	A	iutA traT	NT
204	-	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT	A	iutA traT	O157
217	-	I1 FIA FIB FrepB	-	-	-	CAZ CTX CIP CN	A	iutA traT neuC PAI	NT
231	+	FIA FIB FrepB Y	-	-	+	CAZ CTX CN CIP SXT	B2	fimH sat iutA traT neuC usp PAI	O18
27	-	HI2 FrepB	qnrB	-	+	CAZ CTX CN SXT (CIP)	A	iroN traT	O2a
34	-	HI2	qnrB	-	+	CAZ CTX CIP CN SXT TZP	A	sfaS iroN	O2a
19	-	HI2	qnrB	-	+	CAZ CTX CIP CN SXT	A	iroN	O2a
17	-	HI2	qnrB	-	+	CAZ CTX CN SXT TZP (CIP)	A	iroN	O2a
58	+	FIA FIB Y	-	-	+	CAZ CTX SXT CIP CN (TZP)	B2	fimH papAH papC papGII afa/draBC hylA iutA traT kpsMTII usp PAI	O16
71	+	FIA FIB I1	-	-	+	CAZ CTX SXT CIP (TZP)	B2	fimH papAH papC papGII iutA traT neuC usp PAI	O16
09	+	FIA FIB	-	-	+	CAZ CTX SXT CIP (TZP)	B2	fimH papAH papC papGII iutA traT neuC kpsMTIII usp PAI	O18
32	+	FIA FIB	-	-	+	CAZ CTX SXT CIP (TZP)	B2	fimH papAH papC papGII iutA traT neuC usp PAI	O16
123	+	FIA FIB	-	-	+	CAZ CTX CN CIP SXT	B2	fimH fimA papC papGII iutA traT neuC usp PAI	O16
157	+	FIA FIB FrepB	-	-	-	CAZ CTX CN CIP SXT	B2	fimH papAH papC papGII iutA traT kpsMTII usp PAI	O16
219	+	FIA FIB	-	-	+	CAZ CTX SXT CIP (AK)	B2	fimH papAH papEF papC papGII iutA traT neuC usp PAI	O18
160	+	FIA FIB FrepB	-	-	+	CAZ CTX CN CIP	B2	fimH sat iutA traT kpsMTII usp PAI	O16
232	+	FIA FrepB Y	-	-	+	CTX CIP SXT (TZP)	B2	fimH sat iutA traT neuC usp PAI	O18
18	-	FIB	qnrS	-	-	CAZ CXT SXT	A	--	O75
93	+	FIA FIB FrepB	-	-	+	CAZ CTX CN CIP	B2	fimH papAH papC papGII afa/draBC sat hylA iutA traT usp PAI	O16
159	+	FIA FIB FrepB	-	-	+	CAZ CTX CN CIP SXT (TZP)	B2	fimH papAH papC papGII afa/draBC hylA iutA traT kpsMTII usp PAI	O16
183	+	FIA FIB FrepB	-	-	+	CAZ CTX CN CIP SXT (TZP)	B2	fimH papAH papC papGII afa/draBC sat iutA traT kpsMTII usp PAI	O18
230	-	HI2	qnrB	-	+	CAZ CTX CN	A	--	O18
149	-	FIA FIB A/C FrepB	-	rmtC	-	AK CAZ CTX CIP CN MEM SXT TZP	B1	--	NT
16	-	--	-	-	-	CAZ CTX CN SXT TZP	B1	--	NT
143	-	FIA FIB	-	-	+	CAZ CTX CIP CN SXT TZP	D	fimH papEF sat iutA traT kpsMTII PAI	NT
163	-	FIA FIB	-	-	+	CAZ CTX CIP CN SXT TZP	D	fimH papEF sat iutA traT kpsMTII PAI	NT
168	-	HI1 FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	D	fimH papEF traT	O2a
111	-	FIA FIB Y FrepB	-	-	+	CAZ CTX CIP SXT TZP	A	iutA traT	O157
129	-	FIB	-	-	-	CAZ CTX SXT	B1	fimA fimH bmaE	O6a
69	-	I1 FIB FrepB K	-	-	-	CAZ CTX SXT (TZP)	D	fimH iroN iutA traT ibeA kpsMTII tsh usp PAI	O157
75	+	FIA FIB FrepB	-	-	-	CAZ CTX SXT CIP	B2	fimH iutA kpsMTII usp PAI	O16
221	-	FIA FIB FrepB	-	-	-	CAZ CTX CIP CN SXT (TZP)	B1	fimA fimH iutA	O157
222	-	FIA FIB FrepB	-	-	-	CAZ CTX CIP CN SXT (TZP)	B1	fimA fimH iutA	O157
209	-	FIIs	qnrB	-	-	CAZ CTX CIP CN SXT TZP	A	hylA kspMTIII	O7
191	+	FIA FIB FrepB	-	-	+	CAZ CTX CIP CN SXT TZP	B1	fimH papC iutA traT neuC usp	NT

