Molecular Epidemiology of mcr-1, blaKPC-2, and blaNDM-1 Harboring Clinically Isolated Escherichia coli from Pakistan

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Purpose: The multiple-drug resistant Escherichia coli are among the deadliest pathogens causing life-threatening infections. This study was planned to determine the molecular epidemiology of mcr-1, blaKPC-2, and blaNDM-1 harboring clinically isolated E. coli from Pakistan.

Methods: In total, 545 strains of E. coli from clinical samples were collected from June 2018 to September 2019. All the isolates were screened for colistin-resistance, extended-spectrum-β-lactamases (ESBL), and carbapenemases through the micro-dilution method, Double-Disk-Synergy-Test (DDST), and Modified-Hodge-Test (MHT). The detection, sequence-typing, conjugal transfer, S1-PFGE, plasmid-replicon-typing, and southern-blotting for mcr, ESBL, and carbapenemase-encoding genes were performed.

Findings: A total of four (0.73%) colistin-resistant strains carrying alongside mcr-1 and blaCTX-M-15 genes, three of these strains also had the blaTEM-1 gene. The presence of ESBL genes was detected in 139 (25.5%) isolates harboring blaCTX-M-15 (74.82%), blaTEM (34.53%), blaSHV (28.06%) and blaOXA-1 (28.78%). In 129 carbapenemase-producers, 35.83% possessed blaNDM-1, 26.67% blaKPC-2, 8.3% blaOXA-48, 25% blaVIM-1, and 20.83% blaIMP1 genes. The sequence typing revealed that mcr-1 harboring isolates belonged to ST405, ST117, and ST156. Fifty percent of blaKPC-2 and 48.83% of blaNDM-1 were found on ST131 and ST1196, respectively. Two rare types of STs, ST7584, and ST8671 were also identified in this study. The mcr-1 gene was located on IncI2 (60-kb) plasmid. The blaKPC-2 was present on (140-kb) IncH12, (100-kb) IncN, (90-kb) IncN1, while blaNDM-1 was located on (70-kb) IncFIK, (140-kb) IncH12, (100-kb) IncN, (60-kb) IncA/C, and (45-kb) IncFII plasmids, which were successfully trans-conjugated. Among the plasmid types, the IncI1 carrying blaKPC-2, IncH12 harboring blaKPC-2 and blaNDM-1, and IncFIK carrying blaNDM-1 were for the first time detected in Pakistan.

Conclusion: The mcr-1, blaKPC-2, and blaNDM-1 genes finding in various clonal and plasmids types indicate that a substantial selection of the resistance genes had occurred in our clinical strains.

Keywords: colistin-resistance, extended-spectrum-β-lactamases, carbapenemases, mcr1, blaKPC-2, blaNDM-1, plasmids, E. coli

Introduction

Antimicrobial resistance is a grave concern globally, where the situation is continuously getting worse due to overuse and misuse of antibiotics. Various mechanisms of antimicrobial resistance have been established by pathogens, of which extended-spectrum-β-lactamases (ESBL) enzymes production is the most prominent one.1 ESBL enzymes were first reported in 1980. Until now, more than 350 ESBLs genes are documented, consisting of point mutated variants of TEM, SHV,
and CTX-M. Among these, the bla<sub>CTX-M15</sub> type has been frequently documented from the strains of human origin. ESBLs are prevalent worldwide. A meta-analysis in 2018 reported a 40% pooled proportion of clinically isolated ESBLs positive Enterobactericea from Pakistan.

Carbapenems are considered effective drugs against most ESBL producing pathogens, but due to their extensive practice, various resistance mechanisms have been developed in which carbapenemase production is most threatening. According to Ambler classification, carbapenemases are divided into four groups; class A, B, C, and class D. The bla<sub>KPC-2</sub> is a prominent member of class A, and class D consists of bla<sub>OXA-48</sub> like variants. Class B (MBL) is further subdivided into B1, B2, and B3; among these, B1 contains the largest number of clinically relevant acquired MBLs, like bla<sub>NDM-1</sub>, bla<sub>IMP</sub>, bla<sub>VIM</sub>, and many others. These resistance genes are mostly found on plasmids having inter-generic transmission capability. A meta-analysis in 2020 reported an overall 28% pooled proportion of clinically isolated carbapenem-resistant Gram-negative strains from Pakistan.

Colistin is considered the last optional therapy against multiple drug-resistant (MDR) pathogens. Chromosomally mediated colistin resistance has been recognized for a long time, but it is not as alarming due to their only vertical transmission. For the first time, a study in 2015 reported the mcr-1 gene on IncI2 type plasmid in Klebsiella pneumoniae and E. coli, having resistance to colistin. The mcr-1 gene produces phospho-ethanol-amine transferases that complement phospho-ethanol-amine to the phosphate group of lipopolysaccharide (LPS). Due to this, LPS becomes more cationic, and colistin loses its binding affinity. Until now, the mcr-1 gene has been identified in 47 different countries. The mcr-1 bearing E. coli was first time identified in migratory birds in Pakistan. Later on, the mcr-1 presence was also reported in clinical samples and broiler in Faisalabad, Pakistan.

