

Molecular Epidemiology of *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-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*, *bla_{KPC-2}*, and *bla_{NDM-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 *bla_{CTX-M-15}* genes, three of these strains also had the *bla_{TEM-1}* gene. The presence of ESBL genes was detected in 139 (25.5%) isolates harboring *bla_{CTXM-15}* (74.82%), *bla_{TEM}* (34.53%), *bla_{SHV}* (28.06%) and *bla_{OXA-1}* (28.78%). In 129 carbapenemase-producers, 35.83% possessed *bla_{NDM-1}*, 26.67% *bla_{KPC-2}*, 8.3% *bla_{OXA-48}*, 25% *bla_{VIM-1}*, and 20.83% *bla_{IMP-1}* genes. The sequence typing revealed that *mcr-1* harboring isolates belonged to ST405, ST117, and ST156. Fifty percent of *bla_{KPC-2}* and 48.83% of *bla_{NDM-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 *bla_{KPC-2}* was present on (140-kb) IncH12, (100-kb) IncN, (90-kb) IncI1, while *bla_{NDM-1}* was located on (70-kb) IncFIIK, (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 *bla_{KPC-2}*, IncH12 harboring *bla_{KPC-2}* and *bla_{NDM-1}*, and IncFIIK carrying *bla_{NDM-1}* were for the first time detected in Pakistan.

Conclusion: The *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-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*, *bla_{KPC-2}*, *bla_{NDM-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,

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and CTX-M.² Among these, the *bla*_{CTX-M15} type has been frequently documented from the strains of human origin.³ ESBLs are prevalent worldwide. A meta-analysis in 2018 reported a 40% pooled proportion of clinically isolated ESBLs positive Enterobacteriaceae from Pakistan.⁴

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.⁵ According to Ambler classification, carbapenemases are divided into four groups; class A, B, C, and class D. The *bla*_{KPC-2} is a prominent member of class A, and class D consists of *bla*_{OXA-48} 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*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM}, and many others.⁶ 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.⁷

Colistin is considered the last optional therapy against multiple drug-resistant (MDR) pathogens.⁸ Chromosomally mediated colistin resistance has been recognized for a long time, but it is not as alarming due to their only vertical transmission.⁹ 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.¹⁰ Until now, the *mcr-1* gene has been identified in 47 different countries.¹¹ 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.¹²⁻¹⁶

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 carbapenemase-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*_{KPC-2}, and *bla*_{NDM-1} 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).¹⁷ 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 (www.dovepress.com/get-supplementary-file.php?f=302687.docx Table 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, ceftazidime, 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 carbapenemases and ESBLs producing ability of all the isolates were determined by modified-Hodge-test (MHT) and double-disk-synergy-test (DDST). All of the MHT positive isolates were subjected to Metallo- β -lactamases (MBL)

detection via a combined-disc-test (CDT). CLSI guidelines were used for results interpretations.¹⁸

Antibiotic Resistance Genes

The genomic DNA from all isolates was extracted using the conventional boiling method.¹⁹ The quantity and quality of extracted DNA were analyzed by one drop™ (OD-1000+, Spectrophotometer). The *mcr* genes (*mcr-1* to *mcr-9*) were investigated via PCR from colistin-resistant isolates using specific primers (Table S1, supplementary data). Similarly, *bla*_{CTX-M} group 1, *bla*_{SHV}, *bla*_{TEM}, and *bla*_{OXA} variants of ESBL genes and carbapenemases producing genes (*bla*_{KPC-2}, *bla*_{OXA-48}, *bla*_{NDM-1}, *bla*_{IMB}, and *bla*_{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 *mcr-1*, *bla*_{KPC-2}, and *bla*_{NDM-1} harboring isolates, multi-locus-sequence-typing (MLST) were carried out. The seven alleles, *adh*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *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 *mcr-1*, *bla*_{KPC-2}, and *bla*_{NDM-1} bearing strains, trans-conjugation was carried out. The *E. coli* EC 600 (Nal^R, Rif^R) was selected as recipients, and *mcr-1*, *bla*_{KPC-2}, and *bla*_{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 *mcr-1* carrying conjugants. Similarly, meropenem (4 mg/L) and rifampicin (600 mg/L) were used to select *bla*_{KPC-2} and *bla*_{NDM-1} bearing conjugants. To run the experiment, a protocol already evaluated and published has been used.² The conjugants were identified using antimicrobial-sensitivity testing and *mcr-1*, *bla*_{KPC-2}, and *bla*_{NDM-1} genes specific PCR.

To explore the incompatibility types of plasmids bearing *mcr-1*, *bla*_{KPC-2}, and *bla*_{NDM-1}, PCR-based replicon typing (PBRT) was performed. For this experiment, plasmid DNA from successful transconjugants was obtained by alkaline-lysis procedure.²⁰ 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.

