Genomic characterization of high-risk *Escherichia coli* and *Enterobacter hormaechei* clones recovered from a single tertiary-care hospital in Pakistan

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**Abstract**

**Aims:** Spread of carbapenem-resistant Enterobacterales have become a global problem. We characterized extended-spectrum β-lactamase (ESBL)-producing Enterobacterales from urinary tract infections cases from Allied Hospital Faisalabad, Pakistan.

**Methods and Results:** Eleven (22%, 11/50) ESBL-producing Enterobacterales (*Escherichia coli*; *n* = 10 and *Enterobacter hormaechei*; *n* = 1) were recovered and processed through VITEK-2, PCR, rep-PCR followed by whole-genome sequencing (WGS) of ESBL-producing *Ent. hormaechei* and carbapenem-resistant *E. coli* isolates. Plasmid transferability of *bla*<sub>NDM-1</sub>-producers was assayed by conjugation experiments. All ESBL strains carried the *bla*<sub>CTX-M-15</sub> gene. Of these *bla*<sub>CTX-M-15</sub>-producing *E. coli*, four also carried *bla*<sub>NDM-1</sub> located on transferable plasmids. All *E. coli* strains belonged to ST448 and displayed similar genetic features including genes for antimicrobial resistance, heavy metal, biocides and virulence. Genomic features of a multidrug-resistant (MDR) *Ent. hormaechei* were also reported for the first time in Pakistan.

**Conclusion:** Our findings indicate that *bla*<sub>NDM-1</sub>-producing *E. coli* ST448 is a multidrug, heavy metals and biocides-resistant strain. Therefore, the screening of these isolates may be effective in limiting the MDR bacteria spread in hospitalized patients and within the community.

**Significance and Impact of this Study:** Spread of multi-drug-resistant ESBL-producing bacteria in the clinical settings of Pakistan is a serious challenge and further limiting treatment options in the country. WGS could be used as a tool in the nationwide antibiotic surveillance programme to explore insights of spread and outbreak.

**Keywords**

antimicrobial resistance, *bla*<sub>NDM-1</sub>, Enterobacterales, ESBL, genomics, Pakistan
INTRODUCTION

Antimicrobial resistance (AMR) is an ongoing public health problem of global dimensions. Over the last decades, multidrug-resistant (MDR) Enterobacteriales have become a serious public health issue as treatment options are minimizing day by day due to increased resistance to broad-spectrum antibiotics such as cephalosporins and carbapenems (Bevan et al., 2017; Giske et al., 2009). Mainly, carbapenems have been serving as the last-line drugs for MDR Gram-negative bacteria treatment. An increased frequency of carbapenemase production in Enterobacteriales family members has been recently reported in developing countries such as Bangladesh, Pakistan and India, where 1.9 billion people live under poor healthcare facilities (Choudhury et al., 2018; Hossain et al., 2020; Islam et al., 2012; Kumarasamy et al., 2010; Stewardson et al., 2019).

Extended-spectrum β-lactamases (ESBLs) is a rapidly emerging group of enzymes that can hydrolyze different generations of cephalosporins and carbapenems. Among different ESBLs, the production of ESBL_A and ESBL_CARBA enzymes are usually encoded by genes present on plasmids of members of the Enterobacteriales family, and alongside they can carry several genes conferring resistance to other broad-spectrum antibiotics, for example, aminoglycosides, macrolides and quinolones. The ESBL_A group, known as classical ESBL, contains several blaCTX-M- β-lactamase variants that can hydrolyze several generations of cephalosporins (e.g. third and fourth generations) (Bevan et al., 2017; Giske et al., 2009). The ESBL_CARBA group comprises different types of carbapenemase variants (blaNDM, blaKPC, blaVIM, blaOXA-48 and blaIMP) that can hydrolyze antibiotics belonging to the carbapenem class (Giske et al., 2009). Historically, the Indian subcontinent has been considered as the hotspot for NDM (New Delhi-metallo-β-lactamase [MBL])-producing Escherichia coli (Giske et al., 2009; Kumarasamy et al., 2010). Alarmingly, carbapenem and/or cephalosporin resistance determinants like blaCTX-M and blaNDM have been found in E. coli and Enterobacter species on top of other resistance mechanisms directed against fluoroquinolones and aminoglycosides (Al-Agamy et al., 2019; Huang et al., 2019).