To combat multiple-drugs-resistant superbugs, continuous surveillance studies and molecular typing of bacterial strains, and plasmid typing are required for the current depiction of resistance epidemiology. Aiming this, we in the current study screened 545 E. coli to determine the prevalence of colistin resistance mcr, ESBL, and carbapenemases-encoding genes in Pakistan. We performed antimicrobial susceptibility testing for all isolates. Sequence and plasmid replicon typing, S1-PFGE, and southern blotting were carried out for mcr-1, bla<sub>KPC-2</sub>, and bla<sub>NDM-1</sub> harboring isolates.

**Methods**

**Samples Collection and Species Identification**

A total of 545 clinically isolated E. coli were obtained from the microbiology laboratory of the Pakistan Institute of Medical Sciences (PIMS), located in Islamabad, the capital city of Pakistan. PIMS is a government-assisted tertiary care hospital, and Pakistan is a developing country with half of the population living below the poverty line. Therefore, patients approached to PIMS hospital is comparatively high due to their low-cost treatment services, indicating the locality’s health status.

The inclusion criteria for sampling were the entire routine processing samples having E. coli as a bacterial pathogen collected from June 2018 to September 2019 in the microbiology laboratory of PIMS Islamabad, Pakistan. According to laboratory records, the strains were isolated from stool (n=18), wound (n=73), blood (n=109), and urine (n=345) as per standard microbiological and biochemical procedures such as colony morphology, gram staining, and API 20E strip test (BioMérieux). The growths of isolated strains were evaluated on MacConkey agar and cysteine-lactose-electrolyte-deficient (CLED) media. Each strain was maintained in Luria-Bertani (LB) media for further processing. E. coli identification and confirmation were accomplished through 16S rDNA PCR amplification and sequencing, using specific primers (https://www.dovepress.com/get_supplementary_file.php?f=302687.docxTable S1, supplementary data). The sequencing results were analyzed using the EZ-Biocloud pipeline (https://www.ezbiocloud.net/).

**Antibiotics Sensitivity Testing, Carbapenemase, MBL, and ESBL Detection**

The broth microdilution method for colistin, amikacin ampicillin, cefotaxime, aztreonam, chloramphenicol, gentamycin, ciprofloxacin, cefoxitin, fosfomycin, tetracycline, and meropenem, was performed to assist antibiotic sensitivity of all collected strains. The minimum inhibitory concentrations for fosfomycin-resistant isolates were confirmed via the agar dilution method according to CLSI guidelines. The carbapenemes and ESBLs producing ability of all the isolates were determined by modified-Hodge-test (MHT) and double-desk-synergy-test (DDST). All of the MHT positive isolates were subjected to Metallo-β-lactamases (MBL)
Antibiotic Resistance Genes

The genomic DNA from all isolates was extracted using the conventional boiling method.\(^9\) The quantity and quality of extracted DNA were analyzed by one-dropTM (OD-1000+, Spectrophotometer). The \textit{mcr} genes (\textit{mcr}-1 to \textit{mcr}-9) were investigated via PCR from colistin-resistant isolates using specific primers (Table S1, supplementary data). Similarly, \textit{bla\textsubscript{CTX-M}} group 1, \textit{bla\textsubscript{SHV}}, \textit{bla\textsubscript{TEM}}, and \textit{bla\textsubscript{OXA}} variants of ESBL genes and carbapenemases producing genes (\textit{bla\textsubscript{KPC-2}}, \textit{bla\textsubscript{OXA48}}, \textit{bla\textsubscript{NDM-1}}, \textit{bla\textsubscript{IMI}}, and \textit{bla\textsubscript{VIM}}) were investigated via PCR from their respective genomic DNA using genes-specific primers. (Table S1, supplementary data). A 1.5% agarose gel was used to visualize the amplicons of expected sizes. All of the amplified genes were sequenced and blast via the NCBI blast tool for genes confirmation (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Molecular Typing

To find out the sequence type (ST) and ST complex of \textit{mcr}-1, \textit{bla\textsubscript{KPC-2}}, and \textit{bla\textsubscript{NDM-1}} harboring isolates, multi-locus-sequence-typing (MLST) were carried out. The seven alleles, \textit{adk}, \textit{fumC}, \textit{gyrB}, \textit{icd}, \textit{mdh}, \textit{purA}, and \textit{recA}, were amplified and sequenced. The sequencing results were analysed on the MLST database to find out the sequence types of our isolates (http://enterobase.warwick.ac.uk/species/index/ecoli). The phylogenetic analysis based on MLST sequence types and alleles was performed on BioNumerics (Applied math, Belgium).

