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Genomic epidemiology, antimicrobial resistance and virulence factors of *Enterobacter cloacae* complex causing potential community-onset bloodstream infections in a tertiary care hospital of Nepal

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Objectives: Community-onset bloodstream infections (BSIs) caused by carbapenemase-producing *Enterobacter cloacae* complex (ECC) species are increasing internationally. This observation suggests that ECC are emerging pathogens, requiring for detailed understanding on their genomic epidemiology including transmission dynamics and antimicrobial resistance profiles.

Patients and methods: We performed WGS on 79 *Enterobacter* spp. isolated from the patients with clinically significant BSIs and admitted to emergency department of a major tertiary hospital in Nepal between April 2016 and October 2017.

Results: We identified 5 species and 13 STs of ECC. *Enterobacter xiangfangensis* ST171, one of the globally emerging carbapenem resistant ECC clones with epidemic potential, was the most prevalent (42%). Phylogenetic analysis showed a large (>19 400 SNPs) core genome SNP distance across major STs, which was minimal (<30 SNPs) among the isolates of each prevalent ST, suggesting the relatively recent importation of major STs followed by local clonal expansions. Genomic evidence for resistance to all major antimicrobial classes except for colistin and macrolides was detected. A limited number of isolates also carried $bla_{\text{NDM-1}}$ ($n=2$) and $bla_{\text{OXA-48}}$ ($n=1$) carbapenemase genes. Virulence factors encoding siderophores (24%), T6SSD (25%) and fimbriae (54%) were detected.

Conclusions: Our study highlighted that MDR ECC clones are important pathogens of BSIs in community. Though of low prevalence, carbapenem resistance observed in our ECC isolates raised concern about further community dissemination, underscoring the need for community surveillance to identify MDR ECC clones with epidemic potential.

Introduction

Bacteria belonging to *Enterobacter cloacae* complex (ECC) are opportunistic Gram-negative bacilli that comprise a part of the healthy gut microbiota of humans and animals. ECC are also members of the ESKAPE group of pathogens and are frequently associated with MDR nosocomial infections.[1] ECC bacteria are intrinsically resistant to several β-lactam antimicrobials because of constitutive expression of the chromosomally located AmpC gene.[2,3] Further, a rapid emergence and spread of carbapenem-resistant *E. cloacae* complex (CREC) variants have been widely reported, complicating the therapeutic management of patients infected with MDR infections.[4–6]

MDR strains of ECC bacteria can colonize high-contact surfaces in ICUs, where exposure to several clinically used broad-spectrum antimicrobials is probable.[7,8] Under suboptimal infection control measures in hospitals, the colonized ECC bacteria can cause nosocomial infections in vulnerable patient population.[7,9] In communities, specifically of low- and middle-income countries, the rampant use of broad-spectrum antimicrobials may facilitate

the selection and colonization of MDR ECC in human gut. The resulting reservoir can serve as an endogenous source of invasive community-onset bloodstream infections (CO-BSIs), specifically in individuals with debilitating health conditions, such as reduced immunity, malignancy, diabetes and severe illnesses requiring prolonged hospitalization and antimicrobial therapy.[9]

Bloodstream infection (BSI) is one of prevalent diagnoses among patients with acute febrile illness seeking urgent healthcare in emergency department (ER) of hospitals globally.[10–12] The prevalence of Enterobacterales is high among ER-attending patients with bacterial BSI.[10,13–15] Studies on aetiology of bacterial CO-BSIs among ER-attending patients is limited in Nepal. A retrospective study conducted in a major tertiary hospital reporting on 23 years (1992–2014) of microbiological data constituting >220 000 bacterial isolates from blood samples is one of the comprehensive studies on bacterial BSIs in Nepal.[13] The study evidenced an increasing temporal trend in the proportion of MDR non-*Salmonella* Enterobacterales, particularly those originating from the outpatients. Further, after *Salmonella enterica* Typhi and Paratyphi A, MDR ECC was identified to be the most prevalent non-*Salmonella* Enterobacterales causing BSIs, mostly among ER-attending patients.[13] The findings underscored a potentially emerging role of ECC in causation of BSIs. Other studies have also highlighted MDR ECC as an increasingly important bacterial aetiology of CO-BSIs, thereby threatening the current antimicrobial therapy.[16,17]

Genomic investigation permits an exact identification of species of ECC group, genetic determinants of antimicrobial resistance (AMR) and virulence, and bacterial population structure. Such data can be used to determine the emergence and spread of clinically and epidemiologically relevant *Enterobacter* spp. A WGS-based phylogenetic study conducted on *E. cloacae* isolates causing neonatal sepsis in a tertiary hospital in Nepal elucidated the transmission dynamics of infection, and also identified an environmental source for one of the outbreaks.[7] Genomic studies on clinical isolates of ECC bacteria causing CO-BSIs among ER-attending patients is however lacking in the existing literature. To fulfill this knowledge gap and aiming to characterize the species, AMR genes and phylogenetic relationships of ECC bacteria causing potential CO-BSIs, we performed WGS and comprehensive genomic analyses of ECC isolated from the patients admitted to ER of a major tertiary hospital in Nepal.

