Infection and Drug Resistance

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Molecular Epidemiology of *mcr-1*, bla_{KPC-2}, and bla_{NDM-1} Harboring Clinically Isolated *Escherichia coli* from Pakistan

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Hazrat Bilal[1]
Tayyab Ur Rehman[2]
Muhammad Asif Khan[2]
Fareeha Hameed[2]
Zhang Gao Jian[1]
Jianxiong Han[1]
Xingyuan Yang[1]

[1]Faculty of Health Sciences, Institute of Physical Sciences and Information Technology, Anhui University, Hefei, People's Republic of China; [2]Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan

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 Incl2 (60-kb) plasmid. The bla_{KPC-2} was present on (140-kb) IncH12, (100-kb) IncN, (90-kb) Incl1, 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 Incl1 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,

Correspondence: Xingyuan Yang
Faculty of Health Sciences, Institute of Physical Science and Information Technology, Anhui University, No, 111 Jiulong Road, Hefei, Anhui, 230601, People's Republic of China
Email xingyuan@ahu.edu.cn

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/IDR.S302687

and CTX-M.[2] Among these, the *bla*$_{\text{CTX-M15}}$ 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 Enterobactericea 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*$_{\text{KPC-2}}$ is a prominent member of class A, and class D consists of *bla*$_{\text{OXA-48}}$ 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*$_{\text{NDM-1}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, 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 Incl2 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 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*$_{\text{KPC-2}}$, and *bla*$_{\text{NDM-1}}$ 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.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 carbapenemases 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)

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 dropTM (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, $bla_{\text{CTX-M}}$ group 1, bla_{SHV}, bla_{TEM}, and bla_{OXA} variants of ESBL genes and carbapenemases producing genes ($bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$, bla_{IMP}, 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,* $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ harboring isolates, multi-locus-sequence-typing (MLST) were carried out. The seven alleles, *adk, 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,* $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ bearing strains, trans-conjugation was carried out. The *E. coli* EC 600 (Nal^{R}, Rif^{R}) was selected as recipients, and *mcr-1,* $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ 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 $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ 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,* $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ genes specific PCR.

To explore the incompatibility types of plasmids bearing *mcr-1,* $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$, 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 $bla_{\text{KPC-2}}$, and five from $bla_{\text{NDM-1}}$) were selected for this experiment. Southern blotting was performed to confirm the presence of genes on plasmids. The digoxin-labeled $bla_{\text{KPC-2}}$, *mcr-1*, and $bla_{\text{NDM-1}}$ 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 Incl2 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.