Trans-Conjugation and Plasmid Replicon Typing

To identify the conjugation-ability of \textit{mcr}-1, \textit{bla\textsubscript{KPC-2}}, and \textit{bla\textsubscript{NDM-1}} bearing strains, trans-conjugation was carried out. The \textit{E. coli} EC 600 (Na\textsuperscript{+}, Rif\textsuperscript{r}) was selected as recipients, and \textit{mcr}-1, \textit{bla\textsubscript{KPC-2}}, and \textit{bla\textsubscript{NDM-1}} bearing strains were taken as donors. The sensitivity of all the donor strains was checked for rifampicin. Colistin (2 mg/L) and rifampicin (600 mg/L) were used to select \textit{mcr}-1 carrying conjutants. Similarly, meropenem (4 mg/L) and rifampicin (600 mg/L) were used to select \textit{bla\textsubscript{KPC-2}} and \textit{bla\textsubscript{NDM-1}} bearing conjutants. To run the experiment, a protocol already evaluated and published has been used.\(^2\) The conjutants were identified using antimicrobial sensitivity testing and \textit{mcr}-1, \textit{bla\textsubscript{KPC-2}}, and \textit{bla\textsubscript{NDM-1}} genes specific PCR.

To explore the incompatibility types of plasmids bearing \textit{mcr}-1, \textit{bla\textsubscript{KPC-2}}, and \textit{bla\textsubscript{NDM-1}}. PCR-based replicon typing (PBRT) was performed. For this experiment, plasmid DNA from successful transconjugants was obtained by alkaline-lysis procedure.\(^2\) The PBRT 2.0 kit (MBK0078, Diatheva, Italy) was used for plasmid incompatibility typing. It can detect thirty different plasmid incompatibility types having eight multiplex PCR reactions. The specific amplicon size for plasmid type was visualized on 2% agarose gel, and the results were interpreted according to manufacturer guidelines.

SI-PFGE and Southern Hybridization

To determine the sizes of plasmid, SI Pulse Field Gel Electrophoresis (PFGE) of the trans-conjugants were performed following the protocol.\(^2\) A total of fourteen trans-conjugants (four from \textit{mcr}-1, five from \textit{bla\textsubscript{KPC-2}}, and five from \textit{bla\textsubscript{NDM-1}}) were selected for this experiment. Southern blotting was performed to confirm the presence of genes on plasmids. The digoxin-labeled \textit{bla\textsubscript{KPC-2}}, \textit{mcr}-1, and \textit{bla\textsubscript{NDM-1}} specific probes were used for Southern blotting according to the kit’s instructions (Roche Diagnostics, Germany, Mannheim).

Genetic Context of \textit{mcr}-1, PCR Mapping

The genetic context of \textit{mcr}-1 was determined by PCR mapping following the published data of pHNSHP45 and \textit{mcr}-1 harboring IncI2 plasmid detected in healthy broiler from Pakistan.\(^10,13\) We considered seven genes specific primers for \textit{virD4}, \textit{pilN}, \textit{hp}, \textit{ParA}, \textit{mcr}-1, \textit{tnpA}, and \textit{nikB}, (Table S1 supplementary data). The extracted plasmids DNA were subjected to PCR using the genes specific primers. The amplicons were visualized on 1% agarose gel and sequenced for confirmation. The sequencing results were blasted via the NCBI blast tool.

Results

Bacterial Isolation and Antibiotic Susceptibility Profile

A total of 545 \textit{E. coli} strains were confirmed via colony morphology and 16S rDNA sequence blast. The antibiotic susceptibility testing of isolates for twelve antibiotics was performed through microdilution (Figure 1). Colistin sulphate and fosfomycin were the most susceptible drugs, with 99.27% and 93.03% susceptibility. Only four (0.73%) isolates showed resistance to colistin.
ESBL and Carbapenemases Producing Isolates

The DDST and MHT were performed for all isolates to determine the prevalence of ESBL and carbapenemases-producing strains. Among 545 tested isolates, 139 (25.5%) and 120 (22.02%) isolates were ESBL positive and carbapenemases producers, respectively, based on DDST and MHT results from interpretation. Furthermore, to determine the prevalence of MBL among MHT positive isolates, the CDT was carried out. The results revealed that 80 (66.67%) out of 120 carbapenemases producing strains were also MBL positive. The DDST, MHT, and CDT revealed that four colistin-resistant isolates were ESBL positive but not carbapenemases producers.

Antibiotic-Resistant Genes

Upon molecular confirmation of colistin resistance through PCR, a total of four (0.73%) colistin-resistant strains carrying alongside mer-1 and blaCTX-M-15 genes, three of these strains also had the blaTEM-1 gene (Table 1).