[1] Phylogenetic Group, [2] Not typable with PCR

Fig. 1 Characteristics and relatedness of CTX-M-producing *Escherichia coli*

Table 1 Virulence factors, plasmid replicons, and antimicrobial resistance among phylogroup B2 CTX-M group 1 *E. coli* isolates

Isolate	Origin	ST131[b]	O serotype	Virulence factors	*qnr*/*qepA*	*aac(6')-Ib-cr*	Plasmid replicon	Antimicrobial resistance
9	Urine	+	O18	*fimH papAH papC papG*II *iutA traT neuC kpsMT*III *usp PAI*	−	+	FIA FIB	CAZ CTX SXT CIP (TZP)
32	Pus	+	O16	*fimH papAH papC papG*II *iutA traT neuC usp PAI*	−	+	FIA FIB	CAZ CTX SXT CIP (TZP)
58	Blood	+	O16	*fimH papAH papC papG*II *afa/draBC hylA iutA traT kpsMT*III *usp PAI*	−	+	FIA FIB Y	CAZ CTX SXT CIP CN (TZP)
71	Urine	+	O16	*fimH papAH papC papG*II *iutA traT neuC usp PAI*	−	+	FIA FIB I1	CAZ CTX SXT CIP (TZP)
75[a]	Pus	+	O16	*fimH iutA kpsMT*III *usp PAI*	−	−	FIA FIB FrepB	CAZ CTX SXT CIP
77	−	+	O16	*fimH papAH papC papG*II *afa/draBC hylA iutA traTusp PAI*	−	+	FIB FrepB	CAZ CTX CN CIP
82	Urine	+	O18	*fimH papAH papC papG*II *iutA kpsMT*II *usp*	−	+	FIA FIB FrepB	CAZ CTX SXT CIP (TZP)
93	Urine	+	O16	*fimH papAH papC papG*II *afa/draBC sat hylA iutA traT usp PAI*	−	+	FIA FIB FrepB	CAZ CTX CN CIP
123	Urine	+	O16	*fimA fimH papC papG*II *iutA traT neuC usp PAI*	−	+	FIA FIB	CAZ CTX CN CIP SXT
130	Urine	+	O16	*fimH papAH papC papG*II *afa/draBC sat hylA iutA traT kpsMT*II *kpsMT*III *usp PAI*	−	−	FIA FIB	CAZ CTX CN CIP
131	Urine	−	O157	*iutA traT neuC usp fliCh7 PAI*	−	−	FIA FIB FrepB	CAZ CTX SXT (CIP)
157	Urine	+	O16	*fimH papAH papC papG*II *iutA traT kpsMT*II *usp PAI*	−	−	FIA FIB FrepB	CAZ CTX CN CIP SXT
159	Pus	+	O16	*fimH papAH papC papG*II *afa/draBC hylA iutA traT kpsMT*II *usp PAI*	−	+	FIA FIB FrepB	CAZ CTX CN CIP SXT (TZP)
160	Pus	+	O16	*fimH sat iutA traT kpsMT*II *usp PAI*	−	+	FIA FIB FrepB	CAZ CTX CN CIP
183	Urine	+	O18	*fimH papAH papC papG*II *afa/draBC sat iutA traT kpsMT*II *usp PAI*	−	+	FIA FIB FrepB	CAZ CTX CN CIP SXT (TZP)
219	Ear Swab	+	O18	*fimH papAH papEF papC papG*II *iutA traT neuC usp PAI*	−	+	FIA FIB	CAZ CTX SXT CIP (AK)
223	Urine	−	O16	*fimA fimH sat iutA traT usp PAI*	−	−	FIA FIB FrepB	CAZ CTX CN CIP SXT (TZP)
226	Stool	−	O157	*fimH traT*	−	+	FIB FrepB	CAZ CXT SXT
228	Stool	−	O157	*fimH traT kpsMT*II	−	+	FIA FIB FrepB	CAZ CXT SXT
231	Wound	+	O18	*fimH sat iutA traT neuC usp PAI*	−	+	FIA FIB FrepB Y	CAZ CTX CN CIP SXT
232	Wound	+	O18	*fimH sat iutA traT neuC usp PAI*	−	+	FIA FrepB Y	CTX CIP SXT (TZP)
233	Wound	−	O2a	*fimH papEF sat iutA traT neuC kpsMT*II *PAI*	*qnrB*	+	FIB FIIs FrepB	CAZ CTX CN CIP SXT