S1-PFGE and Southern Hybridization

To determine the sizes of plasmid, S1 Pulse Field Gel Electrophoresis (PFGE) of the trans-conjugants were performed following the protocol.² A total of fourteen trans-conjugants (four from *mcr-1*, five from *bla*_{KPC-2}, and five from *bla*_{NDM-1}) were selected for this experiment. Southern blotting was performed to confirm the presence of genes on plasmids. The digoxin-labeled *bla*_{KPC-2}, *mcr-1*, and *bla*_{NDM-1} specific probes were used for Southern blotting according to the kit's instructions (Roche Diagnostics, Germany, Mannheim).

Genetic Context of *mcr-1*, PCR Mapping

The genetic context of *mcr-1* was determined by PCR mapping following the published data of pHNSHP45 and *mcr-1* harboring IncI2 plasmid detected in healthy broiler from Pakistan.^{10,13} We considered seven genes specific primers for *virD4*, *pilN*, *hp*, *ParA*, *mcr-1*, *tnpA*, and *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 *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.

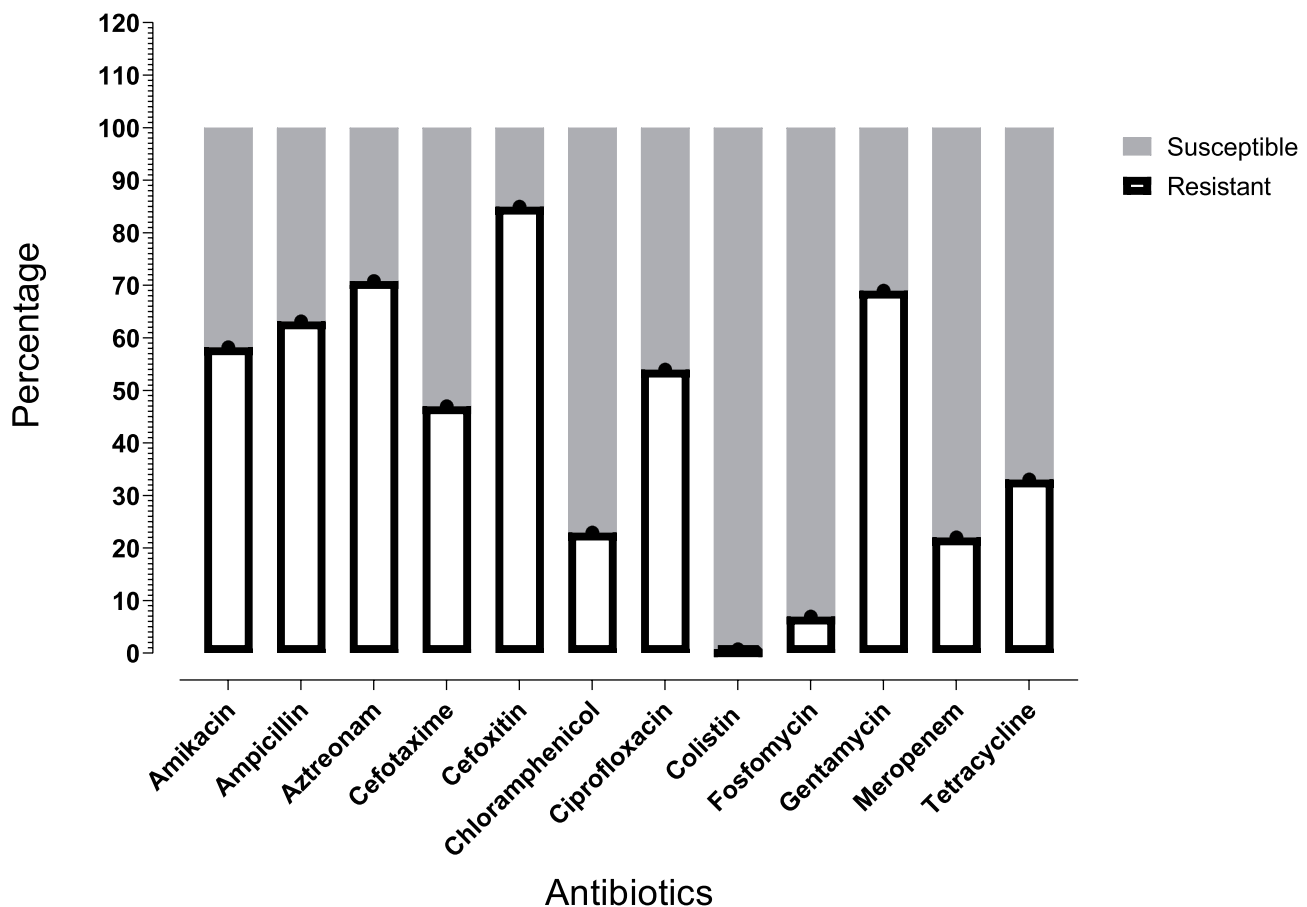


Figure 1 Antibiotic resistance profile of all tested *E. coli* isolates.