Patients and methods

Ethics

Ethical approval for conducting this study was obtained from Nepal Health Research Council (NHRC) under the approval registration number of 120/2012 for the research entitled 'Prevalence of extended spectrum β-lactamase (ESBL) pathogens and factors responsible for ESBL associated infections within Patan Hospital'.

Study setting and design

This was a retrospective study on routine laboratory results and archived bacterial isolates in the microbiology department at Patan Hospital, one of the largest tertiary hospitals in Nepal. Annually, Patan Hospital provides healthcare services to nearly 320 000 outpatients and 20 000 inpatients. The ER provides acute healthcare services to >100 patients per day, and >36 000 patients per year.

Study definitions and case ascertainment

A CO-BSI was defined as a BSI producing positive blood culture result from a blood sample taken from a patient having undifferentiated fever within 48 h of admission. At the ER, patients stayed for a maximum of 12–24 h post-admission. Therefore, all ER patients presenting with an undifferentiated fever at admission and producing a positive blood culture were considered to have a potential CO-BSI. After collection of a blood sample for microbiological culture, the patient was empirically treated with IV third-generation cephalosporins. Patients showing clinical improvement were discharged with a prescription of oral azithromycin and requested to come to a follow-up examination with blood culture report on the fifth day. Alternatively, patients failing to show clinical improvement or undergoing deterioration were admitted to an appropriate hospital ward. This being a retrospective study, information on clinical care taken by the patients in other healthcare facilities before being admitted to the ER of Patan Hospital could not be recorded.

Blood culture

A blood sample was collected at bedside by an attending nurse after an aseptic skin preparation and following a standard protocol. For adults, 8–10 mL of blood was collected in a BD BACTEC Plus aerobic/F culture vial (Becton Dickinson, Sparks, MD, USA); while for children, 1–3 mL of blood was collected in BD BACTEC PedsPlus/F culture vial. The inoculated vials were incubated in an automated BD BACTEC FX40 continuous culture system, and subjected to routine microbiological processing for bacterial isolation, identification and antimicrobial susceptibility testing (AST). The Gram-negative bacilli that were lactose-fermenting, motile, indole-negative, urease-negative, citrate-positive, hydrogen sulphide non-producing, and giving acid/acid reaction with gas production in a triple sugar iron test were presumptively identified as *Enterobacter* spp. AST was performed using the modified Kirby–Bauer disc diffusion method where the antimicrobial discs were placed on Mueller–Hinton agar swabbed with 0.5 McFarland units of overnight bacterial suspension to create a confluent bacterial lawn.[18] After incubation at 35 ± 2°C for 18 h, zones of inhibition were measured, and the results were interpreted referring to the zone size breakpoints as recommended by CLSI.[19] Pure colonies of *Enterobacter* spp. were suspended in Red Protect Bacterial Bead Preservation System (TSC Technical Service Consultants Ltd, Lancashire, UK), and stored at −80°C for genomic analyses.

Isolate ascertainment

The microbiology laboratory information management database of Patan Hospital was interrogated for *Enterobacter* spp. isolated from blood samples originating from the patients attending the ER between April 2016 and October 2017. Replicate *Enterobacter* spp. isolates having an identical AST profile and originating from the same patient were excluded from the analysis. The archived *Enterobacter* isolates stored at −80°C in the freezer were retrieved, revived and processed for genomic analyses.

Bacterial conjugation

The conjugation experiment was performed between the recipient isolate of *Escherichia coli* J53 strain (resistant to amikacin and sodium azide) and each of the donor ECC isolates of ST171, ST134 and ST1377 by combining equal volumes (5 mL) of overnight LB cultures. The bacterial strains were conjugated at 37°C for 12 h in LB broth. The resulting *E. coli* transconjugants were selected on MacConkey agar containing sodium azide (100 mg/L), amikacin (64 mg/L) and ceftriaxone (6 mg/L). The transfer of AMR plasmids was confirmed by plasmid extraction from the *E. coli* transconjugants using a modified Kado and Liu method,[20] followed by gel electrophoresis. AST of *E. coli* transconjugants was performed for ciprofloxacin, ofloxacin, cefotaxime, ceftriaxone, co-trimoxazole, gentamicin, amikacin, chloramphenicol, meropenem, imipenem, colistin,

tetracycline, streptomycin and erythromycin using the modified Kirby–Bauer disc diffusion method.[18]

WGS and genomic analysis

Genomic DNA of *Enterobacter* isolates was extracted using the Wizard Genomic DNA Extraction Kit (Promega, WI, USA) by following manufacturer's recommendations. The dual index-tagged pooled whole genome DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, CA, USA). WGS was performed on a HiSeq X Ten platform (Illumina) to generate 150 bp paired-end reads.