All isolates were negative for 16S methylases

AK amikacin, *CAZ* ceftazidime, *CIP* ciprofloxacin, *CTX* cefotaxime, *CN* gentamicin, *SXT* trimethoprim–sulfametoxazole, *TZP* piperacillin–tazobactam, − negative, + positive, ( ) intermediate

[a] CTX-M 9 group

[b] Variants of ST131 were determined using *pabB* PCR

Table 2 Virulence factors, plasmid replicons, and antimicrobial resistance among phylogroup A CTX-M group 1 *E. coli* isolates

Isolate	Origin	O serotype	Virulence factors	*qnr*/*qepA*	*aac(6')-Ib-cr*	Plasmid replicon	Antimicrobial resistance
4	Pus	O157	*fimA fimH iutA traT*	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT
12	Pus	NT	*fimA fimH iroN traT iss*	−	−	I1 Y FrepB	CAZ CTX CIP SXT
14	Blood	O157	*fimA fimH iutA traT*	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
15	Pus	NT	*fimA fimH traT*	*qepA*	+	FIA FrepB	CAZ CTX CIP CN SXT TZP
17	Urine	O2a	*iroN*	*qnrB*	+	HI2	CAZ CTX CN SXT TZP (CIP)
18	Urine	O75		*qnrS*	−	FIB	CAZ CXT SXT
19	Urine	O2a	*iroN*	*qnrB*	+	HI2	CAZ CTX CIP CN SXT
22	−	NT	*fimH iutA traT PAI*	−	+	FIA FIB Y FrepB	CAZ CTX CIP CN SXT
27	Pus	O2a	*iroN traT*	*qnrB*	+	HI2 FrepB	CAZ CTX CN SXT (CIP)
31	Pus	O157	*fimA fimH iroN traT iss*	−	−	I1 Y FrepB	CAZ CXT CIP SXT
33	Sputum	O2a	*fimH*	*qnrB*	+	HI2 FIA FIB	CAZ CTX CIP CN SXT (TZP)
34	Blood	O2a	*sfaS iroN*	*qnrB*	+	HI2	CAZ CTX CIP CN SXT TZP
45	Pus	NT	*fimH*	*qnrS*	+	L/M FIA FIB	CAZ CTX CIP CN SXT TZP
65	Urine	NT	*iutA traT PAI*	−	+	I1 FIA FIB FrepB	CAZ CTX CIP CN SXT TZP
74	Urine	O157	*fimH iutA traT*	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
92	−	O157	*fimA fimH iutA traT*	−	+	FIA FIB Y FrepB	CAZ CTX CIP SXT (TZP)
104	Urine	NT	*fimA fimH traT*	*qepA*	+	FIA FrepB	CAZ CTX CIP CN SXT TZP
111	Pus	O157	*iutA traT*	−	+	FIA FIB Y FrepB	CAZ CTX CIP SXT TZP
115	Throat Swab	NT	*fimA fimH traT*	*qepA*	+	FIA FrepB	CAZ CTX CIP CN SXT TZP
201	Urine	NT	*iutA traT*	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT
204	Urine	O157	*iutA traT*	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT
206	Urine	NT	*iutA traT PAI*	−	−	FIA FIB FrepB	CIP
209	Urine	O7	*hylA kpsMT*III	*qnrB*	−	FIIs	CAZ CTX CIP CN SXT TZP
214	Urine	NT	*fimA fimH traT*	−	−	FIA FIB	CAZ CTX CIP CN SXT
217	Urine	NT	*iutA traT neuC PAI*	−	−	I1 FIA FIB FrepB	CAZ CTX CIP CN
220	Urine	NT	*fimA iutA traT PAI*	−	+	I1 FIA FIB FrepB	CAZ CTX CIP CN
225	Urine	O157	*fimA fimH traT*	−	+	FIA FIB Y	CIP SXT
229	Stool	NT	*iutA traT*	−	−	FIA FIB FrepB	CAZ CTX CIP CN SXT
230	Stool	O18		*qnrB*	+	HI2	CAZ CTX CN SXT