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 *mcr-1* and *bla_{CTX-M-15}* genes, three of these strains also had the *bla_{TEM-1}* gene (Table 1).

Among 139 ESBL producer strains, 74.82% (104 of 139) harbored *bla_{CTXM-15}* gene, 34.53% (48 of 139) carried *bla_{TEM}* gene (n=29 *bla_{TEM-1}*, n=19 *bla_{TEM-116}*), 28.78% (40 of 139) contained *bla_{OXA-1}* gene and 5.75% (8 of 139) harbored *bla_{SHV-75}* gene. The *bla_{SHV-11}*, a non ESBL variant of SHV was identified in 31 isolates. In 129 carbapenemase-producers, 35.83% (43 of 129) possessed *bla_{NDM-1}*, 26.67% (32 of 129) *bla_{KPC-2}*, 8.3% (10 of 129) *bla_{OXA-48}*, 25% (30 of 129) *bla_{VIM-1}*, and 20.83% (25 of 129) possessed *bla_{IMP-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 *bla_{NDM-1}*, *bla_{CTXM-15}*, and *bla_{OXA-1}* were 16.9% (12 of 71), *bla_{NDM-1}* and *bla_{CTXM-15}* were 15.49% (11 of 71), *bla_{IMP-1}*, *bla_{CTXM-15}*, and *bla_{TEM-1}* were 11.27% (8 of 71), *bla_{IMP-1}* and *bla_{OXA-1}* were 11.27% (8 of 71), *bla_{KPC-2}*, *bla_{CTXM-15}*, and *bla_{TEM}* were 9.86% (7 of 71), *bla_{VIM-1}* and *bla_{SHV}* were 8.45% (6 of 71), *bla_{KPC-2}* and *bla_{CTXM-15}* were 7.04% (5 of 71), *bla_{KPC-2}*, *bla_{TEM}*, and *bla_{SHV}* were 5.63% (4 of 71), *bla_{OXA-48}*

Table 1 Antimicrobial Susceptibility Profile of *mcr-1* Harbored Isolates

Isolate ID	MIC of Antibiotics in mg/L												ESBL	ESBL Genes	CRE
	AMP	FOX	CAP	CIP	COL	FOM	AZT	GEN	AN	MEM	CTX	TET	DDST		MHT
PKE211	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)	+ve	CTXM-15/TEM-1	-ve
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	CTXM-15	-ve
PKE196	>256 (R)	32 (R)	2 (S)	1 (S)	16 (R)	128 (I)	128 (R)	64 (R)	128 (R)	0.032 (S)	64 (R)	128 (R)	+ve	CTXM-15 TEM-1	-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	CTXM-15 TEM-1	-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, gentamycin; An, amikacin; TET, tetracycline; AZT, aztreonam; DDST, double desk synergy test; CDT, combined desk test; CTX-M, cefotaximase Munich β -lactamase of ESBL-A type; TEM, temoneira, β -lactamase of ESBL-A type; CR, carbapenem resistant; (R), resistant; (S), susceptible; (I), moderate resistant.

and *bla_{SHV}* were 5.63% (4 of 71), *bla_{VIM-1}* and *bla_{CTXM-15}* were 2.82% (2 of 71), *bla_{KPC-2}*, *bla_{VIM-1}* and *bla_{TEM}* were 2.82% (2 of 71), *bla_{NDM-1}*, *bla_{OXA-48}*, and *bla_{TEM}* 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*, *bla_{KPC-2}*, and *bla_{NDM-1}* harboring isolates. The clonality of *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-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 *bla_{KPC-2}* harboring isolates were ST131 (n=16), ST1196 (n=7), ST405 (n=5), and ST7584 (n=4). Likewise, the sequence types of *bla_{NDM-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*, *bla_{KPC-2}*, and *bla_{NDM-1}* harboring isolates.