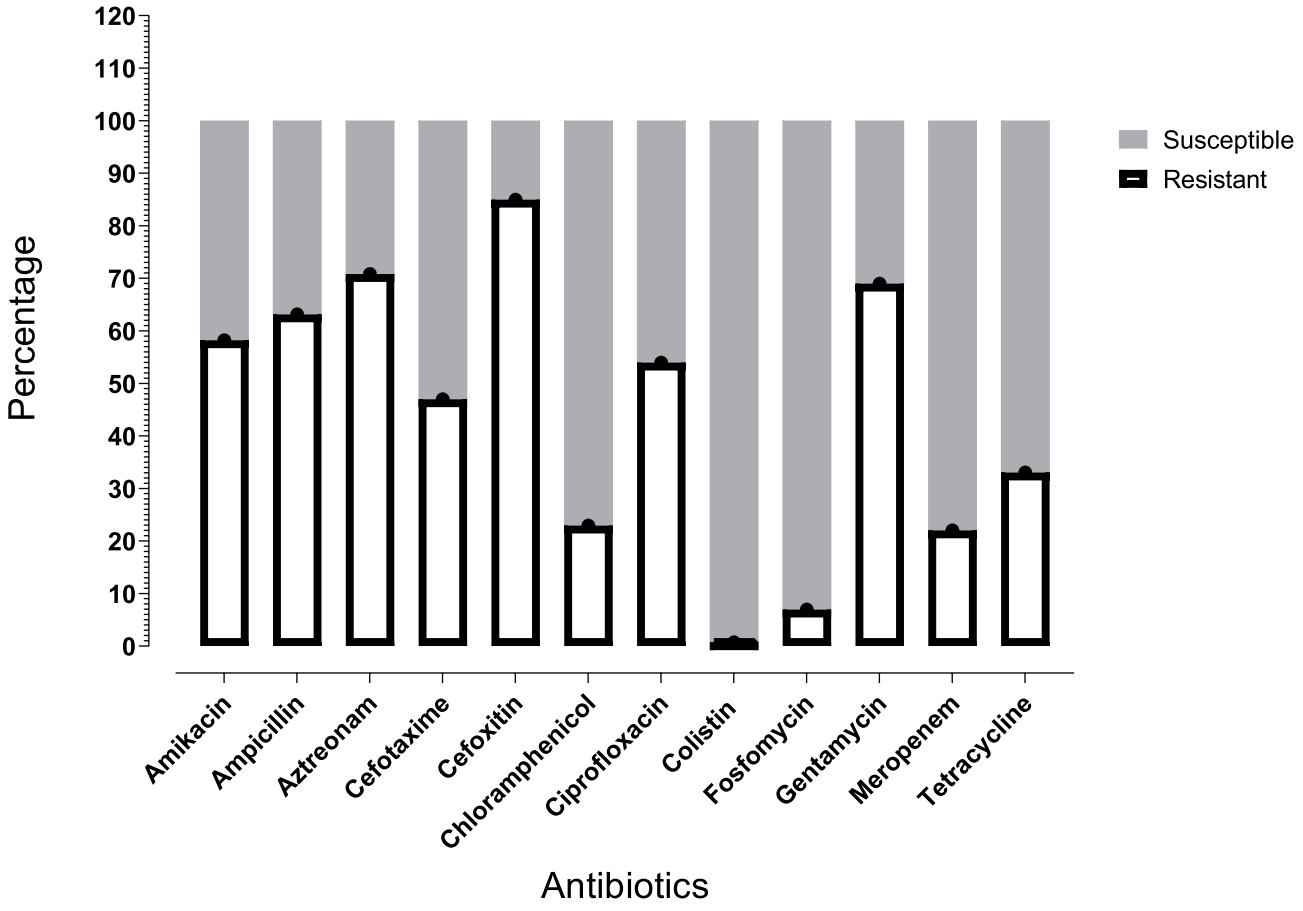

Figure 1 Antibiotic resistance profile of all tested *E. coli* isolates.

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 $bla_{\text{CTX-M-15}}$ genes, three of these strains also had the $bla_{\text{TEM-1}}$ gene (Table 1).

Among 139 ESBL producer strains, 74.82% (104 of 139) harbored $bla_{\text{CTXM-15}}$ gene, 34.53% (48 of 139) carried bla_{TEM} gene (n=29 $bla_{\text{TEM-1}}$, n=19 $bla_{\text{TEM-116}}$), 28.78% (40 of 139) contained $bla_{\text{OXA-1}}$ gene and 5.75% (8 of 139) harbored $bla_{\text{SHV-75}}$ gene. The $bla_{\text{SHV-11}}$, a non ESBL variant of SHV was identified in 31 isolates. In 129 carbapenemase-producers, 35.83% (43 of 129) possessed $bla_{\text{NDM-1}}$, 26.67% (32 of 129) $bla_{\text{KPC-2}}$, 8.3% (10 of 129) $bla_{\text{OXA-48}}$, 25% (30 of 129) $bla_{\text{VIM-1}}$, and 20.83% (25 of 129) possessed $bla_{\text{IMP-1}}$ gene. The 51.07% (71 0f 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 $bla_{\text{NDM-1}}$, $bla_{\text{CTXM-15}}$, and $bla_{\text{OXA-1}}$ were 16.9% (12 of 71), $bla_{\text{NDM-1}}$ and $bla_{\text{CTXM-15}}$ were 15.49% (11 of 71), $bla_{\text{IMP-1}}$, $bla_{\text{CTXM-15}}$, and $bla_{\text{TEM-1}}$ were 11.27% (8 of 71), $bla_{\text{IMP-1}}$ and $bla_{\text{OXA-1}}$ were 11.27% (8 of 71), $bla_{\text{KPC-2}}$, $bla_{\text{CTXM-15}}$, and bla_{TEM} were 9.86% (7 of 71), $bla_{\text{VIM-1}}$ and bla_{SHV} were 8.45% (6 of 71), $bla_{\text{KPC-2}}$ and $bla_{\text{CTXM-15}}$ were 7.04% (5 of 71), $bla_{\text{KPC-2}}$, bla_{TEM}, and bla_{SHV} were 5.63% (4 of 71), $bla_{\text{OXA-48}}$

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 Incl2 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 Incl1 (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 Incl1 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 Incl1 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 (Incl1), 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)
mcr-1	*bla*$_{KPC-2}$	*bla*$_{OXA-48}$	*bla*$_{NDM-1}$	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV-75}$	*bla*$_{OXA}$	*bla*$_{SHV-11}$	
+						+	+				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%)
			+			+			+		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 *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