All isolates were negative for ST131 and 16S methylases

NT non-typable, *AK* amikacin, *CAZ* ceftazidime, *CIP* ciprofloxacin, *CTX* cefotaxime, *CN* gentamicin, *SXT* trimethoprim–sulfametoxazole, *TZP* piperacillin–tazobactam, − negative, + positive, ( ) intermediate

extent among all isolates included in this study. Plasmids replicons harboring IncFIA, IncFIB, FrepB, and IncFIIs were identified in all B2 *E .coli* with different combinations. Plasmids IncFIA and IncFIB were most commonly recognized in all isolates, FrepB was the second most commonly identified plasmid among B2 *E. coli*, replicon IncY was present in three isolates, one of the isolates was positive for IncFIIs plasmids with IncFIB and FrepB plasmids, and only one isolate harbored IncI1 plasmids with IncFIA and IncFIB plasmids (Table 1). Plasmids IncFIA, IncFIB, and FrepB were recognized in combinations and alone in almost all non-B2 isolates. As the plasmid profiles in Tables 2, 3, and 4 showed, IncY plasmids were the second most commonly detected with combinations of IncFIA, IncFIB, and/or FrepB. Plasmids types IncI1 and IncHI2 were much less frequently observed in *E. coli* of non-B2 isolates. IncHI1, IncA/C, IncL/M, IncK, IncFIIs, and IncN replicon types were identified particularly at least once in different isolates.

Determination of resistance genes and *aac(6')-lb-cr*

Among CTX-M-producing *E. coli* isolates, 72.4 % harbored genes encoding plasmid-mediated quinolones resistance (PMQR), whereas *aac(6')-lb-cr* was observed among 67.3 % isolates and *qnr* alleles were distributed in 19.3 % isolates. Only 4.5 % (1/22) of B2 *E. coli* harbored *qnrB* quinolones resistance genes (Table 1), whereas 13 % (10/76) ($p = 0.4471$) of isolates were positive for these genes in the non-B2 group. No isolates of the B2 group carried other *qnr* genes tested in this study, whereas 7.8 % of isolates linked to the non-B2 group hosted *qepA* and 4 % harbored *qnrS* (Tables 2, 3, and 4). Similarly, plasmid-mediated fluoroquinolone resistance genes

Table 3 Virulence factors, plasmid replicons, and antimicrobial resistance among phylogroup B1 CTX-M group 1 *E. coli* isolates