Transconjugation and PBRT

The transconjugation experiment was performed to confirm the transferability and localization of *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-1}* genes on plasmids. The conjugation experiments succeeded for all isolates except for five ST131 strains carrying *bla_{KPC-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 *bla_{KPC-2}*, and *bla_{NDM-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 *bla_{KPC-2}*, and *bla_{NDM-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 *bla_{KPC-2}* harboring isolates were IncH12 (n=15), IncN (n=8), and IncI1 (n=4). For *bla_{NDM-1}* harboring isolates were IncFIIK (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 *bla_{KPC-2}*, IncH12 harboring *bla_{KPC-2}*, and *bla_{NDM-1}*, and IncFIIK carrying *bla_{NDM-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 *bla_{KPC-2}* transconjugants (two having IncI1 plasmid, two having IncN plasmid, and one having IncH12 plasmid), and five *bla_{NDM-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 *bla_{KPC-2}* (Figure 4). The plasmid sizes for *bla_{NDM-1}* were 70-kb (IncFIIK), 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 *virD4*, *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)
	<i>mcr-1</i>	<i>bla_{KPC}</i> 2	<i>bla_{OXA}</i> 48	<i>bla_{NDM}</i> 1	<i>bla_{IMP}</i>	<i>bla_{VIM}</i>	<i>bla_{CTX-M}</i>	<i>bla_{TEM}</i>	<i>bla_{SHV}</i> 75		
+						+	+				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%)
			+			+			+		12 (21.8%)
			+			+					11 (2.02%)
			+		+						3 (0.55%)
			+	+							1 (0.18%)
			+		+				+		1 (0.18%)
			+	+							3 (0.55%)
			+								7 (1.28%)
				+		+	+				8 (1.47%)
				+					+		8 (1.47%)
				+	+						2 (0.37%)
				+							3 (0.55%)
					+	+					2 (0.37%)
					+						7 (1.28%)
						+					24 (4.40%)
						+	+				9 (1.65%)
						+	+		+		3 (0.55%)
						+			+		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%)	32 (26.6%)	10 (8.3%)	43 (35.8%)	25 (20.8%)	30 (25%)	104 (74.8%)	48 (34.5%)	8 (5.75%)	40 (28.7%)	31 (5.69%)	<< No. of Isolates having Particular gene

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 *IsAplI* loci) were designed to clear this ambiguity. The PCR result and sequencing for new *tnpA* primers revealed that *IsAplI* 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

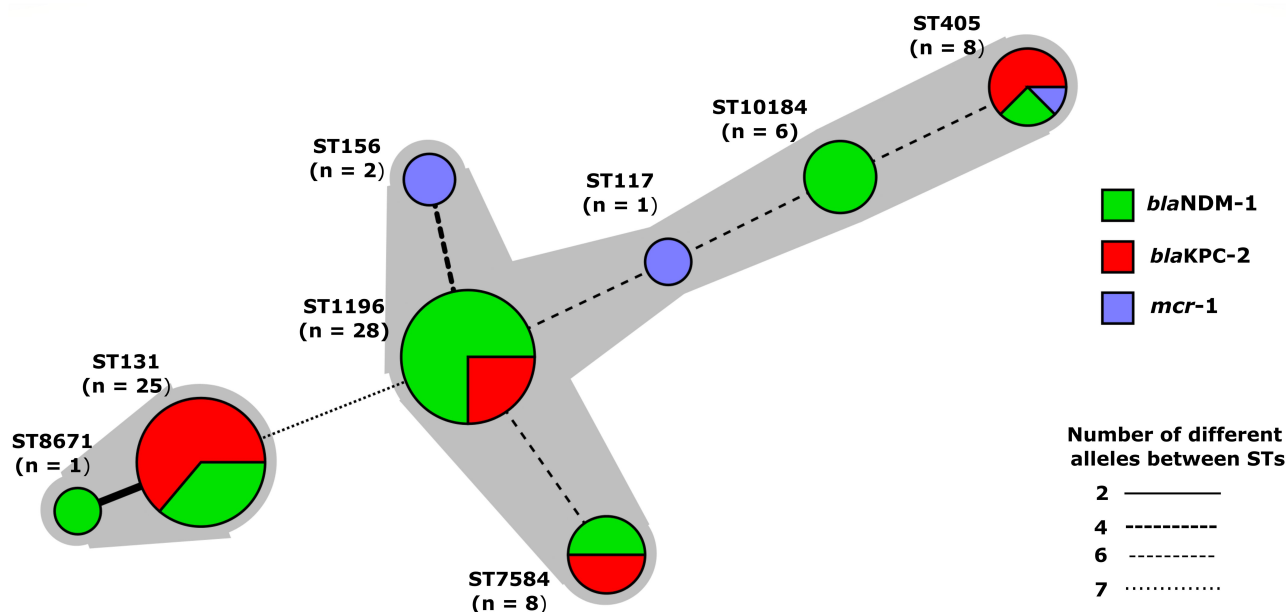


Figure 2 Minimum spanning tree of *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-1}* harboring *E. coli* by MLST type and alleles. Each node represent a sequence type, size of node represent the number of isolates, length of branches represent number of different alleles out of seven MLST alleles. Nodes are labeled with corresponding sequence type.

prevalence of plasmid-mediated colistin resistance, ESBL, and carbapenemases producing genes in clinically isolated *E. coli* from Pakistan. The molecular epidemiology of *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-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.^{21,22}