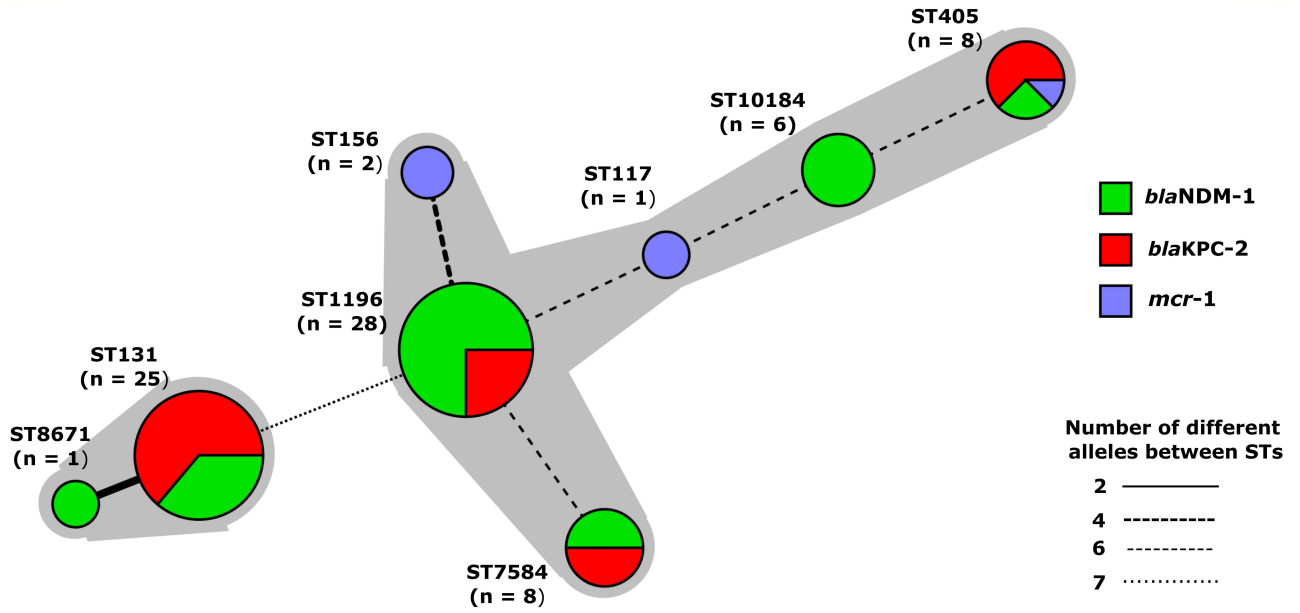

Figure 2 Minimum spinning tree of *mcr-1*, bla_{KPC-2}, and bla_{NDM-1} harboring *E. coli* by MLST type and alleles. Each node represent a sequence type, size of node represent the number of isolates, length of branches represent number of different alleles out of seven MLST alleles. Nodes are labeled 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
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	bla_{KPC-2}	11/32 (34.38%)
IncN	~ 100-kb	ST 1196	None	bla_{KPC-2}	5/32 (15.63%)
IncI1α	~ 90-kb	ST 7584	None	bla_{KPC-2}	4/32 (12.5%)
IncN	~ 100-kb	ST 405	ST405 Cplx	bla_{KPC-2}	3/32 (9.37%)
IncH12	~ 140-kb	ST 1196	None	bla_{KPC-2}	2/32 (6.25%)
IncH12	~ 140-kb	ST 405	ST405 Cplx	bla_{KPC-2}	2/32 (6.25%)
IncFIIK	~ 70-kb	ST 1196	None	bla_{NDM-1}	17/43 (39.53%)
IncH12	~ 140-kb	ST 131	ST131 Cplx	bla_{NDM-1}	9/43 (20.93%)
IncH12	~ 140-kb	ST 10,184	ST69 Cplx	bla_{NDM-1}	5/43 (11.63%)
IncN	~ 100-kb	ST 1196	None	bla_{NDM-1}	4/43 (9.30%)
IncN	~ 100-kb	ST 8671	None	bla_{NDM-1}	1/43 (2.32%)
IncA/C	~ 60-kb	ST 7584	None	bla_{NDM-1}	4/43 (9.3%)
IncN	~ 100-kb	ST 405	ST405 Cplx	bla_{NDM-1}	2/43 (4.65%)
IncFII	~ 45-kb	ST 10,184	ST69 Cplx	bla_{NDM-1}	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.

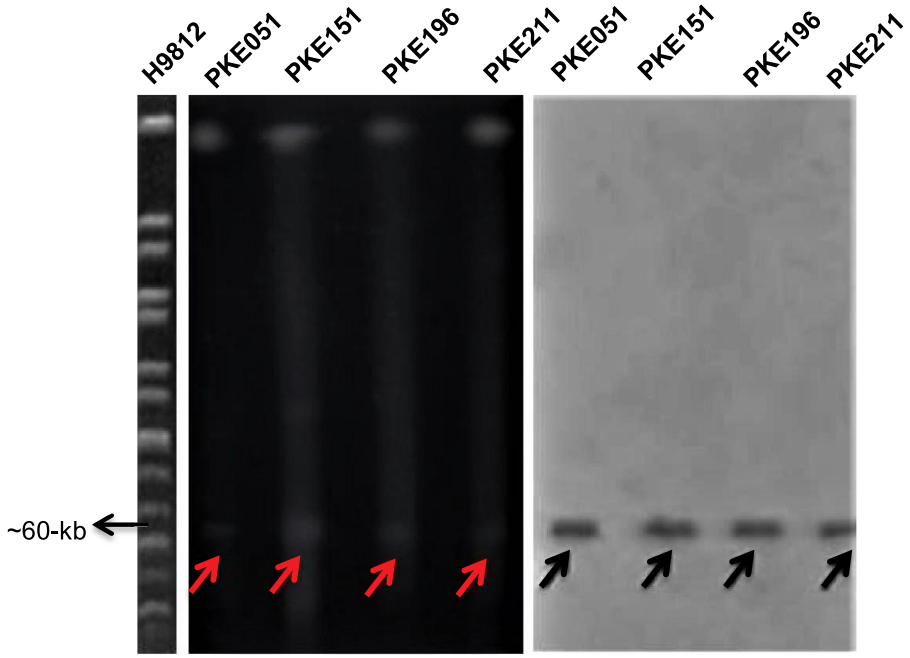

Figure 3 S1 PFGE and Southern blot of *mcr-1* harboring strains. H9812 is a molecular size marker (1135 to 20.5-kb). PKE211, PKE196, and PKE14, PKE051 are the isolates; digested with S1 enzymes. All isolates show 60-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.