Among 139 ESBL producer strains, 74.82% (104 of 139) harbored blaCTXM-15 gene, 34.53% (48 of 139) carried blatEM gene (n=29 blatEM-1, n=19 blatEM-116), 28.78% (40 of 139) contained blaOXA-1 gene and 5.75% (8 of 139) harbored blaSHV-75 gene. The blaSHV-11, a non ESBL variant of SHV was identified in 31 isolates. In 129 carbapenemase-producers, 35.83% (43 of 129) possessed blanDM-1, 26.67% (32 of 129) blakPC-1, 8.3% (10 of 129) blaOXA-48, 25% (30 of 129) blavIM-1, and 20.83% (25 of 129) possessed blaimp-1 gene. The 51.07% (71 of 139) ESBL positive isolates were also positive for carbapenemases. Co-harboring ESBL and carbapenem-resistant genes were detected in these isolates. The prevalence of the co-occurrence of blanDM-1, blactXM-15, and blaOXA-1 were 16.9% (12 of 71), blanDM-1 and blactXM-15 were 15.49% (11 of 71), blaimp-1, blactXM-15 and blatEM-1 were 11.27% (8 of 71), blaimp-1 and blaOXA-1 were 11.27% (8 of 71), blakPC-1, blactXM-15, and blatEM were 9.86% (7 of 71), blavIM-1 and blashv were 8.45% (6 of 71), blakPC-1 and blactXM-15! were 7.04% (5 of 71), blakPC-1, blactXM-15, and blashv were 5.63% (4 of 71), blaoXA-48

![Antibiotic resistance profile of all tested E. coli isolates.](https://www.dovepress.com/)

**Figure 1** Antibiotic resistance profile of all tested E. coli isolates.
**Table 1** Antimicrobial Susceptibility Profile of mcr-1 Harbored Isolates

| Isolate ID | MIC of Antibiotics in mg/L | ESBL Genes | CRE |
|------------|----------------------------|-------------|-----|
|            | AMP | FOX | CAP | CIP | COL | FOM | AZT | GEN | AN | MEM | CTX | TET | DDST | MHT |
| PKE11      | 256 (R) | 16 (R) | 8 (I) | 4 (R) | 4 (R) | 32 (S) | 128 (R) | 4 (S) | 64 (R) | 0.064 (S) | 256 (R) | 1 (S) |         |         |
| PKE141     | 256 (R) | 32 (R) | 2 (S) | 0.032 (S) | 4 (R) | 4 (S) | 128 (R) | 16 (R) | 128 (R) | 0.032 (S) | 64 (R) | 256 (R) |         | +ve |
| PKE196     | >256 (R) | 32 (R) | 2 (S) | 1 (S) | 16 (R) | 128 (R) | 64 (R) | 128 (R) | 0.032 (S) | 64 (R) | 128 (R) |         | +ve |
| PKE051     | >256 (R) | 64 (R) | 1 (S) | 0.064 (S) | 8 (R) | 8 (S) | 256 (R) | 32 (R) | 256 (R) | 0.032 (S) | 128 (R) | 128 (R) |         | +ve |

**Abbreviations:** ID, identification; ESBL, extended-spectrum β-lactamases; MIC, minimum inhibitory concentration; MBL, metallo-β-lactamases; AMP, ampicillin; COL, colistin; CTX, cefotaxime; CAP, chloramphenicol; CIP, ciprofloxacin; FOX, cefoxitin; FOM, fosfomycin; GEN, gentamicin; AN, amikacin; TET, tetracycline; AZT, aztreonam; DDST, double disk synergy test; CDT, combined disk test; CTX-M, cephalosporin Munich β-lactamase of ESBL-A type; TEM, temoneira; β-lactamase of ESBL-A type; CR, carbapenem resistant; (R), resistant; (S), susceptible; (I), intermediate.

and blaSHV were 5.63% (4 of 71), blaVIM-1, and blaCTXM-15 were 2.82% (2 of 71), blaKPC-2, blaVIM-1 and blaTEM were 2.82% (2 of 71), blaNDM-1, blaOXA-48, and blaTEM were 2.82% (2 of 71) (Table 2).

**Molecular Typing**

The MLST was performed according to Warwick MLST Database to find out the sequence types of mcr-1, blaKPC-2, and blaNDM-1 harboring isolates. The clonality of mcr-1, blaKPC-2, and blaNDM-1 carrying isolates was assessed as a minimum spanning tree based on MLST sequence types and alleles (Figure 2). According to MLST results, the sequence types for mcr-1 harboring isolates were ST156 (n=2), ST117 (n=1), and ST405 (n=1). The sequence types of blaKPC-2 harboring isolates were ST131 (n=16), ST1196 (n=7), ST405 (n=5), and ST7584 (n=4). Likewise, the sequence types of blaNDM-1 harboring isolates were ST1196 (n=21), ST131 (n=9), ST10184 (n=6), ST7584 (n=4), ST405 (n=2) ST8671 (n=1) (Table S2, supplementary data). Overall, the MLST result indicates the diversity in clonal type for mcr-1, blaKPC-2, and blaNDM-1 harboring isolates.