After an initial quality assessment of raw Illumina reads by FastQC v0.11.5,[21] the adapters were removed using Trimmomatic v0.36.[22] SRST2 v0.2.0[23] was used to identify the AMR genes using the ARG-ANNOT[24] database; virulence factors using BIGSdb[25] and VFDB (http://www.mgc.ac.cn/VFs/) databases; plasmid replicons using the PlasmidFinder[26] database, and MLST using MLST typing scheme (https://pubmlst.org/organisms/enterobacter-cloacae). SRST2 used Bowtie2 to map the raw reads against the reference databases, and SAMtools to detect the gene variants. Raw reads were *de novo* assembled using Unicycler v0.48[27] to generate the contigs, followed by a genome annotation using Prokka v1.5.[28] Bandage[29] was used to visualize the assembly graph of plasmid-carrying isolates. Subsequently, BLASTn which was integrated in Bandage, was used to identify and extract the assembled sequences containing the detected AMR genes and plasmid replicons of IncF/IncR. The extracted sequences were used to search for the best-matching plasmids in NCBI database using BLASTn.

The taxonomic assignment of *Enterobacter* spp. is complex. Recently, whole genome based average nucleotide identity (ANI) (defined as a mean nucleotide identity of all orthologous genes shared between two microbial genomes) has been used to generate an updated taxonomy of the *Enterobacter* genus.[30] Here, we used a fast approximate ANI tool, called MASH v1.1.1,[31] to generate a pairwise ANI-based distance matrix between our *Enterobacter* isolates and the published reference collection of 22 *Enterobacter* species,[30] comprising *Enterobacter quasiroggenkampii, Enterobacter quasimori, Enterobacter wuhouensis, Enterobacter quasihormaechei, Enterobacter huaxiensis, Enterobacter chuandaensis, Enterobacter sichuanensis, Enterobacter chengduensis, Enterobacter soli, E. cloacae, Enterobacter mori, Enterobacter bugandensis, Enterobacter ludwigii, Enterobacter cancerogenus, Enterobacter asburiae, Enterobacter hormaechei, Enterobacter xiangfangensis, Enterobacter kobei, Enterobacter timonensis, Enterobacter oligotrophica, Enterobacter dissolvens* and *Enterobacter roggenkampii*. A pairwise ANI with a cut-off threshold of 96% was used to identify the appropriate species.

Pairwise SNP distance

We sought to compare the genetic distance between the three predominant *Enterobacter* STs (ST171, ST134 and ST1377) in our collection. Initially, Roary[32] was used to construct a pan genome for the three STs with a blastp percentage identity of 99% and a core definition of 99%. SNP-sites v2.1.3[33] was then used to extract the SNP sites from the core gene alignment (1012 genes), resulting in an alignment of 47 601 SNPs. Pairwise genetic distances (difference in the number of SNPs) within and between the three STs were estimated from the SNP alignment using the ape (v4.1) and adegenet (v2.0.1) packages in R (v3.3.2).[34]

Phylogenetic analysis

A pairwise SNP distance analysis showed significant SNP differences between the three predominant STs (ST171, ST134 and ST1377), but limited SNP differences within individual STs. To obtain a further phylogenetic resolution for each ST, we reconstructed a pan genome for individual STs using Roary[32] (blastp percentage identity of 99%, and core definition of 99%). The core gene alignment of each ST was subjected to Gubbins (v2.3.2)[35] to remove the regions of recombination, followed by an extraction of SNPs using SNP-sites v2.1.3.[33] The number of core genes for each ST was 3833 (ST171), 3542 (ST134) and 3742 (ST1377). The SNP alignment of each ST was used to construct a maximum likelihood (ML) phylogenetic tree using IQ-TREE v1.4.4,[36] with the best-fit evolutionary model (K3Pu + ASC) identified based on the Bayesian Information Criterion in jModelTest implemented in this software. The support for the ML tree was assessed via 100 pseudo-replicates. Further, to examine the phylogeny of ECC ST171 in a global context, we combined genomic data from our study together with 93 publicly available assembled genomes of ST171 isolates from NCBI.[37] Core gene alignment was re-analysed using Roary v3.9.1 (roary -e –mafft -p 8 *.gff). The number of core genes were 3689 genes, corresponding to an alignment length of 3 602 642 bp (~75% of genome size). Subsequently, Gubbins v1.4.5 (default settings) was used to identify and to remove the recombinant SNPs. A final alignment of 1217 core SNPs was used to construct the ML phylogenetic tree using IQ-TREE v1.4.4,[36] with the K3Pu + ASC model of nucleotide substitution. Support for the ML tree was assessed via 100 pseudo-replicates.