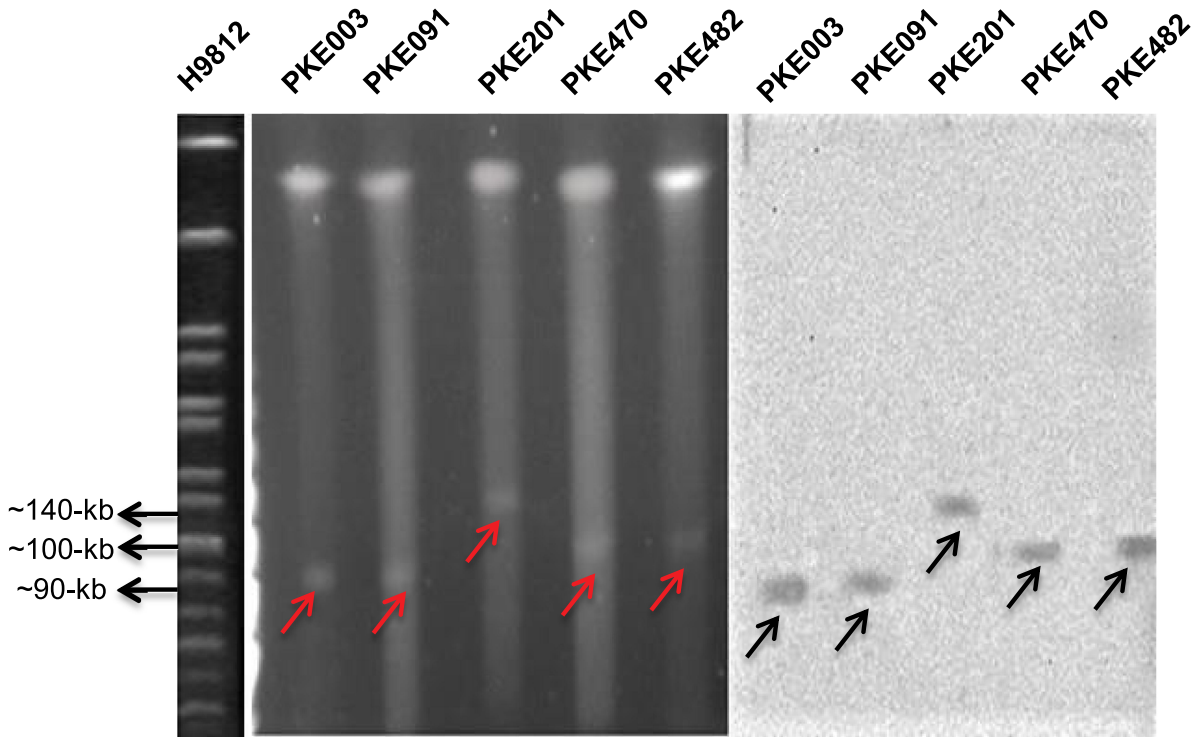

Figure 4 S1 PFGE and Southern blot of bla_{KPC-2} harboring strains. H9812 is a molecular size marker. PKE003 and PKE091 isolates have 90-kb plasmid, PKE201 isolate has 140-kb plasmid, and PKE470 and PKE482 isolates have 100-kb plasmid. The red arrows represent plasmids on PFGE gel. Black arrows represent plasmids on nylon membrane.

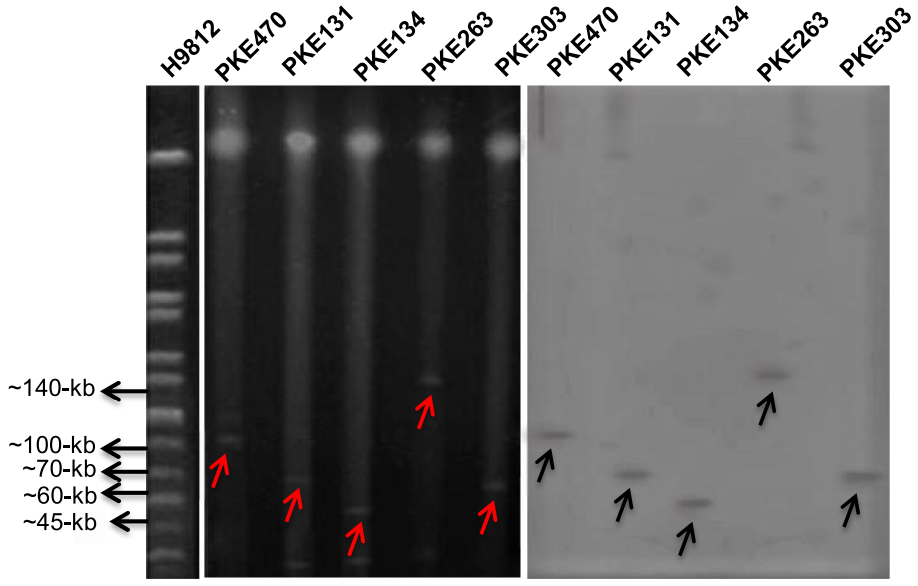

Figure 5 S1 PFGE and Southern blot of bla_{NDM-1} harboring strains. H9812 is a molecular size marker. PKE470 isolate have 100-kb plasmid, PKE131 isolate have 70-kb plasmid, PKE134 isolate have 45-kb plasmid, PKE263 isolate have 140-kb plasmid, and PKE303 isolate have 60-kb plasmid. The red arrows represent 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,