**Transconjugation and PBRT**

The transconjugation experiment was performed to confirm the transferability and localization of mcr-1, blaKPC-2, and blaNDM-1 genes on plasmids. The conjugation experiments succeeded for all isolates except for five ST131 strains carrying blaKPC-2. The transconjugants were evaluated for antibiotic sensitivity testing and detection of relevant resistant genes via PCR. In mcr-1 harboring transconjugants, only the mcr-1 gene was detected; however, PCR did not detect the ESBL genes. Similarly, the transconjugants of blaKPC-2, and blaNDM-1 showed two to four-fold increases in meropenem MIC than donor strains, though remaining resistance profiles were similar to that of the donor strains (Table S3, supplementary data).

To find out the plasmids type for blaKPC-2, and blaNDM-1, and mcr-1, the PBRT was carried out from the transconjugants strains. The IncI2 type plasmid was detected in all the mcr-1 harboring isolates. The plasmids incompatibility types for blaKPC-2 harboring isolates were IncH12 (n=15), IncN (n=8), and IncI1 (n=4). For blaNDM-1 harboring isolates were IncFI1K (n=17), IncH12 (n=14), IncN (n=5), IncA/C (n=4), and IncFII (n=1) (Table 3). Among the plasmid types, the IncI1 carrying blaKPC-2, IncH12 harboring blaKPC-2, and blaNDM-1, and IncFI1K carrying blaNDM-1 were for the first time detected in Pakistan.

**S1 PFGE and Southern Hybridization**

S1 PFGE and southern-hybridization were performed for four mcr-1 transconjugants, five blaKPC-2 transconjugants (two having IncI1 plasmid, two having IncN plasmid, and one having IncH12 plasmid), and five blaNDM-1 (one from each type of plasmid). The S1 PFGE and southern-blotting results revealed that all the mcr-1 harboring transconjugants have a plasmid size near 60-kb (Figure 3). Similarly, plasmids sizes near to 90-kb (IncI1), 100-kb (IncN) and 140-kb (IncH12) were detected for blaKPC-2 (Figure 4). The plasmid sizes for blaNDM-1 were 70-kb (IncFI1K), 140-kb (IncH12), 100-kb (IncN), 60-kb (IncA/C), and 45-kb (IncFII) (Figure 5).

**mcr-1 Genetic Context, PCR Mapping**

For PCR mapping of mcr-1 genetic background, seven genes closed to mcr-1 were amplified and sequenced. Among these, six genes virA4, pilN, hp, mcr-1, nikB, and ParA showed 100% similarity with pHNSHP45 upon
Table 2 Prevalence of Solo or in Co-Existence of Antibiotic Resistance Genes Detected in This Study

| Colistin Resistance Gene | Carbapenem Resistance Genes Among 120 MHT Positive Isolates | ESBL Producing Genes Among 139 ESBL Positive Strains | Non-ESBL SHV | No. of Isolates Having Multiples Genes (Total n=545) |
|--------------------------|-------------------------------------------------------------|-----------------------------------------------------|--------------|-----------------------------------------------------|
|                          | blaqKPC. | blaqOXA. | blaqNDM. | blaqIMP | blaqVIM | blaqCTX. | blaqTEM | blaqSHV. | blaqOXA | blaqSV.11 |                          |
| +                        |           |          |          | +       | +       | +        | +       | +        | +       | +        | 3 (0.55%) |
| +                        | +        |          | +       | +       | +       | +        | +       | +        | +       | +        | 1 (0.18%) |
| +                        | +        | +       | +       | +       | +       | +        | +       | +        | +       | +        | 7 (1.28%) |
| +                        | +        | +       | +       | +       | +       | +        | +       | +        | +       | +        | 4 (0.73%) |
| +                        | +        | +       | +       | +       | +       | +        | +       | +        | +       | +        | 5 (0.92%) |
| +                        | +        | +       | +       | +       | +       | +        | +       | +        | +       | +        | 6 (1.1%)  |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 4 (0.73%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 6 (1.1%)  |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 4 (0.73%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 4 (0.73%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 7 (1.28%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 8 (1.47%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 8 (1.47%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 3 (0.55%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 7 (1.28%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 9 (1.65%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 11 (2.02%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 12 (21.8%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 3 (0.55%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 3 (0.55%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 7 (1.28%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 8 (1.47%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 8 (1.47%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 7 (1.28%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 24 (4.40%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 24 (4.40%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 10 (1.85%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 10 (1.85%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 6 (1.1%)  |
| +                        |           |          |          |          |          |          |          |          |          |          | 5 (0.92%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 8 (1.47%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 3 (0.55%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 5 (0.92%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 8 (1.47%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 4 (0.73%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 6 (1.1%)  |
| +                        |           |          |          |          |          |          |          |          |          |          | 2 (0.37%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 4 (0.73%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 1 (0.18%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 7 (1.28%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 12 (21.8%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 9 (1.65%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 11 (2.02%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 24 (4.40%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 9 (1.65%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 10 (1.85%) |
| +                        |           |          |          |          |          |          |          |          |          |          | 10 (1.85%) |

Notes: Cell having (+) represent presence of particular gene in each cell. Empty cell represent the absence of particular gene. The underneath row represent the percentage of each gene.

sequence alignment. Though, the amplicon size of the tnpA gene was unexpected (Figure 6). New primers for tnpA (having no IsApl1 loci) were designed to clear this ambiguity. The PCR result and sequencing for new tnpA primers revealed that IsApl1 is absent in our isolates.