However, some other studies from Pakistan reported different results that are 53.4% and 87.50%.^{23,24} The contradictions in results might be due to different phenotypic detection approaches.⁴ The occurrence of ESBL producing genes in the current study was 74.82% for *bla_{CTXM-15}*. The *bla_{CTXM-15}* is a worldwide dominated genotype in clinically isolated ESBL producing strain.³ Some other studies

Table 3 Number of *mcr-1*, *bla_{KPC-2}*, and *bla_{NDM-1}* Harboring Plasmids Incompatibility Type Prevalent on Various Sequence Types

Plasmid Type	Plasmid Sizes	ST	ST Cplx	Resistant Gene	No. of Isolates
Incl2	~ 60-kb	ST405	ST405 Cplx	<i>mcr-1</i>	¼ (25%)
Incl2	~ 60-kb	ST117	ST23Cplx	<i>mcr-1</i>	¼ (25%)
Incl2	~ 60-kb	ST156	ST156Cplx	<i>mcr-1</i>	2/4 (50%)
IncH12	~ 140-kb	ST 131	ST131 Cplx	<i>bla_{KPC-2}</i>	11/32 (34.38%)
IncN	~ 100-kb	ST 1196	None	<i>bla_{KPC-2}</i>	5/32 (15.63%)
Incl1α	~ 90-kb	ST 7584	None	<i>bla_{KPC-2}</i>	4/32 (12.5%)
IncN	~ 100-kb	ST 405	ST405 Cplx	<i>bla_{KPC-2}</i>	3/32 (9.37%)
IncH12	~ 140-kb	ST 1196	None	<i>bla_{KPC-2}</i>	2/32 (6.25%)
IncH12	~ 140-kb	ST 405	ST405 Cplx	<i>bla_{KPC-2}</i>	2/32 (6.25%)
IncFIIK	~ 70-kb	ST 1196	None	<i>bla_{NDM-1}</i>	17/43 (39.53%)
IncH12	~ 140-kb	ST 131	ST131 Cplx	<i>bla_{NDM-1}</i>	9/43 (20.93%)
IncH12	~ 140-kb	ST 10,184	ST69 Cplx	<i>bla_{NDM-1}</i>	5/43 (11.63%)
IncN	~ 100-kb	ST 1196	None	<i>bla_{NDM-1}</i>	4/43 (9.30%)
IncN	~ 100-kb	ST 8671	None	<i>bla_{NDM-1}</i>	1/43 (2.32%)
IncA/C	~ 60-kb	ST 7584	None	<i>bla_{NDM-1}</i>	4/43 (9.3%)
IncN	~ 100-kb	ST 405	ST405 Cplx	<i>bla_{NDM-1}</i>	2/43 (4.65%)
IncFII	~ 45-kb	ST 10,184	ST69 Cplx	<i>bla_{NDM-1}</i>	1/43 (2.33%)

Note: 5 ST131 *bla_{KPC-2}* strains plasmid were not detected.

Abbreviations: kb, kilo base; ST, sequence type; ST Cplx, sequence type complex.

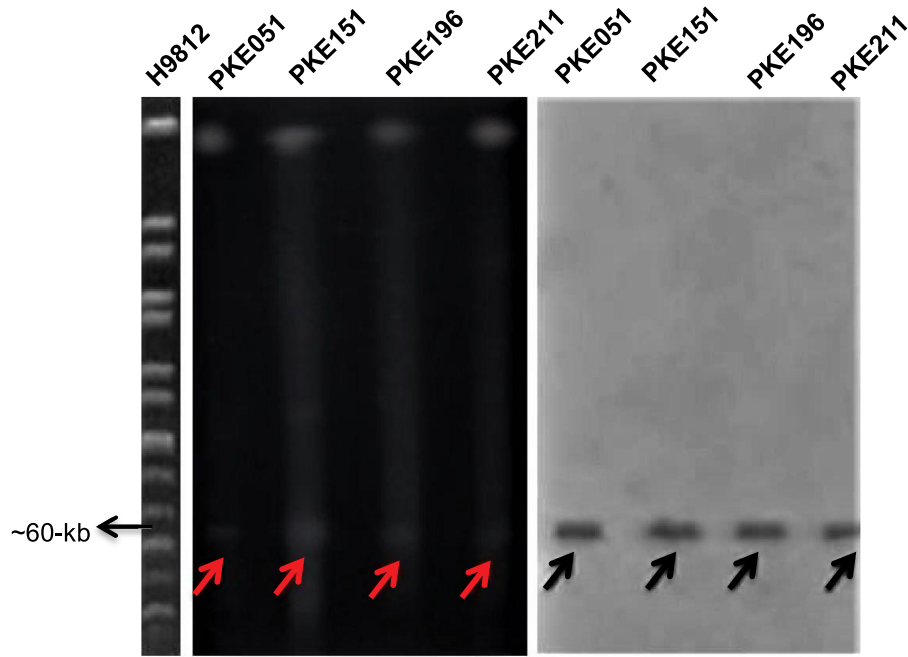


Figure 3 SI PFGE and Southern blot of *mcr-1* harboring strains. H9812 is a molecular size marker (1135 to 20.5-kb). PKE211, PKE196, and PKE151, PKE051 are the isolates; digested with SI enzymes. All isolates show 60-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.