Isolate	Origin	ST131[a]	O serotype	Virulence factors	*qnr*/*qepA*	16S methylases	*aac(6')-Ib-cr*	Plasmid replicon	Antimicrobial resistance
7	Urine	–	O157	*fimA fimH iutA traT tsh*	*qepA*	–	+	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
16	Pus	–	NT		–	–	–	–	CAZ CTX CN SXT TZP
39	Pus	–	O157		*qnrS*	–	–	FIB	CAZ CTX SXT (TZP)
56	Sputum	–	O157	*fimH iutA traT*	–	–	+	FIA FIB Y FrepB	CAZ CTX CIP CN SXT
89	Urine	–	O157	*fimH sat traT*	–	–	+	FrepB	CAZ CTX CIP SXT (TZP)
129	Stool	–	O6a	*fimA fimH bmaE*	–	–	–	FIB	CAZ CTX SXT
136	Urine	–	O157	*fimH sfa/focDE traT*	–	–	–	FrepB	CAZ CTX CIP SXT
149	Urine	–	NT		–	*rmtC*	–	FIA FIB A/C FrepB	AK CAZ CTX CIP CN MEM SXT TZP
191	Sputum	+	NT	*fimH papC iutA traT neuC usp*	–	–	+	FIA FIB FrepB	CAZ CTX CIP CN SXT TZP
221	Urine	–	O157	*fimA fimH iutA*	–	–	–	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
222	Urine	–	O157	*fimA fimH iutA*	–	–	–	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
224	Urine	–	O6a	*fimA fimH sfa/focDE traT*	–	–	–	FIB P FrepB	CAZ CTX SXT TZP

NT non-typable, *AK* amikacin, *CAZ* ceftazidime, *CIP* ciprofloxacin, *CTX* cefotaxime, *CN* gentamicin, *SXT* trimethoprim–sulfamethoxazole, *TZP* piperacillin–tazobactam, − negative, + positive, ( ) intermediate

[a] Variants of ST131 were determined using *pabB* PCR

aac(6')-Ib-cr were detected in 82 % (18/22) of isolates associated with B2 (Table 1), while these genes were identified in 65 % (49/76) ($p=0.1922$) of non-B2 *E. coli* isolates (Tables 2, 3, and 4).

Only 4 % of isolates from the non-B2 phylogenetic group were found to be non-susceptible to aminoglycosides, whilst all isolates of the B2 group were susceptible to aminoglycosides. The isolates positive for 16S rRNA methylases genes hosted *rmtB* (2.6 %) and *rmtC* (1.3 %) among the non-B2 phylogenetic group of *E. coli* isolates (Tables 3 and 4). Two isolates among non-B2 *E. coli* harbored both *qepA* and *rmtB* (Table 4). In the phylogroup B2 isolates belonging to CTX-M group 9, no PMQR or 16S rRNA methylase encoding genes were detected (Table 1). Most of the isolates carrying *qnrB* genes hosted IncHI2 plasmids alone or in combination with IncFIA, IncFIB, and/or FrepB replicons, whereas isolates positive for *qnrS*, *qepA*, and 16S rRNA methylases genes co-harbored IncFIA, IncFIB, and/or FrepB (Tables 1, 2, and 4).

Prevalence of the ST131 clone

Among all ESBL-producing *E. coli* isolates, 18 (18 %) were positive in the *pabB* PCR. In the B2 phylogroup, 17/22 (72 %) isolates harbored the *pabB* gene, and only 1/76 B1 (1.3 %) isolates were positive ($p=0.0001$). The *pabB* variant indicative of the ST131 clone was not detected in any other phylogroup (Tables 1 and 3).

Prevalence of virulence factors

Thirty-four fitness factors or virulence genes were assessed, which broadly characterize the general mechanisms of pathogenesis. The prevalence of these virulence factors was significantly higher in the B2 phylogenetic groups of *E. coli* as compared to the other phylogenetic groups. Twenty-four out of 34 genes studied were recognized in at least one isolate.

More than 95 % (21/22) of B2 *E. coli* possessed adhesins, type 1 fimbriae encoded by *fimH* (Table 1), as compared to 67.1 % (51/76) ($p=0.0063$) of non-B2 *E. coli* isolates (Tables 2, 3, and 4). The type 1 fimbriae encoding gene *fimA* was detected in 22.3 % (17/76) of non-B2 isolates (Tables 2, 3, and 4), while only 9 % (2/22) ($p=0.2271$) of *E. coli* in the B2 phylogenetic group possessed this adhesin type (Table 1). *papAH*, *papC*, and *papG* allele II genes encoding the structural subunit of pyelonephritis-associated pili which mediate adherence to host cells were prevalent in 54.5 % (12/22) ($p=0.0001$), 59 % (13/22) ($p=0.0001$), and 59 % (13/22) ($p=0.0001$) of B2 *E. coli* isolates, respectively (Table 1), while among non-B2 *E. coli* isolates, only one isolate harbored *papC* (Table 3). In the B2 phylogenetic group, 27.2 % (6/22) ($p=0.0001$) of isolates harbored *afa*/*draBC* (afimbrial Dr-binding adhesins). Among toxins, two genes, *hylA* (α-hemolysin) and *sat* (secreted autotransporter toxins), were