Discussion
Antimicrobial-resistance is a global matter of concern, but developing countries like Pakistan, where there are poor hygienic conditions and deprived clinical structures, are at risk. The present study was designed to determine the
prevalence of plasmid-mediated colistin resistance, ESBL, and carbapenemases producing genes in clinically isolated E. coli from Pakistan. The molecular epidemiology of mcr-1, blaKPC-2, and blaNDM-1 was determined.

In the present study, the prevalence of ESBL was 25%, which agreed with previous studies from Pakistan that reported 23.56% and 23.91% ESBL positive isolates. However, some other studies from Pakistan reported different results that are 53.4% and 87.50%. The contradictions in results might be due to different phenotypic detection approaches. The occurrence of ESBL producing genes in the current study was 74.82% for blaCTXM-15. The blaCTXM-15 is a worldwide dominated genotype in clinically isolated ESBL producing strain. Some other studies

Table 3 Number of mcr-1, blaKPC-2, and blaNDM-1 Harboring Plasmids Incompatibility Type Prevalent on Various Sequence Types

| Plasmid Type | Plasmid Sizes | ST       | ST Cplx      | Resistant Gene | No. of Isolates |
|--------------|---------------|----------|--------------|----------------|-----------------|
| IncI2        | ~60-kb        | ST405    | ST405 Cplx   | mcr-1          | ¼ (25%)         |
| IncI2        | ~60-kb        | ST117    | ST23Cplx     | mcr-1          | ¼ (25%)         |
| IncI2        | ~60-kb        | ST156    | ST156Cplx    | mcr-1          | 2/4 (50%)       |
| IncH12       | ~140-kb       | ST 131   | ST131 Cplx   | blaKPC-2       | 11/32 (34.38%)  |
| IncN         | ~100-kb       | ST 1196  | None         | blaKPC-2       | 5/32 (15.63%)   |
| IncIa        | ~90-kb        | ST 7584  | None         | blaKPC-2       | 4/32 (12.5%)    |
| IncN         | ~100-kb       | ST 405   | ST405 Cplx   | blaKPC-2       | 3/32 (9.37%)    |
| IncH12       | ~140-kb       | ST 1196  | None         | blaKPC-2       | 2/32 (6.25%)    |
| IncH12       | ~140-kb       | ST 405   | ST405 Cplx   | blaKPC-2       | 2/32 (6.25%)    |
| IncFlik      | ~70-kb        | ST 1196  | None         | blaNDM-1       | 17/43 (39.53%)  |
| IncH12       | ~140-kb       | ST 131   | ST131 Cplx   | blaNDM-1       | 9/43 (20.93%)   |
| IncH2        | ~140-kb       | ST 10,184| ST69 Cplx    | blaNDM-1       | 5/43 (11.63%)   |
| IncN         | ~100-kb       | ST 1196  | None         | blaNDM-1       | 4/43 (9.30%)    |
| IncN         | ~100-kb       | ST 8671  | None         | blaNDM-1       | 1/43 (2.32%)    |
| IncA/C       | ~60-kb        | ST 7584  | None         | blaNDM-1       | 4/43 (9.3%)     |
| IncN         | ~100-kb       | ST 405   | ST405 Cplx   | blaNDM-1       | 2/43 (4.65%)    |
| IncFli       | ~45-kb        | ST 10,184| ST69 Cplx    | blaNDM-1       | 1/43 (2.33%)    |

Note: 5 ST131 blaKPC-2 strains plasmid were not detected.
Abbreviations: kb, kilo base; ST, sequence type; ST Cplx, sequence type complex.
Figure 3 S1 PFGE and Southern blot of mcr-1 harboring strains. H9812 is a molecular size marker (1135 to 20.5-kb). PKE211, PKE196, and PKE14, PKE051 are the isolates; digested with S1 enzymes. All isolates show 60-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.