Accession numbers

All raw Illumina sequence reads of 79 *Enterobacter* spp. generated in this study on HiSeq X Ten platform (Illumina) were deposited in the European Nucleotide Archive under the accession numbers from ERS6485160 to ERS6485239 (Table S1, available as Supplementary data at *JAC-AMR* Online). The whole genome sequences of two *Enterobacter* spp. used as reference strains for BLAST Ring Image Generator (BRIG)[38] were retrieved from NCBI (accession numbers: CP031569 and AP021911.1).

Results

Demographics

Between April 2016 and October 2017, we recovered a total of 127 *Enterobacter* spp. isolated from blood cultures across different departments at Patan Hospital. Of these, 79 (62%) isolates originated from the ER-attending patients having clinically suspected BSIs. The median age of these ER-attending patients was 41 years (IQR: 30–59.5), with a male to female ratio of 0.92:1.

Genome-based species and ST identification of *Enterobacter* spp.

Enterobacter spp. of 5 different species and 13 STs were identified (Figure 1a). *E. xiangfangensis* (73.4%, 58/79) and *E. hormaechei* (20.2%, 16/79) were the two most common species, followed by *E. hoffmannii* (3.8%, 3/79), *E. asburiae* (1.3%, 1/79), and *E. quasihormaechei* (1.3%, 1/79). Among the identified STs, ST171 was found to be the most predominant (41.8%, 33/79), followed by ST134 (22.8%, 18/79), ST1377 (15.2%, 12/79), ST not identified (7.5%, 6/8) and several diverse singleton STs ($n = 8$). The three prevalent STs (ST171, ST134, and ST1377) collectively accounted for 80% of the total isolates. ST171 and ST134 isolates belonged to *E. xiangfangensis*, and ST1377 isolates were identified as *E. hormaechei*.

Antimicrobial susceptibility profile

All 79 *Enterobacter* isolates were resistant to cefotaxime (Figure 1b). A high proportion of isolates were phenotypically resistant to gentamicin (82.3%; 65/79), chloramphenicol (82.3%; 65/79), co-trimoxazole (92.4%; 73/79) and ciprofloxacin