Table 4 Virulence factors, plasmid replicons, and antimicrobial resistance among phylogroup D CTX-M group 1 *E. coli* isolates

Isolate	Origin	O serotype	Virulence factors	*qnr*/*qepA*	16S methylases	*aac(6')-Ib-cr*	Plasmid replicon	Antimicrobial resistance
5	Pus	O2a	*fimH iutA traT kpsMT*III *PAI*	−	−	+	HI2 FIA FrepB	CAZ CTX CIP CN SXT TZP
6	Blood	O2a	*fimH iutA traT kpsMT*III *PAI*	*qnrB*	−	+	HI2 FIA FrepB	CAZ CTX CIP SXT TZP
8	Asp. fluid	NT	*fimH traT kpsMT*II *PAI*	−	−	+	I1 FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
11	Urine	NT	*iutA traT kpsMT*III *PAI*	*qepA*	*rmtB*	−	FIA FrepB	AK CAZ CTX CIP CN SXT TZP
21	Asp. fluid	NT	*fimH traT kpsMT*III *PAI*		−	+	FIA FIB FrepB	CAZ CTX CIP SXT (TZP)
43	Pus	NT	*fimH traT kpsMT*III *PAI*	−	−	+	FIA FIB FrepB	CAZ CTX CIP SXT (TZP)
46	Pus	O2a	*fimH iroN traT kpsMT*II *PAI*	*qnrB*	−	+	HI2 FIA FIB FrepB	CAZ CTX CIP CN SXT
60	Pus	O1	*fimA sat iutA traT PAI*	−	−	+	N FIA FIB Y FrepB	CAZ CTX CIP CN SXT (TZP)
61	Urine	O1	*fimH traT kpsMT*III *PAI*	−	−	+	FIA FIB FrepB	CAZ CTX CIP SXT TZP
62	Urine	O25a	*fimH traT kpsMT*II	−	−	+	FrepB	CAZ CTX CIP CN SXT (TZP)
68	Urine	O1	*iutA traT PAI*	−	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT
69	Urine	O157	*fimH iroN traT ibeA kpsMT*II *tsh usp PAI*	−	−	−	I1 FIB FrepB K	CAZ CTX SXT (TZP)
78	Urine	NT	*fimH iutA traT kpsMT*III *PAI*	−	−	−	FIB FrepB	CAZ CTX CIP CN SXT TZP
80	Urine	NT	*fimH sat hylA traT kpsMT*II *PAI*	−	−	+	FrepB	CAZ CTX CIP CN SXT TZP
95	Urine	O1	*iutA traT PAI*	−	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT
126	Pus	NT	*fimH iutA traT kpsMT*II	−	−	+	FrepB	CAZ CTX CIP CN SXT TZP
132	Urine	NT	*fimH iutA traT PAI*	−	−	−	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
133	Urine	NT	*fimH iutA traT kpsMT*II	−	−	−	FrepB	CAZ CTX CIP CN SXT
139	Urine	NT	*fimH iutA traT kpsMT*III *PAI*	*qepA*	*rmtB*	−	FIA FrepB	AK CAZ CTX CIP CN TZP
143	Urine	NT	*fimH papEF sat iutA traT kpsMT*II *PAI*	−	−	+	FIA FIB	CAZ CTX CIP CN SXT TZP
154	−	NT	*fimH traT PAI*	−	−	+	FrepB	CAZ CTX CIP
163	Urine	NT	*fimH papEF sat iutA traT kpsMT*II *PAI*	−	−	+	FIA FIB	CAZ CTX CIP CN SXT TZP
168	Pus	O2a	*fimH papEF traT*	−	−	+	HI1 FrepB	CAZ CTX CIP CN SXT TZP
202	Urine	NT	*fimH traT kpsMT*III *PAI*	−	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
203	Urine	NT	*fimH traT kpsMT*III *PAI*	−	−	+	FIA FIB FrepB	CAZ CTX CIP CN SXT (TZP)
205	Pus	O1	*fimH traT kpsMT*II *PAI*	−	−	−	FIA FIB Y	CAZ CTX CIP SXT
207	Pus	O1	*fimH traT kpsMT*II *PAI*	−	−	−	FIA FIB Y	CAZ CTX CIP SXT
208	Urine	O1	*fimH traT kpsMT*II *PAI*	−	−	−	FIA FIB Y	CAZ CTX CIP SXT
211	Urine	NT	*fimH traT kpsMT*III *PAI*	−	−	+	FIA Y FrepB	CAZ CTX CIP SXT (TZP)
212	Urine	NT	*fimH traT kpsMT*III *PAI*	−	−	+	FIA Y FrepB	CAZ CTX CIP SXT
213	Pus	O1	*iutA traT PAI*	−	−	+	FIA FIB Y FrepB	CAZ CTX CIP CN SXT
215	Urine	O1	*fimH traT kpsMT*III *PAI*	−	−	−	FIA FIB Y	CAZ CTX CIP SXT
216	Pus	O1	*iutA traT PAI*	−	−	+	FIA FIB Y FrepB	CAZ CTX CIP CN SXT
218	Pus	O1	*iutA traT PAI*	−	−	+	FIA FIB Y FrepB	CAZ CTX CIP CN SXT TZP
227	Stool	O157	*fimH traT kpsMT*II	−	−	−	FIA FIB FrepB	CAZ CTX SXT