Biocide substances like silver and quaternary ammonium compounds (benzylikonium chloride, chlorhexidine and cetylpyridinium chloride) may also affect antibiotic resistance directly by selecting for porin deficiency and thereby it mediates a beta-lactams cross-resistance (Li et al., 1997; Shafaati et al., 2016). Alarmingly, blaCTX-M- producing E. coli started to show resistance to heavy metals and biocide compounds as these compounds are frequently used in hospitals, farms and agriculture to ensure medication, hygiene and biosecurity (Adamse et al., 2017; Shafaati et al., 2016; Silver, 2003; Sütterlin et al., 2014).

The increased prevalence of carbapenem-resistant Enterobacteriales has been reported sporadically from major hospitals in Pakistan in several studies and suggests spread in clinical settings in Pakistan where patients are coming from several locations of the country (Kumarasamy et al., 2010; Nahid et al., 2013; Qamar et al., 2019; Stewardson et al., 2019). However, there is also a lack of detailed genomic epidemiology of carbapenem-resistant strains in Pakistani hospitals. Currently, little is known about the prevalence and genetic characteristics of clinical ESBL producing E. coli and Enterobacter strains co-harbouring carbapenem resistance in Pakistan. In this study, we investigated the detailed molecular and genomic epidemiology of carbapenem-resistant Enterobacteriales isolated from hospitalized patients in a tertiary care hospital of Pakistan.

MATERIALS AND METHODS

Sample collection and isolation of ESBL producers

From January to March 2016, 50 clinical samples (urine, n = 40 and wound, n = 10) were collected in an ongoing study for the screening of ESBL producing Enterobacteriales from Allied Hospital Faisalabad, Pakistan. Samples were streaked on CHROMagar-ESBL (CHROMagarCo.) and incubated overnight at 37°C. One potential ESBL-producer was selected from each plate for further investigations. Species identification of the ESBL-producers was confirmed by API 20E biochemical test (bioMérieux) followed by MALDI-TOF/MS (Bruker Daltonics) according to the manufacturer’s instruction.

The Institutional Bioethics/Biosafety Committee of the University of Agriculture, Faisalabad, has approved this study (D. No. 109/ORIC).

Antimicrobial susceptibility testing

Confirmation of the ESBL production was done by double-disc synergy test according to the CLSI guidelines (CLSI, 2016) followed by Vitek-2 compact system (AST-card GN38; bioMérieux). The Vitek-2 system was also used for the detection of additional phenotypic AMR. MDR was defined as resistance to three or more different classes of antimicrobials. Carbapenemase production and their types were determined phenotypically by MBL&KPC&OXA-48 discs kit (Liofilchem) according to the manufacturer’s instruction.
Detection of ESBL and MBL producing genes by PCR

A Maxwell® 16 Cell DNA Purification Kit (Promega) was used with the automated Maxwell 16 SEV instrument in order to get purified genomic DNA from freshly harvested bacteria. Genomic DNA extracted from both methods was centrifuged for 10 min at 13,000 rpm and the supernatant was taken to be stored at −20°C. A PCR protocol was used to detect ESBL genes blaTEM, blaSHV, and blaCTX-M (Tofteland et al., 2007). DNA sequencing was performed on blaCTX-M positive samples to determine the blaCTX-M genotype. The screening of MBL genes was also performed by various real time-PCRs for blaVIM, blaNDM, blaIMP, blaGIM, blaSPM and blaSIM (Swayne et al., 2013). ESBL-producing isolates were screened for blaOXA-48 using a method described previously (Poirot et al., 2011).