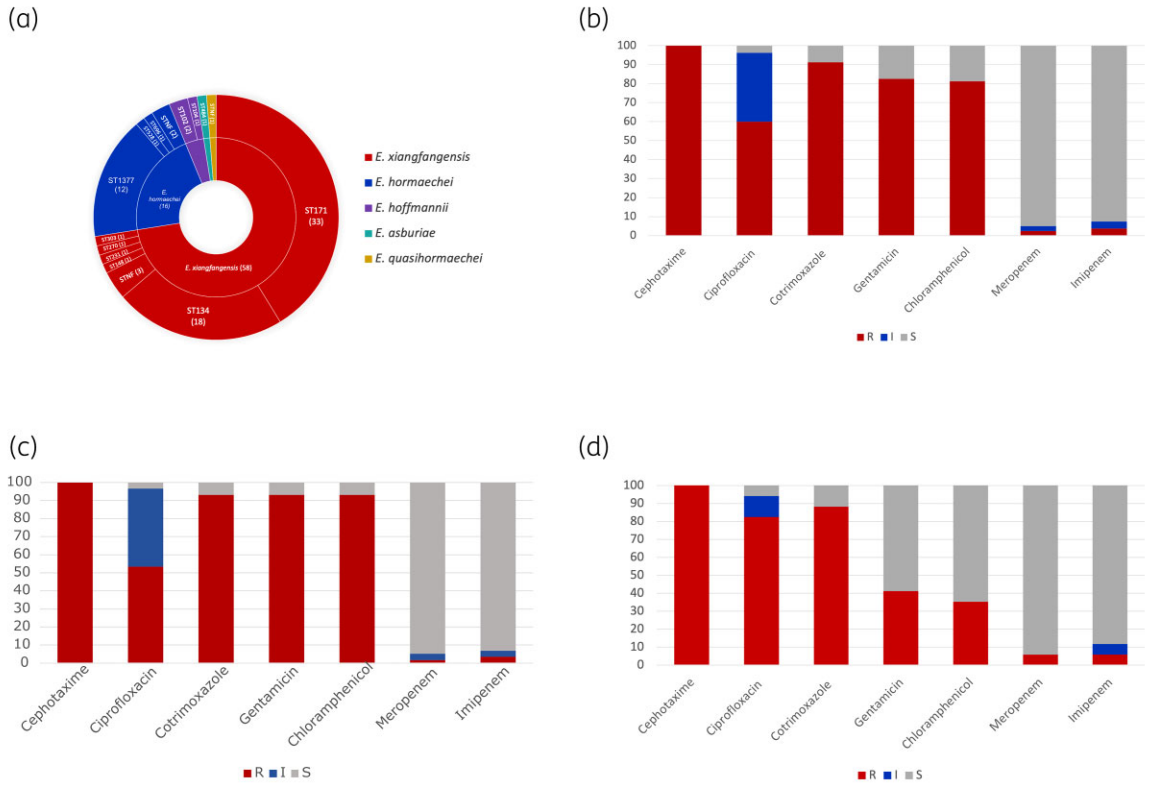

Figure 1. Species, ST and phenotypic AST profiles of *Enterobacter* isolates. (a) The species distribution of our *Enterobacter* isolates and the distribution of STs within each species. (b, c and d) The phenotypic AST profile of all isolates, of *E. xiangfangensis* and of *E. hormaechei*, respectively. The *y*-axis indicates the percentage of isolates. R, resistant; I, intermediate; S, susceptible.

(60.8%; 48/79). Alternatively, resistance to carbapenems was low, with 5.1% (4/79) and 7.5% (6/79) of isolates being resistant to meropenem and imipenem, respectively. At species level, in comparison to *E. hormaechei* isolates ($n=16$), *E. xiangfangensis* isolates ($n=58$) were more frequently resistant to gentamicin (95% versus 38%) and chloramphenicol (93% versus 38%), but less resistant to ciprofloxacin (54% versus 81%) (Figure 1c and d). The proportion of resistant isolates of *E. xiangfangensis* and *E. hormaechei* were comparable for cefotaxime (100% versus 100%), meropenem (5.2% versus 5.8%) and co-trimoxazole (92% versus 94%).

Genes for AMR and virulence

A high proportion of *Enterobacter* spp. isolates tested positive for a diverse set of AMR genes conferring resistance to all classes of antimicrobial agents except carbapenems, macrolides and colistin (Figure 2). Of note, *E. xiangfangensis* isolates had the most extensive array of AMR genes, including $bla_{\text{CTX-M-1}}$, bla_{ACT}, *qnrB, dfrA5, strA/B, aac(3)-IIa, aac-aad,* $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1D}}$, *catA, tet*(A), *sulII* and *fosA2*. Three isolates of *E. xiangfangensis* additionally had carbapenem resistance genes including $bla_{\text{OXA-48}}$ (ST171, $n=1$) and $bla_{\text{NDM-1}}$ (ST134, $n=1$; ST231, $n=1$). In comparison to *E. xiangfangensis*, the *E. hormaechei* isolates had less extensive AMR gene profiles, namely bla_{ACT}, *qnrB*, $bla_{\text{CTX-M-1}}$ and *dfrA5*. One *E. hormaechei* isolate (ST528) also carried a $bla_{\text{NDM-1}}$ gene.

By ST, all ST171 and ST134 isolates carried a comparable group of AMR genes, namely *aac(3)-IIa, aac-aad,* $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1D}}$, *dfrA5*, *fosA2*, *qnrB*, *strAB* and *tet*(A), predicted to confer resistance to aminoglycosides, third-generation cephalosporins, fosfomycin, trimethoprim, quinolones, streptomycin and tetracyclines (Figure 3). Relatively, ST1377 isolates had fewer AMR genes (*aac-aad,* $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, *dfrA5* and *qnrB*). The distribution of virulence factors was distinct across the three STs. A majority of ST171 isolates had flagella-encoding gene (*fliA*) and type-3 fimbriae biosynthetic cluster (*mrkA-F*), whereas ST134 isolates predominantly carried a salmochelin gene cluster, and an *hcp/tssD* gene associated with type VI secretion system (T6SS), and ST1377 isolates had a siderophore yersiniabactin gene cluster and *lpfB-lpfC* genes encoding for long polar fimbriae (Figure 3).

Plasmid replicons

All ST171 isolates carried a large IncFIB plasmid showing a substantial genetic similarity to a non-AMR IncFIB plasmid of a clinical *E. hormaechei* isolate detected in USA (accession number: CP031569) (coverage: 90%, identity: 99%). The plasmid had self-transfer modules and multiple heavy metal resistance gene