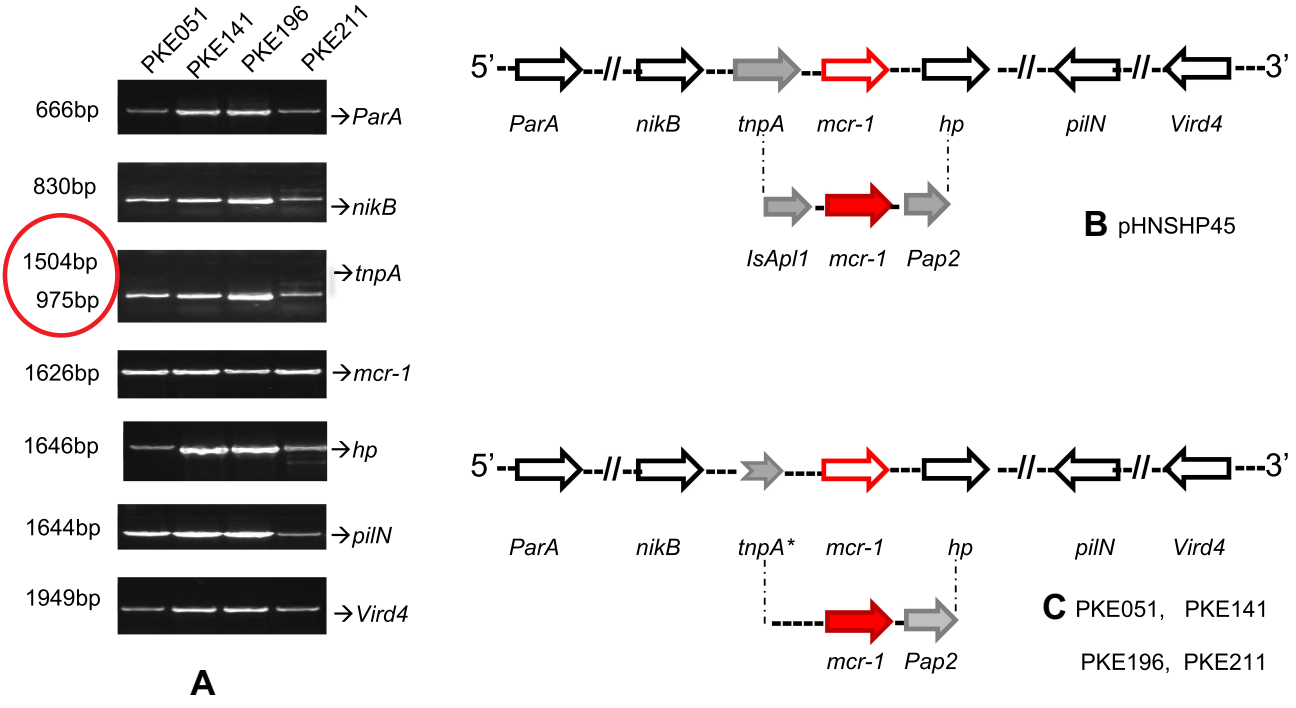

Figure 6 PCR mapping of *mcr-1* genetic background. (**A**) PCR amplified *vird4*, *pilN*, *hp*, *ParA*, *mcr-1*, *tnpA*, and *nikB*, and *tnpA* genes, red circle show the shortened band of *tnpA*. (**B**) pHNSPH45 reference map, indicating *Pap2* on downstream of *mcr-1* and *IsApl1* on upstream and. (**C**) *mcr-1* genetic setting in our strains, *IsApl1* 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 $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ 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 $bla_{\text{CTXM-15}}$) 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 ($bla_{\text{NDM-1}}$, bla_{IMP}, bla_{VIM}) and ESBL ($bla_{\text{CTXM-15}}$, 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 $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ harboring isolates. The ST131 and ST1196 are the most prevalent sequence types for $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ 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 $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ genes are reported for the first time in the current study. However, a study from Myanmar detected other carbapenemase-encoding gene ($bla_{\text{NDM-5}}$) on ST1196.[32] The finding of ST405 carrying $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ 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 $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$) and ST10184 ($bla_{\text{NDM-1}}$ harboring), are identified in this study.

All the $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ harboring isolates have successfully trans-conjugated except five ST131 $bla_{\text{KPC-2}}$ carrying strains. The failure of $bla_{\text{KPC-2}}$ conjugation in these strains might be due to its localization on the chromosome. A study from China recently determined the occurrence of $bla_{\text{KPC-2}}$ 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 $bla_{\text{KPC-2}}$ and IncFIIK for $bla_{\text{NDM-1}}$. 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, Incl1, 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 Incl2 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 $bla_{\text{CTXM-15}}$ gene was detected in all of these, while three had the $bla_{\text{TEM-1}}$ 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 $bla_{\text{CTXM-15}}$ 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 multiclonal 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. coil*. 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 Incl2 plasmid, having transferability. The PCR results of *mcr-1* transconjugants revealed that the Incl2 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 Incl2 plasmid having only *mcr-1* gene in ST155 isolated from a healthy broiler. The previously reported ST155 incl2 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 Incl2 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 Incl2 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.

Acknowledgment

The authors are thankful to the Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan and Institute of Health sciences, Anhui University, China for supporting and facilitating this work.

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.