Bacterial conjugation experiments

ESBL-CARBA-producing E. coli strains were tested to check their plasmid transferability by conjugation. As donors, four blaNDM-1-producing E. coli strains were analysed for conjugation. E. coli DA11782 (mcrA, Δ(mrr-hsdRMS-mcrBC), ΔlacX74, deoR, recA1, araD139Δ ara-leu)7697, galK, rpsL, endA1, nurG, rifR) was used as the recipient. Equal amounts of donor and recipient from overnight cultures in Luria-Bertani broth were mixed and incubated without shaking overnight at 37°C. Approximately 109 colony forming units of conjugation mixture were plated on selective culture media plates containing 8 μg/ml meropenem and 100 μg/ml rifampin, and incubated overnight at 37°C.

Whole-genome sequencing and bioinformatics analysis

Enterobacter hormaechei and all blaNDM positive E. coli strains were subjected to whole-genome sequencing (WGS). Extraction of genomic DNA was performed with EZ1 Advanced XL system (QIAGEN) and fresh overnight cultures in blood agar plates were used for extraction. The extracted genomic DNA was measured using Qubit dsDNA assay kit (Life Technologies). Extracted genomic DNA was sequenced on Illumina platform (Illumina HiSeq 2500, 2 × 100 paired-end) at the Science for Life laboratory (SciLifelab), generating 2 × 100 paired-end sequences. De novo assembly was done using CLC Genomics Workbench version 12.0.3. WGS analysis was performed using open access bioinformatics web tools at Center for Genomic Epidemiology (CGE; www.genomicepidemiology.org) for detection of antibiotic resistance and biocide resistance genes, virulence genes and plasmid replicon types. Multilocus sequence typing was performed web-tools from www.enterobase.warwick.ac.uk E. coli Multilocus sequence typing (MLST) Database. Detection of metal (Mercury; mer gene) and biocide (Silver; sil) resistance genes were performed using BLASTn search from the assembled whole-genome sequence data using web-based open source platform named Galaxy (https://usegalaxy.org/). Geneious Prime (v. February 3, 2019) was used to get information about the structure of blaNDM-1 gene and the reference sequence used to annotate the blaNDM-1 contigs was KP770033. Single nucleotide polymorphisms (SNPs) in the isolates core genome were identified by Parsnp (Harvest suite v.1.0) (Treangen et al., 2014) using internal reference (EC8). All the blaNDM-1 positive E. coli isolates were analysed and compared with 41 global ST448 E. coli genomic sequences of human clinical origin taken from Enterobase database (www.enterobase.warwick.ac.uk). The phylogenetic analyses of these genomic sequences were performed with CSI-Phylogeny v 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny). The acquired antibiotic resistance genes of these isolates were analysed using ResFinder. The phylogenetic tree was visualized using interactive tree of life (iTOL v 6) (Letunic & Bork, 2016).

RESULTS

In total, 11 strains were found as ESBL positive phenotypically consisting of E. coli (n = 10) and Ent. hormaechei (n = 1). ESBL-producers were found as MDR and displayed phenotypic resistances from 4 to 6 different classes of antibiotics. All strains were resistant to ampicillin, enrofloxacin and marbofloxacin (Table 1). Only four E. coli strains were resistant to carbapenems for example, meropenem and imipenem. Phenotypic diversity of antibiotic resistance is presented in Table 1. All ESBL producing isolates were positive for blaCTX-M-1 and genes encoding for the blaCTX-M-II, blaCTX-M-III and blaCTX-M-IV groups were not detected. Sanger sequencing of blaCTX-M-1 positives PCR amplicons revealed the blaCTX-M-15 genotype. Four E. coli strains were positive for the blaNDM gene, but none of the samples were positive for blaIM, blaIMP, blaGIM, blaSPE, blaSIM and blaOXA-48. Sanger sequencing of blaNDM positives PCR amplicons revealed the blaNDM-1 genotype. Strong co-relation exists between antibiotic resistance phenotypes and genotypes among the resistant strains. One of the ESBL-producing E. coli isolates was found positive by the O25b-ST113 PCR assay. The rep-PCR analysis of all E. coli strains identified six different clonal types (A, B, C, D, E, F). All
blaNDM-1-producing *E. coli* strains belonged to type A and appeared as the dominant clonal type (Table 1). WGS analysis of *blaNDM* positive strains revealed *blaNDM-1* in addition to *blaCTX-M-15* type. All *blaNDM-1*-positive *E. coli* strains were carrying similar antibiotic resistance markers (Figure 1). Resistance markers in *E. coli* strains included β-lactams (*blaOXA-16, blaTEM-18*), carbapenems (*blaNDM-1*), quinolones (*qnrS1, aac[6’]-Ib-cr*), aminoglycosides (*aadA1, aad16, aac[3]-IIa*), tetracycline (*tetD*), macrolide (*mphA*), sulfonamides (*sul1, sul2*) and trimethoprim (*dfrA27*). Analysis of genetic environment confirmed the *blaNDM-1* gene was adjacent to the *bleMBL* gene (a belomycin resistance protein) followed by a truncated *trpF* gene downstream, on the other hand, *aadA1* conferring aminoglycoside resistance and a truncated narrow-spectrum β-lactamase, *blaOXA-10* was located in the upstream direction (Figure 1).