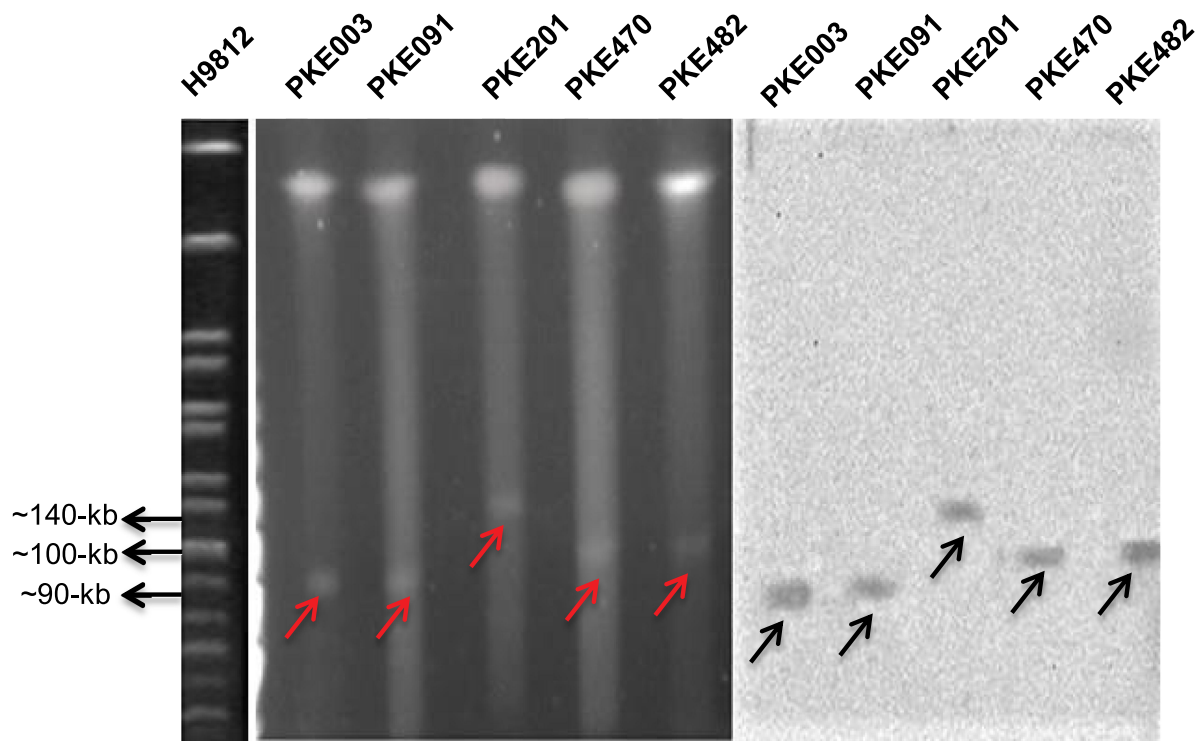


Figure 4 SI PFGE and Southern blot of *bla_{KPC-2}* harboring strains. H9812 is a molecular size marker. PKE003 and PKE091 isolates have 90-kb plasmid, PKE201 isolate has 140-kb plasmid, and PKE470 and PKE482 isolates have 100-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.