Funding

This work was supported by grants from Natural Science Foundation of China (number 31771310 to Xingyuan Yang) and Anhui Province Natural Science Foundation (number 1708085MC67 to Xingyuan Yang).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Theuretzbacher U, Bush K, Harbarth S. Critical analysis of antibacterial agents in clinical development. *Nat Rev Microbiol*. 2020;18(5):286–298. doi:10.1038/s41579-020-0340-0
2. Shafiq M, Huang J, Ur Rahman S, et al. High incidence of multidrug-resistant Escherichia coli coharboring mcr-1 and bla (CTX-M-15) recovered from pigs. *Infect Drug Resist*. 2019;12:2135–2149. doi:10.2147/IDR.S209473
3. Woerther PL, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. *Clin Microbiol Rev*. 2013;26(4):744–758. doi:10.1128/CMR.00023-13

4. Abrar S, Hussain S, Khan RA, Ul Ain N, Haider H, Riaz S. Prevalence of extended-spectrum-β-lactamase-producing Enterobacteriaceae: first systematic meta-analysis report from Pakistan. *Antimicrob Resist Infect Control*. 2018;7(1):26. doi:10.1186/s13756-018-0309-1
5. Codjoe FS, Donkor ES. Carbapenem Resistance: a Review. *Med Sci (Basel)*. 2017;6(1). doi:10.3390/medsci6010001
6. Suay-García B, Pérez-Gracia MT. Present and Future of Carbapenem-resistant Enterobacteriaceae (CRE) Infections. *Antibiotics (Basel)*. 2019;8(3). doi:10.3390/antibiotics8030122
7. Ain N, Abrar S, Sherwani RAK, Hannan A, Imran N, Riaz S. Systematic surveillance and meta-analysis on the prevalence of metallo-β-lactamase producers among carbapenem resistant clinical isolates in Pakistan. *J Glob Antimicrob Resist*. 2020;23:55–63. doi:10.1016/j.jgar.2020.07.024
8. Karaiskos I, Lagou S, Pontikis K, Rapti V, Poulakou G. The "Old" and the "New" antibiotics for MDR gram-negative pathogens: For Whom, When, and How. *Front Public Health*. 2019;7:151.
9. Zhai YJ, Sun HR, Luo XW, et al. CpxR regulates the colistin susceptibility of Salmonella typhimurium by a multitarget mechanism. *J Antimicrob Chemother*. 2020;75(10):2780–2786. doi:10.1093/jac/dkaa233
10. Liu YY, Wang Y, Walsh TR, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis*. 2016;16(2):161–168. doi:10.1016/S1473-3099(15)00424-7
11. Nang SC, Li J, Velkov T. The rise and spread of mcr plasmid-mediated polymyxin resistance. *Crit Rev Microbiol*. 2019;45(2):131–161. doi:10.1080/1040841X.2018.1492902
12. Mohsin M, Azam M, Ur Rahman S, et al. Genomic background of a colistin-resistant and highly virulent MCR-1-positive Escherichia coli ST6395 from a broiler chicken in Pakistan. *Pathog Dis*. 2019;77(7). doi:10.1093/femspd/ftz064.
13. Lv J, Mohsin M, Lei S, et al. Discovery of a mcr-1-bearing plasmid in commensal colistin-resistant Escherichia coli from healthy broilers in Faisalabad, Pakistan. *Virulence*. 2018;9(1):994–999. doi:10.1080/21505594.2018.1462060
14. Azam M, Ehsan I, Sajjad Ur R, Saleemi MK, Javed MR, Mohsin M. Detection of the colistin resistance gene mcr-1 in avian pathogenic Escherichia coli in Pakistan. *J Glob Antimicrob Resist*. 2017;11:152–153. doi:10.1016/j.jgar.2017.10.012
15. Mohsin M, Raza S, Roschanski N, Guenther S, Ali A, Schierack P. Description of the First Escherichia coli clinical isolate harboring the colistin resistance gene mcr-1 from the Indian Subcontinent. *Antimicrob Agents Chemother*. 2017;61(1). doi:10.1128/AAC.01945-16
16. Mohsin M, Raza S, Roschanski N, Schaufler K, Guenther S. First description of plasmid-mediated colistin-resistant extended-spectrum β-lactamase-producing Escherichia coli in a wild migratory bird from Asia. *Int J Antimicrob Agents*. 2016;48(4):463–464. doi:10.1016/j.ijantimicag.2016.07.001
17. Lupindu AM. Isolation and Characterization of Escherichia coli from animals, humans, and environment. In: Samie A, editor. *Escherichia Coli - Recent Advances on Physiology, Pathogenesis and Biotechnological Applications*. London, United Kingdom: IntechOpen Limited; 2017:187–206.
18. Wayne P. *Performance Standards for Antimicrobial Susceptibility Testing*. Clinical and laboratory standards institute, CLSI supplement M100; 2020.
19. Dilhari A, Sampath A, Gunasekara C, et al. Evaluation of the impact of six different DNA extraction methods for the representation of the microbial community associated with human chronic wound infections using a gel-based DNA profiling method. *AMB Express*. 2017;7(1):179. doi:10.1186/s13568-017-0477-z