All isolates were negative for ST131

NT non-typable, *AK* amikacin, *CAZ* ceftazidime, *CIP* ciprofloxacin, *CTX* cefotaxime, *CN* gentamicin, *SXT* trimethoprim–sulfametoxazole, *TZP* piperacillin–tazobactam, − negative, + positive, ( ) intermediate

prevalent in 22.7 % (5/22) and 36.3 % (8/22) of B2 *E. coli* isolates (Table 1), respectively, as compared to lower prevalences in the other phylogenetic groups of 2.6 % (2/76) ($p = 0.0058$) and 6.5 % (5/76) ($p = 0.0012$) of isolates (Tables 2, 3, and 4). Of the siderophores studied, ferric aerobactin receptors encoded by *iutA* were distributed among 90 % (20/22) of B2 *E. coli* isolates (Table 1) and 46 % (35/76) ($p = 0.0002$) of isolates belonging to other phylogenetic groups (Tables 2, 3, and 4).

Virulence markers encoding protectins, *traT* genes (serum survival), were highly distributed among B2 and other phylogenetic groups of *E. coli*, 90 % (20/22) and 81.5 % (62/76) ($p = 0.5124$) of isolates, respectively (Tables 1, 2, 3, and 4). Genes PAI (pathogenicity-associated island marker) and *usp* (uropathogenic-specific protein) were disseminated among 86.3 % (19/22) isolates of the B2 phylogenetic group (Table 1), while among non-B2 isolates, 46 % (35/76) ($p = 0.0012$) and 2.6 % (2/76) ($p = 0.0001$), respectively, were carrying these genes (Tables 2, 3, and 4). *kpsMT*II ((group 2 capsule LPS) were prevalent in 17.1 % (13/76) of isolates of non-B2 phylogenetic groups (Tables 2, 3, and 4), while 45.4 % (10/22) ($p = 0.0097$) of B2 isolates harbored these genes (Table 1).

Discussion

Among the ESBL-producing *E. coli* from Pakistan, almost all of them produced ESBLs belonging to CTX-M phylogroup 1. The genetic diversity was high among the multidrug-resistant isolates, as demonstrated by PFGE. Co-resistance to other antimicrobial agents was highly prevalent, but almost all isolates were susceptible to meropenem and amikacin. Phylogenetic group D (36 %) was the most prevalent among CTX-M-producing *E. coli* isolates, followed by group A (30 %), group B2 (22 %), and group B1 (12 %). Among all phylogroups, isolates belonging to group B2 carried the highest contents of virulence genes.