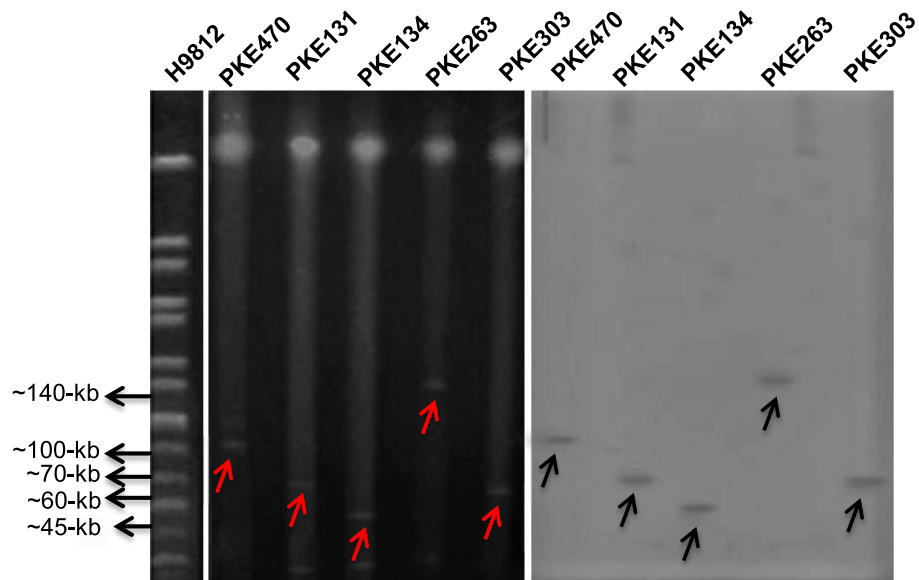


Figure 5 SI PFGE and Southern blot of *bla_{NDM-1}* harboring strains. H9812 is a molecular size marker. PKE470 isolate have 100-kb plasmid, PKE131 isolate have 70-kb plasmid, PKE134 isolate have 45-kb plasmid, PKE263 isolate have 140-kb plasmid, and PKE303 isolate have 60-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.

from Pakistan indicated 72% and 76% *bla_{CTXM-15}* harbored isolates.^{4,25} We reported less prevailing rate for *bla_{TEM}*, *bla_{SHV}*, and *bla_{OXA}* genes than *bla_{CTXM}*. A study from Qatar stated that the *bla_{CTXM}* is supposed to be

evolved through mutations in *bla_{SHV}* and *bla_{TEM}* genes in the current endemic.²⁶

The cumulative prevalence of carbapenemases producers was 22.02%, of which 13.03% were also ESBL positive,

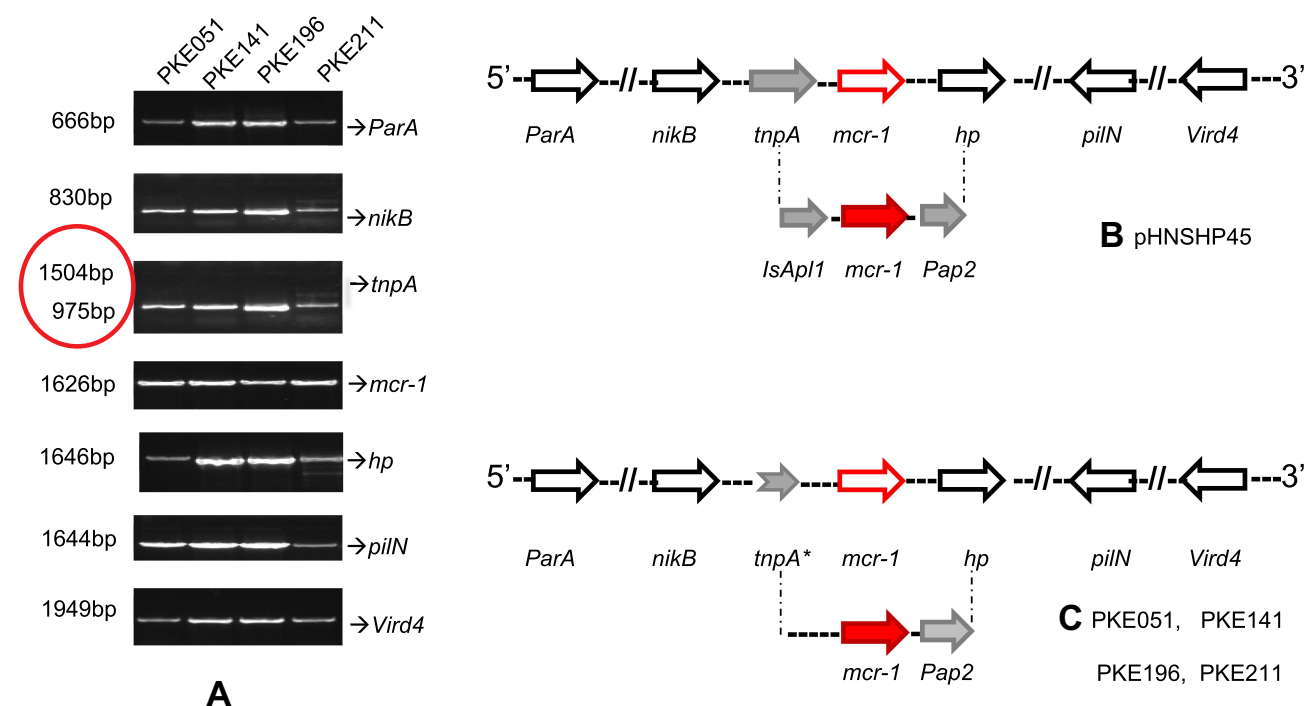


Figure 6 PCR mapping of *mcr-1* genetic background. (A) PCR amplified *vird4*, *pilN*, *hp*, *ParA*, *mcr-1*, *tnpA*, and *nikB*, and *tnpA* genes, red circle show the shortened band of *tnpA*. (B) pHNSHP45 reference map, indicating *Pap2* on downstream of *mcr-1* and *IsApI1* on upstream and. (C) *mcr-1* genetic setting in our strains, *IsApI1* is missing due to truncated *tnpA*. The black, red, and grey arrows represent particular genes having a name written underneath of each arrow. (*) on *tnpA* indicates truncation in *tnpA* gene.