20. Feliciello I, Chinali G. A modified alkaline lysis method for the preparation of highly purified plasmid DNA from Escherichia coli. *Anal Biochem*. 1993;212(2):394–401. doi:10.1006/abio.1993.1346
21. Rahman H, Naeem M, Khan I, et al. Molecular prevalence and antibiotics resistance pattern of class A bla CTX-M-1 and bla TEM-1 beta lactamases in uropathogenic Escherichia coli isolates from Pakistan. *Turk J Med Sci*. 2016;46(3):897–902. doi:10.3906/sag-1502-14
22. Ali F, Niaz Z, Shah PT, Shakeela Q, Uzma B, Ahmed S. Antibiogram of ESBL and MBL producing Pseudomonas aeruginosa among the population of Hazara division, KPK, Pakistan. *J Pak Med Assoc*. 2020;70(11):1979–1984. doi:10.5455/JPMA.19089
23. Ahmed I, Sajed M, Sultan A, et al. The erratic antibiotic susceptibility patterns of bacterial pathogens causing urinary tract infections. *EXCLI J*. 2015;14:916–925.
24. Nahid F, Khan AA, Rehman S, Zahra R. Prevalence of metallo-β-lactamase NDM-1-producing multi-drug resistant bacteria at two Pakistani hospitals and implications for public health. *J Infect Public Health*. 2013;6(6):487–493. doi:10.1016/j.jiph.2013.06.006
25. Abrar S, Vajeeha A, Ul-Ain N, Riaz S. Distribution of CTX-M group I and group III β-lactamases produced by Escherichia coli and klebsiella pneumoniae in Lahore, Pakistan. *Microb Pathog*. 2017;103:8–12. doi:10.1016/j.micpath.2016.12.004
26. Sid Ahmed MA, Bansal D, Acharya A, et al. Antimicrobial susceptibility and molecular epidemiology of extended-spectrum beta-lactamase-producing Enterobacteriaceae from intensive care units at Hamad Medical Corporation, Qatar. *Antimicrob Resist Infect Control*. 2016;5(4). doi:10.1186/s13756-016-0103-x.
27. Xu Y, Gu B, Huang M, et al. Epidemiology of carbapenem resistant Enterobacteriaceae (CRE) during 2000–2012 in Asia. *J Thorac Dis*. 2015;7(3):376–385. doi:10.3978/j.issn.2072-1439.2014.12.33
28. Ain NU, Iftikhar A, Bukhari SS, et al. High frequency and molecular epidemiology of metallo-β-lactamase-producing gram-negative bacilli in a tertiary care hospital in Lahore, Pakistan. *Antimicrob Resist Infect Control*. 2018;7(1):128. doi:10.1186/s13756-018-0417-y
29. Safari M, Mozaffari Nejad AS, Bahador A, Jafari R, Alikhani MY. Prevalence of ESBL and MBL encoding genes in Acinetobacter baumannii strains isolated from patients of intensive care units (ICU). *Saudi J Biol Sci*. 2015;22(4):424–429. doi:10.1016/j.sjbs.2015.01.004
30. Maurya N, Jangra M, Tambat R, Nandanwar H. Alliance of Efflux Pumps with β-lactamases in multidrug-resistant Klebsiella pneumoniae Isolates. *Microb Drug Resist*. 2019;25(8):1155–1163. doi:10.1089/mdr.2018.0414
31. Kim YA, Park YS, Youk T, Lee H, Lee K. Trends in South Korean antimicrobial use and association with changes in Escherichia coli resistance rates: 12-year ecological study using a nationwide surveillance and antimicrobial prescription database. *PLoS One*. 2018;13(12):e0209580. doi:10.1371/journal.pone.0209580
32. Aung MS, San N, Maw WW, et al. Prevalence of extended-spectrum beta-lactamase and carbapenemase genes in clinical isolates of Escherichia coli in Myanmar: dominance of bla(NDM-5) and emergence of bla(OXA-181). *Microb Drug Resist*. 2018;24(9):1333–1344. doi:10.1089/mdr.2017.0387
33. Qamar MU, Walsh TR, Toleman MA, et al. Dissemination of genetically diverse NDM-1, −5, −7 producing-Gram-negative pathogens isolated from pediatric patients in Pakistan. *Future Microbiol*. 2019;14(8):691–704. doi:10.2217/fmb-2019-0012
34. Cai JC, Zhang R, Hu YY, Zhou HW, Chen GX. Emergence of Escherichia coli sequence type 131 isolates producing KPC-2 carbapenemase in China. *Antimicrob Agents Chemother*. 2014;58(2):1146–1152. doi:10.1128/AAC.00912-13
35. Abd El Ghany M, Sharaf H, Al-Agamy MH, Shibl A, Hill-Cawthorne GA. Genomic characterization of NDM-1 and 5, and OXA-181 carbapenemases in uropathogenic Escherichia coli isolates from Riyadh, Saudi Arabia. *PLoS One*. 2018;13(8):e0201613. doi:10.1371/journal.pone.0201613
36. Wang D, Mu X, Chen Y, et al. emergence of a clinical escherichia coli sequence Type 131 strain carrying a chromosomal bla (KPC-2) Gene. *Front Microbiol*. 2020;11:586764. doi:10.3389/fmicb.2020.586764