In this comparative study of phylogenetic groups of *E. coli*, F replicons were by far the most frequently detected. Unlike most of the previous studies, we could not find an association of IncFIA and/or IncFIB with IncFII, except in one isolate [28], which is similar to what has been described in a report from Germany [29]. Most of the CTX-M type ESBL-producing *E. coli* isolates investigated in this study also harbored IncFIA and/or IncFIB in association with FrepB replicons, as described in previous studies [28, 29]. All types of ESBLs are not disseminated by IncF replicons, but CTX-M enzymes are spread by various replicons and most of them were among IncF replicons (IncFIA, IncFIB, and IncFII) [30]. These enzymes are linked on the same plasmids to other antimicrobial resistance genes, such as *qnr* encoding genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qepA*), 16S rRNA methylase encoding genes (*armA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD*), and *aac(6')-Ib-cr*, encoding a fluoroquinolone-modifying enzyme. Together, these arrays of resistance genes confer a multidrug resistance phenotype that can be transferred horizontally [29, 31]. A few of the isolates belonging to CTX-M group 1 were found to be positive for IncHI2 replicons in this study, whilst previous reports from the UK and Spain showed their involvement in the dissemination of CTX-M-9 [28]. Of the PMQR tested in this study, the incidence of *aac(6')-Ib-cr* and *qnr* genes was higher than previously reported [16], while the rarely found *qepA* genes in *E. coli* were also relatively prevalent at 6 % of the isolates included in this study [8, 16].

The ST131 clone, frequently associated with phylogroup B2, has been reported all over the world, but for the first time from Pakistan in this study. However, the incidence of ST131 in the current study was lower than that previously reported for the UK, France, Canada, Turkey, and Cambodia, but higher than that of reports from Thailand [5]. The relationship between IncF replicons, CTX-M 15, and ST131 clones, which has already been described in the literature, was also observed in this study [1].

A high prevalence of extraintestinal virulence genes was observed among ESBL-producing *E. coli* isolates tested in this study, which may be important in the pathogenesis of clinically important infections such as BSI, pyelonephritis, cystitis, and neonatal meningitis [1, 21, 27, 32]. Isolates associated with the B2 phylogenetic groups were highly virulent due to the presence of adhesins (*fimA*, *fimH*, *papAH*, *papC*, *papEF*, *papG*, *afa*/*draBC*), protectins (*traT* and *neuC*), siderophores (*iutA*), toxins (*hylA* and *sat*), and some other virulence factors (*kpsMT*II, *kpsMT*III, *usp*, *fliCh7*, and *PAI*). A relationship between phylogenetic group, serotypes, and virulence factors has been described previously [1, 32]. Mostly, ST131 isolates in the B2 phylogenetic group were O16 and O18 serotypes, as shown in cluster VIII in Fig. 1, while non-ST131 B2 isolates were O157. ST131 B2 *E. coli* also carried *pap*, *fim*, and *PAI* markers. In contrast to a previous study from Spain, ST131 isolates carrying *afa*/*draBC* associated with *PAI* were gentamicin-resistant according to our findings [1]. Among non-B2 isolates, group D had the highest content of virulence factors. The isolates of phylogroup D showed a very low prevalence of *pap* alleles and most of the isolates were associated with *PAI*, as shown in Table 4. However, one ST131 *E. coli* isolate, coupled to phylogroup B1, exhibited a different profile of virulence contents from other members of the group, as shown in Table 3. Overall, virulence factors *fimH*, *iutA*, *traT*, *PAI*, and *kpsMT*II and III were detected in most ESBL CTX-M type *E. coli* isolates included in this study.

In conclusion, these data from Pakistan suggest that highly virulent and genetically divergent isolates with different types of resistance mechanisms were involved in hospital- and community-acquired infections in the region, but amikacin and meropenem still retained their activities. The presence of 16S rRNA methylase genes in the population of CTX-M-producers might, however, over time affect amikacin

susceptibility, as was observed in the, so far, few isolates featuring this mechanism of resistance.

Conflict of interest The authors declare that they have no conflict of interest.

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