Figure 4 S1 PFGE and Southern blot of bla_KPC-2 harboring strains. H9812 is a molecular size marker. PKE003 and PKE091 isolates have 90-kb plasmid, PKE201 isolate has 140-kb plasmid, and PKE470 and PKE482 isolates have 100-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.
from Pakistan indicated 72% and 76% bla\textsubscript{CTXM-15} harbored isolates.\textsuperscript{4,25} We reported less prevailing rate for bla\textsubscript{TEM}, bla\textsubscript{SHV}, and bla\textsubscript{OXA} genes than bla\textsubscript{CTXM}. A study from Qatar stated that the bla\textsubscript{CTXM} is supposed to be evolved through mutations in bla\textsubscript{SHV} and bla\textsubscript{TEM} genes in the current endemic.\textsuperscript{26} The cumulative prevalence of carbapenemases producers was 22.02%, of which 13.03% were also ESBL positive,
much higher than a formerly reported 1.9–2.4% prevalence of carbapenem resistance in Asian countries.\textsuperscript{27} However, some other studies from Pakistan reported a 20.3% and 56% prevalence of carbapenem-resistant isolates.\textsuperscript{24,28} The difference between the prevalence ratios might be due to differences in sample size, source type, and studies’ objectives.\textsuperscript{24} Among the carbapenem-resistant genes, 35.83% and 26.6% isolates had blaNDM-1 and blaKPC-2 genes, 25 and 18 isolates among them, co-harbored ESBL genes, respectively. Similarly, the co-existence of other MBL genes like blaIMP and blaVIM with ESBL genes (especially with blaCTXM-15) has been detected in this study. The co-existence of MBL and ESBL genes had also been detected in another study lastly reported from Pakistan.\textsuperscript{28} Scientifically, there is no significant association between ESBL and carbapenemase production.\textsuperscript{29} The co-occurrence of MBL (blaNDM-1, blaIMP, blaVIM) and ESBL (blaCTXM-15, blaTEM) genes in our strains shows the emergence of various variants of β-lactamases in these clinical isolates. It proposes that carbapenem resistance among our strains is due to carbapenemases besides ESBL’s overproduction, as stated by another study.\textsuperscript{30}

A very little data about the clonal diversity of carbapenemases producing isolates are available from Pakistan. Therefore, we performed the MLST of blaKPC-2, and blaNDM-1 harboring isolates. The ST131 and ST1196 are the most prevalent sequence types for blaKPC-2, and blaNDM-1 detected in the current study. ST131 exhibits multiple virulences and antibiotic resistance genes considering public health threats globally.\textsuperscript{31} The findings of ST1196 carrying the blaKPC-2 and blaNDM-1 genes are reported for the first time in the current study. However, a study from Myanmar detected other carbapenemase-encoding gene (blaNDM-5) on ST1196.\textsuperscript{32} The finding of ST405 carrying blaKPC-2 and blaNDM-1 in the present study seem approved by some other studies reported from Pakistan, China, and Saudi Arabia.\textsuperscript{33–35} Two rare types of STs, ST7584 (in association with blaKPC-2 and blaNDM-1) and ST10184 (blaNDM-1 harboring), are identified in this study.

All the blaKPC-2 and blaNDM-1 harboring isolates have successfully trans-conjugated except five ST131 blaKPC-2 carrying strains. The failure of blaKPC-2 conjugation in these strains might be due to its localization on the chromosome. A study from China recently determined the occurrence of blaKPC-2 on the chromosomal DNA of ST131 \textit{E. coli}.\textsuperscript{36} Various plasmid incompatibility types are detected in the present study in which IncHI2 was prominent for blaKPC-2 and IncFIK for blaNDM-1. The association of carbapenemase-encoding genes in an association of these plasmids is detected from various world regions.\textsuperscript{37,38} Previous studies from Pakistan reported IncN, IncA/C, IncL/M, IncH1, IncFII, and IncR type plasmids in association with carbapenem-resistant genes.\textsuperscript{39–41} Other plasmid types detected in this study are IncN, IncI1, IncA/C, and IncFII. The dissemination of carbapenem-resistant genes through diverse plasmids and clone types is a matter of concern, indicating a substantial selection of resistance genes in our locality’s clinical strains.

This study identified the first mcr-1 bearing IncI2 plasmid in clinically isolated \textit{E. coli} from Islamabad, Pakistan. Four (0.73%) isolates harbored the mcr-1 gene out of 545 tested isolates in our study. To date, only two studies reported mcr-1 harboring clinically isolated \textit{E. coli} from Pakistan. In Peshawar, Pakistan, a study reported 5% mcr-1 out of 120 multiple drug-resistant \textit{E. coli}.\textsuperscript{42} Similarly, a study from Faisalabad, Pakistan, detected one mcr-1 bearing \textit{E. coli} out of 29 ESBL producing strains.\textsuperscript{15} The two earlier reported studies and our current study are from different geographical regions in Pakistan. The nation-wide surveillance might be present the best depiction of mcr-1 prevalence in Pakistan. The mcr-1 harboring isolates in our study were ESBL positive and MDR. The blaCTXM-15 gene was detected in all of these, while three had the blaTEM-1 gene. The co-occurrence of ESBL and mcr-1 has been frequently reported.\textsuperscript{43–45} The researchers propose an evolutionary association between mcr-1 and ESBL since 1980. However, this argument requires additional validation by searching of mcr-1 gene in archived ESBL strains, which might be offer some evidence about the kinetics between mcr-1 and ESBLs.\textsuperscript{46} Another research recommends that the co-harboring of blaCTXM-15 and mcr-1 is maybe due to complex genetic selections that happened under the antibiotics pressure.\textsuperscript{47}