No silver resistance gene was found; however, several mercury resistance genes (*merC* and *merR*) were found. All *blaNDM-1*-producing *E. coli* strains carried *qacE* genes responsible for resistance to different quaternary ammonium compounds such as benzylkonium chloride, chlorhexidine, and cetylpyridinium chloride (Table S1).

All *blaNDM-1*-producing *E. coli* strains belonged to phylogenogroup B1. Characterization of *fimH* is an important typing scheme in *E. coli* as this is an adhesin-related gene that has virulence potential. All sequenced *blaNDM-1*-producing *E. coli* carried *fimH*35. In total, three different clinically associated important virulence genes were found in the *blaNDM-1*-positive *E. coli* strains: *gad* (glutamate decarboxylase), *terC* (tellurium iron resistance) and *lpfA* (long polar fimbriae) (Table S1). The wgSNP analysis of *blaNDM-1*-producing *E. coli* strains showed that EC-6, EC-7, EC-8 and EC-9 were highly closely related with SNP differences of ≤9. Phylogenetic comparison of *E. coli* ST448 with global collection of human origin ST448 showed that these strains are phylogenetically distinct (Figure 1).

MLST analysis of the *blaNDM-1*-producing strains identified them as ST448 complex (Achtman scheme), however, core-genome MLST revealed three different STs (74,416, 76,469 and 100,030) indicating genome-wide diversity. All *blaNDM-1*-producing *E. coli* strains had transferable plasmids as confirmed by the conjugation experiment. Plasmid replicon types IncX3, IncFII(Yp), and ColpVC were found in all *blaNDM-1*-producing *E. coli* isolates.

*Enterobacter hormaechei* carried resistant markers for cephalosporins (*blaCTX-M-15, β*-lactams (*blaOXA-1, *blaACT-24*), quinolones (*qnrB6, qnrB32, aac[6’]-Ib-cr*), aminoglycosides (*aac[3]-IIa*), phenicol (*catB3*), sulfonamides (*sul1*), trimethoprim (*dfrA27*) and fosfomycin (*fossA2*). On the other hand, *Ent. hormaechei* had 2 different plasmid replicon types: IncX3 and IncFIB (pHCM2).

**DISCUSSION**

During winter months complicated urinary tract and wound infections produced by the members of the Enterobacterales is considered a serious challenge in hospital settings in Pakistan because of the amount of failed treatment. Increased prevalence of ESBL-producing Enterobacterales has been reported in Pakistani hospitals.
involving mostly *E. coli* (Abrar et al., 2018). Alarmingly some of the ESBL-producing *E. coli* isolates were resistant to carbapenems, aminoglycosides and quinolones. The ESBL situation was getting worse because of the MDR features to other non-β-lactams antibiotics displayed by these pathogens, ultimately limiting the treatment options available in Pakistani hospitals. Both, hospital and non-hospital uses of antibiotics in Pakistan are not regulated but indiscriminated; some essential antibiotics like cephalosporins, carbapenems, macrolides, quinolones and aminoglycosides are extensively used regularly in clinical practice (Saleem et al., 2019). As a consequence, *E. coli* isolates from our study were resistant to these essential antibiotics indicating how overuse and misuse of essential antibiotics potentially impacted on clinical isolates.