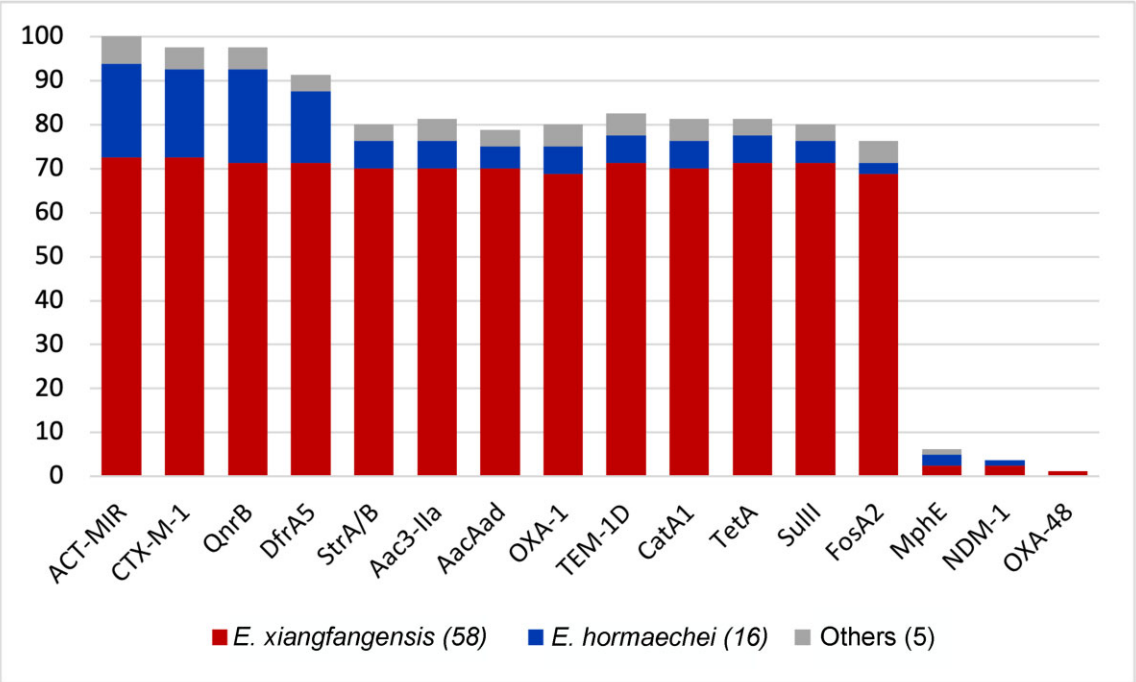

Figure 2. The distribution of AMR genes by *Enterobacter* species. The results for minor species *E. asburiae* ($n=1$), *E. hoffmannii* ($n=3$) and *E. quasihormaechei* ($n=1$) were grouped together as 'Others'. The *y*-axis indicates the percentage of isolates carrying each AMR gene.

clusters for mercury, copper, iron and arsenic (Figure S1). The conjugation experiments confirmed that the IncFIB plasmid was transferable conferring resistance to ceftriaxone, cefotaxime, erythromycin, streptomycin and co-trimoxazole to the otherwise susceptible recipient bacteria. Although ST134 and ST171 displayed an identical AMR gene profile, we were unable to detect any specific plasmid replicon type in our ST134 isolates by plasmid mapping. However, the conjugation experiments determined that ST134 also carried a self-transferable plasmid conferring resistance to ceftriaxone, cefotaxime, erythromycin, streptomycin and co-trimoxazole. Further, we found that all ST1377 isolates had an IncR plasmid with high synteny and similarity to an IncR plasmid from an environmental *Klebsiella pneumoniae* isolate reported from Japan (accession number: AP021911.1) (coverage 73%, identity 99%, Figure S2). This plasmid was also transmissible and mediated resistance to ceftriaxone, cefotaxime, erythromycin, streptomycin and co-trimoxazole.

Phylogenetic relatedness and spatiotemporal distribution of the three predominant Enterobacter *STs*

We sought to investigate the phylogenetic relatedness of the three predominant STs (ST171, ST134, and ST1377). The average pairwise SNP distance between ST171 and ST134 (*E. xiangfangensis*) was 19 429 SNPs, indicating that the two STs were genetically distant though being belonged to the same species. Predictably, ST171 and ST134 were more distantly related to ST1377 (*E. hormaechei*) than each another, with average pairwise SNP differences of 35 698 SNPs (ST171 versus ST1377) and 34 632 SNPs (ST134 versus ST1377). In contrast, the total number of SNPs identified within each ST was very limited; the ST171 isolates varied by only 27 SNPs, while the isolates within ST134 and ST1377 each differed by only 6 SNPs. The *Enterobacter* isolates within each ST formed a distinct cluster (Figure 3). We performed further phylogenetic analysis of our ST171 isolates together with a global collection of carbapenem-resistant ST171 isolates. We found that our ST171 isolates belonged to a single cluster (median pairwise SNP distance: 5 SNPs, range: 0–13 SNPs) separate from the global isolates. The nearest neighbour to our cluster was an ST171 isolate identified in Guatemala in 2013. The medium pairwise SNP distances between the Nepalese clade and the outbreak clade of ST171 from the USA was 288 SNPs (IQR: 283–293 SNPs). These data suggest that our Nepalese ST171 isolates are probably resulting from a single importation followed by local transmission events (Figure S3).