37. Pérez-Vazquez M, Oteo-Iglesias J, Sola-Campoy PJ. Characterization of carbapenemase-producing Klebsiella oxytoca in Spain, 2016–2017. *Antimicrob Agents Chemother*. 2019;63(6):e025029–025018. doi:10.1128/AAC.02529-18
38. Mohamed ER, Ali MY, Waly N, Halby HM, El-Baky RMA. The Inc FII plasmid and its contribution in the transmission of bla(NDM-1) and bla(KPC-2) in Klebsiella pneumoniae in Egypt. *Antibiotics (Basel)*. 2019;8(4):266.
39. Sartor AL, Raza MW, Abbasi SA, et al. Molecular epidemiology of NDM-1-producing Enterobacteriaceae and Acinetobacter baumannii isolates from Pakistan. *Antimicrob Agents Chemother*. 2014;58 (9):5589–5593. doi:10.1128/AAC.02425-14
40. Nahid F, Zahra R, Sandegren L. A blaOXA-181-harbouring multi-resistant ST147 Klebsiella pneumoniae isolate from Pakistan that represent an intermediate stage towards pan-drug resistance. *PLoS One*. 2017;12(12):e0189438. doi:10.1371/journal.pone.0189438
41. Gondal AJ, Saleem S, Jahan S. Novel carbapenem-resistant Klebsiella pneumoniae ST147 Coharboring bla (NDM-1), bla (OXA-48) and extended-spectrum β-lactamases from Pakistan. *Infect Drug Resist*. 2020;13:2105–2115. doi:10.2147/IDR.S251532
42. Hameed F, Khan MA, Bilal H, Muhammad H, Tayyab Ur R. Detection of MCR-1 gene in multiple drug resistant escherichia coli and klebsiella pneumoniae in human clinical samples from Peshawar, Pakistan. *Comb Chem High Throughput Screen*. 2020.
43. Berrazeg M, Hadjadj L, Ayad A, Drissi M, Rolain JM. First detected human case in algeria of mcr-1 plasmid-mediated colistin resistance in a 2011 Escherichia coli isolate. *Antimicrob Agents Chemother*. 2016;60(11):6996–6997. doi:10.1128/AAC.01117-16
44. Doumith M, Godbole G, Ashton P, et al. Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of Salmonella enterica and Escherichia coli in England and Wales. *J Antimicrob Chemother*. 2016;71(8):2300–2305. doi:10.1093/jac/dkw093
45. Quan J, Li X, Chen Y, et al. Prevalence of mcr-1 in Escherichia coli and Klebsiella pneumoniae recovered from bloodstream infections in China: a multicentre longitudinal study. *Lancet Infect Dis*. 2017;17 (4):400–410. doi:10.1016/S1473-3099(16)30528-X
46. Rhouma M, Letellier A. Extended-spectrum β-lactamases, carbapenemases and the mcr-1 gene: is there a historical link? *Int J Antimicrob Agents*. 2017;49(3):269–271. doi:10.1016/j.ijantimicag.2016.11.026
47. Jeannot K, Bolard A, Plésiat P. Resistance to polymyxins in Gram-negative organisms. *Int J Antimicrob Agents*. 2017;49(5):526–535. doi:10.1016/j.ijantimicag.2016.11.029
48. Baloch Z, Lv L, Yi L, et al. Emergence of almost identical F36: a-: B32Plasmids Carrying bla (NDM-5) and qepA in Escherichia coli from both Pakistan and Canada. *Infect Drug Resist*. 2019;12:3981–3985. doi:10.2147/IDR.S236766
49. Mediavilla JR, Patrawalla A, Chen L, et al. Colistin- and carbapenem-resistant Escherichia coli Harboring mcr-1 and blaNDM-5, causing a complicated urinary tract infection in a patient from the United States. *mBio*. 2016;7(4). doi:10.1128/mBio.01191-16.
50. Bachiri T, Lalaoui R, Bakour S, et al. First Report of the plasmid-mediated colistin resistance gene mcr-1 in Escherichia coli ST405 isolated from Wildlife in Bejaia, Algeria. *Microb Drug Resist*. 2018;24(7):890–895. doi:10.1089/mdr.2017.0026
51. Yang RS, Feng Y, Lv XY, et al. Emergence of NDM-5- and MCR-1-producing Escherichia coli clones ST648 and ST156 from a Single Muscovy Duck (Cairina moschata). *Antimicrob Agents Chemother*. 2016;60(11):6899–6902. doi:10.1128/AAC.01365-16
52. Rossi F, Girardello R, Morais C, et al. Plasmid-mediated mcr-1 in carbapenem-susceptible Escherichia coli ST156 causing a blood infection: an unnoticeable spread of colistin resistance in Brazil? *Clinics (Sao Paulo)*. 2017;72(10):642–644. doi:10.6061/clinics/2017(10)09
53. Azam M, Mohsin M, Johnson TJ, et al. Genomic landscape of multidrug resistant avian pathogenic Escherichia coli recovered from broilers. *Vet Microbiol*. 2020;247:108766. doi:10.1016/j.vetmic.2020.108766
54. Manges AR, Johnson JR. Food-borne origins of Escherichia coli causing extraintestinal infections. *Clin Infect Dis*. 2012;55(5):712–719. doi:10.1093/cid/cis502
55. Elbediwi M, Wu B, Pan H, Jiang Z, Biswas S, Li Y. Genomic characterization of mcr-1-carrying Salmonella enterica Serovar 4, [5],12:i:- ST 34 clone isolated from Pigs in China. *Front Bioeng Biotechnol*. 2020;8:663. doi:10.3389/fbioe.2020.00663
56. Kuo SC, Huang WC, Wang HY, Shiau YR, Cheng MF, Lauderdale TL. Colistin resistance gene mcr-1 in Escherichia coli isolates from humans and retail meats, Taiwan. *J Antimicrob Chemother*. 2016;71 (8):2327–2329. doi:10.1093/jac/dkw122
57. Snesrud E, He S, Chandler M, et al. A model for transposition of the colistin resistance gene mcr-1 by ISApl1. *Antimicrob Agents Chemother*. 2016;60(11):6973–6976. doi:10.1128/AAC.01457-16
58. Mohsin M, Van Boeckel TP, Saleemi MK, et al. Excessive use of medically important antimicrobials in food animals in Pakistan: a five-year surveillance survey. *Glob Health Action*. 2019;12 (sup1):1697541. doi:10.1080/16549716.2019.1697541