much higher than a formerly reported 1.9–2.4% prevalence of carbapenem resistance in Asian countries.²⁷ However, some other studies from Pakistan reported a 20.3% and 56% prevalence of carbapenem-resistant isolates.^{24,28} The difference between the prevalence ratios might be due to differences in sample size, source type, and studies' objectives.²⁴ Among the carbapenem-resistant genes, 35.83% and 26.6% isolates had *bla*_{NDM-1} and *bla*_{KPC-2} genes, 25 and 18 isolates among them, co-harbored ESBL genes, respectively. Similarly, the co-existence of other MBL genes like *bla*_{IMP} and *bla*_{VIM} with ESBL genes (especially with *bla*_{CTXM-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.²⁸ Scientifically, there is no significant association between ESBL and carbapenemase production.²⁹ The co-occurrence of MBL (*bla*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM}) and ESBL (*bla*_{CTXM-15}, *bla*_{TEM}) 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.³⁰

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

All the *bla*_{KPC-2} and *bla*_{NDM-1} harboring isolates have successfully trans-conjugated except five ST131 *bla*_{KPC-2} carrying strains. The failure of *bla*_{KPC-2} conjugation in these strains might be due to its localization on the chromosome. A study from China recently determined the occurrence of *bla*_{KPC-2} on the chromosomal DNA of ST131 *E. coli*.³⁶ Various plasmid incompatibility types are detected in the present study in which IncHI2 was prominent for *bla*_{KPC-2} and IncFIIK for *bla*_{NDM-1}. The

association of carbapenemase-encoding genes in an association of these plasmids is detected from various world regions.^{37,38} Previous studies from Pakistan reported IncN, IncA/C, IncL/M, IncH1, IncFII, and IncR type plasmids in association with carbapenem-resistant genes.^{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 clonal 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 *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 *E. coli* from Pakistan. In Peshawar, Pakistan, a study reported 5% *mcr-1* out of 120 multiple drug-resistant *E. coli*.⁴² Similarly, a study from Faisalabad, Pakistan, detected one *mcr-1* bearing *E. coli* out of 29 ESBL producing strains.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 *bla*_{CTXM-15} gene was detected in all of these, while three had the *bla*_{TEM-1} gene. The co-occurrence of ESBL and *mcr-1* has been frequently reported.^{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.⁴⁶ Another research recommends that the co-harboring of *bla*_{CTXM-15} and *mcr-1* is maybe due to complex genetic selections that happened under the antibiotics pressure.⁴⁷

Diverse sequence types of *mcr-1* harboring *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 *bla*_{NDM-1} are previously detected from chicken meat and clinical isolates.^{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.^{49–52} ST156 and ST405 belong to avian-pathogenic *E. coli* lineage and extra-intestinal pathogenic *E. coli*. They are predominantly involved in the worldwide spread of *bla*_{CTXM-15} type

ESBL.^{49,51} Recently, a study from Pakistan detected *mcr-1* bearing ST117 in chickens.⁵³ ST117 are from avian pathogenic *E. coli* lineage and may form a reservoir for antibiotic resistance as a human extra-intestinal pathogenic *E. coli*.⁵⁴ The dissemination of *mcr-1* in extra-intestinal pathogenic and avian pathogenic *E. coli* in samples of human origin suggests food chain transmission of *mcr-1*.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*_{CTXM15} and *bla*_{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 *IsApl1* insertion sequence.¹³ Similarly, in our isolates, the PCR mapping of *mcr-1* genetic background showed similarity with pHNSHP45, except the missing *IsApl1*, due to truncated *tnpA* gene. Usually, the IncX4 type plasmid lacks *IsApl1*, while in IncI2 type plasmid, it either present or absent.¹¹ The *IsApl1* missing might be due to its quick mobilization of the *mcr-1* gene into a new plasmid and self-remaining in the parental plasmid.⁵⁶ Alternatively, in the new plasmids, due to missing *IsApl1*, the *mcr-1* mobilization decreases and strengthens its firmness in the plasmid.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.¹³ In Pakistan's poultry farms, colistin solely or in combination with other drugs is widely used to treat clostridial enteritis and colibacillosis.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.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 *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 *bla*_{NDM-1}

and *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 *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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Disclosure

The authors report no conflicts of interest in this work.

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