We found a notable trend in temporal distribution of the three above STs over the study period. Although both ST171 and ST134 belonged to *E. xiangfangensis* species, they were circulating in two distinct time periods. ST134 was exclusively found between April and July 2016 and subsequently disappeared, while ST171 was first detected in September 2016 and until then became a predominant *E. xiangfangensis* genotype circulating in Nepal (Figure S4). Furthermore, almost all ST1377 (*E. hormaechei*) isolates were identified between September and December 2016, followed by a substantial decline in the following months with only two isolates detected in February and March 2017. We performed spatial mapping and analysis of the three STs from our limitedly available geographical locations, but we did not find any significant clustering (Figure S5).

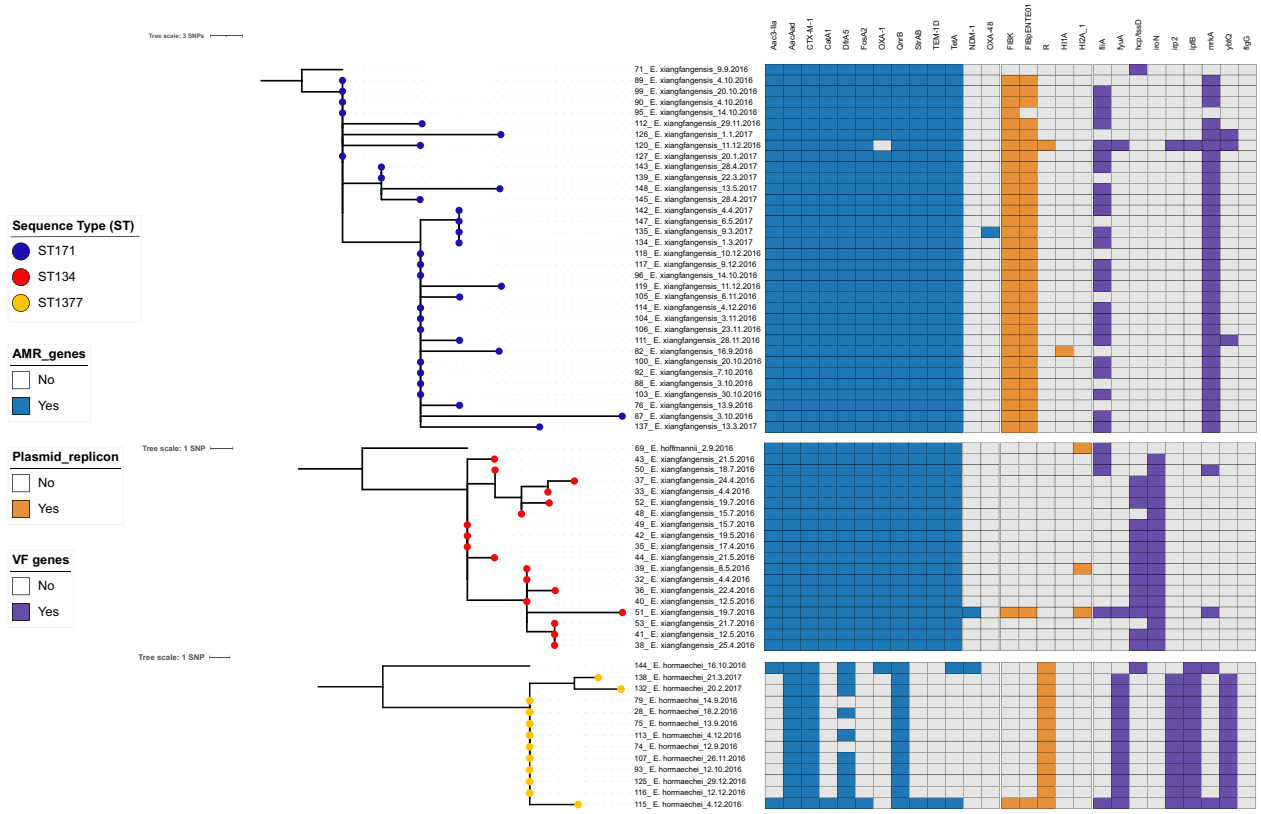

Figure 3. Recombination-filtered core genome-based ML phylogenetic trees for ECC isolates of ST171 (blue dots), ST134 (red dots) and ST1377 (yellow dots). The phylogenetic trees of ST171, ST134 and ST1377 were rooted using genetically closely related outgroups of ST303 (*E. xiangfangensis*), ST202 (*E. hoffmannii*) and ST528 (*E. hormaechei*), respectively. The tip label indicates the bacterial identification number, the species of ECC and the date of isolation. The presence of an array of AMR genes (blue), plasmid replicons (brown) and virulence genes (purple) for each isolate are also shown in coloured square boxes to the right of the trees. Grey shading indicates the absence of the character state. The scale bar indicates nucleotide substitutions per site.