Diverse sequence types of mcr-1 harboring \textit{E. coli} ST156 (n=2), ST405 (n=1), and ST117 (n=1) are detected in this study. The sequence type’s diversity suggests multi-clonal dissemination of colistin resistance mcr-1 from 2018 to 2019 in Pakistan. The ST156 and ST405 having mcr-1 are for the first time reported in the present study from Pakistan. The ST156 and ST405 having blaNDM-1 are previously detected from chicken meat and clinical isolates.\textsuperscript{33,48} ST156 and ST405 bearing mcr-1 have been detected in other countries like ST156 in Brazil and China and ST405 in Algeria and United States.\textsuperscript{49–52} ST156 and ST405 belong to avian-pathogenic \textit{E. coli} lineage and extra-intestinal pathogenic \textit{E. coli}. They are predominantly involved in the worldwide spread of blaCTXM-15 type
ESBL.\textsuperscript{49,51} Recently, a study from Pakistan detected mcr-1 bearing ST117 in chickens.\textsuperscript{53} ST117 are from avian pathogenic \textit{E. coli} lineage and may form a reservoir for antibiotic resistance as a human extra-intestinal pathogenic \textit{E. coli}.\textsuperscript{54} The dissemination of mcr-1 in extra-intestinal pathogenic and avian pathogenic \textit{E. coli} in samples of human origin suggests food chain transmission of mcr-1.\textsuperscript{55}

In this study, the colistin resistance isolates harbor a 60-kb IncI2 plasmid, having transferability. The PCR results of mcr-1 transconjugants revealed that the IncI2 plasmids harbor only the mcr-1 gene, and the ESBLs genes (bla\textsubscript{CTXM15} and bla\textsubscript{TEM}) were not detected in the transconjugants. Another study from Pakistan reported a similar scenario for IncI2 plasmid having only mcr-1 gene in ST155 isolated from a healthy broiler. The previously reported ST155 incI2 plasmid from a healthy broiler had a similar genetic context to pHNSHP45, except for the absence of \textit{IsaP}l insertion sequence.\textsuperscript{13} Similarly, in our isolates, the PCR mapping of mcr-1 genetic background showed similarity with pHNSHP45, except the missing \textit{IsaP}l, due to truncated \textit{tpd}A gene. Usually, the IncX4 type plasmid lacks \textit{IsaP}l, while in IncI2 type plasmid, it either present or absent.\textsuperscript{11} The \textit{IsaP}l missing might be due to its quick mobilization of the mcr-1 gene into a new plasmid and self-remaining in the parental plasmid.\textsuperscript{56} Alternatively, in the new plasmids, due to missing \textit{IsaP}l, the mcr-1 mobilization decreases and strengthens its firmness in the plasmid.\textsuperscript{57}

The detection of mcr-1 on ST117 and similar plasmid features with a previously reported plasmid from healthy broilers suggest that poultry-origin mcr-1 harboring IncI2 plasmid is circulating in Pakistan.\textsuperscript{13} In Pakistan’s poultry farms, colistin solely or in combination with other drugs is widely used to treat clostridial enteritis and colibacillosis.\textsuperscript{58} This extensive use emerge plasmid-mediated colistin-resistant bacteria in the broilers, transferring via the food web into human and horizontally to other bacterial species.\textsuperscript{13} If this practice continues at the same rate, the widespread colistin resistance, and post-antibiotic era will arrive. The guidelines of antibiotics use for human well-being, and animal husbandry should be followed to reduce the hazard of antibiotic resistance.

**Conclusion**

In this study, we report the prevalence of mcr-1, ESBL, and carbapenemase-encoding genes in clinically isolated \textit{E. coli} collected from PIMS hospital Islamabad, Pakistan. The isolates were belonging to diverse clonal and plasmid types. The rare clonal types were identified for \textit{bla}_{NDM-1} and \textit{bla}_{KPC-2}. This indicates that strong selection regarding the resistance genes had occurred in our clinical strains. Moreover, the mcr-1 gene is of the avian pathogenic \textit{E. coli} lineage. Sequence typing and plasmid analysis of mcr-1 suggests its dissemination via horizontal transfer and food chain. Implementation of antibiotics guidelines for animal farming and human well-being are must, to tackle antibiotic resistance at appropriate levels.

**Ethical Approval**

This study was approved by Institutional review board of Anhui University; the ethical approval number is 2020KYNO. 19.

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**Author Contributions**

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, took part in drafting, revising, gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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The authors report no conflicts of interest in this work.

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