Genetic characterization of ESBL-producing *E. coli* isolates confirmed the presence of *bla*_{CTX-M-15} genotype in all isolates. *E. coli* isolates phenotypically resistant to meropenem and imipenem carried the *bla*_{NDM-1} genotype in addition to *bla*_{CTX-M-15}. Epidemiology typing by rep-PCR confirmed all *E. coli* harbouring *bla*_{NDM-1} to belong to one clonal profile (A). The rest of the *E. coli* isolates were completely different from each other. Several reports previously confirmed the abundance of *bla*_{CTX-M-15} in clinical and environmental isolates in Pakistan (Rafaque et al., 2018; Umair et al., 2019). Also, *bla*_{NDM-1} has started to be endemic among ESBL-producing Gram negatives including *E. coli* (Qamar et al., 2019). Interestingly, the *bla*_{NDM-1} positive *E. coli* isolates were carrying other extended-spectrum β-lactams resistance genes, for example, *bla*_{OXA-10} which we reported for the first time in *E. coli* from Pakistan. The *bla*_{OXA-10} gene was reported in *Pseudomonas aeruginosa* from Pakistan and Turkey (Danel et al., 1998; Ullah et al., 2017). *E. coli* isolates carrying *bla*_{NDM-1} also carried several-resistant markers for quinolones, aminoglycosides, macrolides, biocides and antifolates, typical for multidrug resistance plasmids. Generally, *bla*_{NDM-1} producing bacteria display resistance to the members of antibiotics classes carbapenems, aminoglycosides and quinolones (Kumarasamy et al., 2010). All *bla*_{NDM-1} positive *E. coli* belonged to B1 confirming the increased clinical importance of this phylogroup.
Interestingly, which could potentially impact the clinical outcomes. Urine samples are uropathogenic virulent E. coli NDM-1 genotype in clinical isolation between plasmid and resistance markers, that is \( \text{bla}_{\text{CTX-M-15}}, \text{bla}_{\text{OXA-1}}, \text{bla}_{\text{ACT-24}}, \text{qnrB6}, \text{qnrB32}, \text{aac(6')-Ib-cr}, \text{aac(3)-IIa}, \text{catB3}, \text{sul1}, \text{dfrA27} \) and \( \text{fosA2} \). This is the first study from Pakistan reporting the presence of MDR \textit{Ent. hormaechei} isolated from the clinical samples. The ESBL-producing \textit{Ent. hormaechei} carried two different plasmid replicon types (IncX3 and IncFIB) that are strongly associated with carbapenemase-producing genes like \( \text{bla}_{\text{NDM-1}} \) and \( \text{bla}_{\text{KPC}} \).

One of the main limitations of our study is that it was conducted in only single tertiary care hospital in Faisalabad using a small sample size which may not represent the whole population. Seasonal trend may also affect the result. These limitations could be resolved in future studies of CRE in Pakistan.

**CONCLUSIONS**

Based on the findings of the present study, it can be concluded that ESBL\textsubscript{Carba} is gradually increasing in Pakistan with co-resistance to other classes of essential antibiotic classes. The \textit{E. coli} strains carrying \( \text{bla}_{\text{NDM-1}} \) and \textit{Ent. hormaechei} carrying \( \text{bla}_{\text{ACT-24}} \) are high-risk clones that are present in the hospitals of Pakistan and these strains are resistant to biocides. Antibiotic resistance is a serious challenge in Pakistani hospitals and there is no easy solution in sight.

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**CONFLICT OF INTEREST**

The authors declare no competing financial interest exists.

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SUPPORTING INFORMATION
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