Discussion

The genomic characterization of BSIs causing *Enterobacter* spp. is important for understanding the epidemiological landscape and AMR trends in these organisms. Here, in this genomic epidemiological study, we found diverse species and STs of *Enterobacter* isolates causing potential CO-BSI. *E. xiangfangensis* (predominantly ST171 and ST134) followed by *E. hormaechei* (ST1377) were the most prevalent species and STs circulating in this setting. The dominance of these two species of *Enterobacter* species in clinical samples was in concordance with a genomic study comprising a global collection of 170 CREC isolated between 2008 and 2014.[37] Notably, we found that *E. xiangfangensis* isolates carried more AMR genes than *E. hormaechei*. Additionally, we observed that the majority of BSI-causing *Enterobacter* isolates, irrespective of their exact species, were largely susceptible to carbapenem (>94%), but excessively resistant to third-generation cephalosporins (100%). This was an important finding of the study for designing an optimal empirical antimicrobial therapy in our setting.

The prevalence of CO-BSIs caused by MDR ECC bacteria has increased over the last decade, reducing the therapeutic options.[13,16] The fact that all ECC isolates from our study were ESBL-positive showing an absolute phenotypic resistance to third-generation cephalosporins, with a few isolates additionally carrying carbapenem-resistance genes, raises a serious concern. A high prevalence of BSIs caused by ESBL-producing ECC may prompt for an increased use of carbapenems in clinical settings, which will consequently lead to an enrichment of CREC variants. Earlier studies have suggested that widespread use of carbapenems has produced an antimicrobial pressure, which served as a key factor for the global emergence and proliferation of ST171 as a dominant CREC clone.[39] Routine AMR surveillance with the integration of WGS data rapidly captures the emergence and tracks the transmission of novel *Enterobacter* species/genotypes, including the carbapenem-resistant variants.

We found limited genetic diversity among each population of the three predominant ECC STs in Nepal. There are several potential explanations for our observations, including a recent strain importation followed by a rapid clonal expansion, a recent population bottleneck, or community outbreaks. Here, we did not find sufficient evidence for community outbreaks with regards to temporal and/or geographical clustering (Figure S5); instead, we found evidence that the ST171 population likely resulted from a single importation and subsequent local transmission. Furthermore, the fact that we observed temporal changes in

transmission of the three predominant ECC STs suggests for potential influence of environmental factors, such as seasonal variation. Such a phenomenon has been observed in the transmission dynamics of isolates of *S. enterica* Typhi, a major waterborne pathogen that also causes BSIs.[40] Collectively, we surmise that the strain importation followed by clonal expansion and environmental factors are the probable factors shaping the observed population genetic structure of ECC in our setting.

The factors that drove the transmission of such clones are largely unknown. Several studies have reported that asymptomatic carriage of ECC bacteria in body parts such as gut, anterior nares, skin and upper respiratory tract may act as an endogenous source of invasive infections in susceptible individuals.[41] The ability of ECC to colonize humans may play an important role in its transmission and the development of drug-resistant phenotypes.[3,42] We also cannot rule out the possible role of environmental transmission given what we observed in temporal changes of ECC genotypes. We must improve our understanding on the sources and transmission chains of these organisms to improve public health control measures.

The presence of a wide repertoire of virulence factors and AMR genes among the ECC isolates may offer important adaptive mechanisms and contribute to their spread, given the sustained use of antimicrobials in the community.[39,43,44] However, the natural reservoir of resistance-virulence determinants in ECC is not well described. In this study, we showed that the resistance-virulence attributing plasmids of ECC are transferable between and across the bacterial genus. We hypothesize that bacteria sharing the same ecological niches may be a major reservoir for the horizontal transmission of antimicrobial resistance/virulence genes in ECC bacteria. The interaction and plasmid/gene transfer dynamics between *Enterobacter* and other Gram-negative organisms warrants further investigation.

Being a retrospective study, complete demographic and clinical data for patients before admission to ER were lacking. We considered that all clinically significant BSIs among patients presenting to ER were potentially CO-BSIs. As we had no prior information on whether these patients recently received any healthcare services from another health institution before presenting to our ER, we could not exclude that some or all of these infections were healthcare-associated being acquired in other hospitals. If the patients had received recent medical care from the same health institution(s), then they may have acquired the *Enterobacter* infection from the corresponding institution instead from the community. This discrepancy may explain the low genetic diversity of *Enterobacter* isolates within specific STs, particularly of ST134 and ST1377, which showed fewer than 10 SNP differences. This warrants a further genomic investigation on clinical ECC isolates of the hospitals accompanied by a systematically collected demographic, pre-clinical and clinical data including prior recent exposure to healthcare settings, underlying predisposing factors, use of antimicrobials and final outcome of the patients.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S5 are available as Supplementary data at *JAC-AMR* Online.

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