Discovery of mcr-1 Harboring Incl2 Plasmids from Clinical Isolates of Multiclonal E. Coli prevalent in Pakistan.

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Research

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Abstract

Background: Colistin is the last resort antibiotic against multiple drug-resistant (MDR) bacteria found in clinical infections; however, the emergence of plasmid-mediated mcr-1 gene annulled the efficacy of Colistin. This study was planned to determine the prevalence of mcr genes in clinical isolates collected in Pakistan. The molecular types and plasmids of *mcr-1* bearing isolates were analysed.

Methods: A total of 545 *E. coli* isolates collected from two major cities of Pakistan were screened for colistin-resistance and mcr genes from June 2018 to September 2019. All positive strains were subjected to antimicrobial susceptibility testing, ESBL, and MBL detection via DDST and CDT. ESBL genes detection, molecular typing, conjugation experiment, plasmid replicon typing, S1 PFGE, and southern hybridization were performed.

Results: Four (0.73%) strains of mcr-1 positive isolates were susceptible to meropenem, fosfomycin, and chloramphenicol, including one that showed moderate level resistance to chloramphenicol and fosfomycin. All four strains were ESBL positive and harbored the *bla*CTX-M-15 gene, while three of the isolates also harbored the *bla*TEM-1 gene. Molecular typing revealed that four isolates belonged to diverse clonal types, mostly (75%) from avian pathogenic *E. coli* lineage. The *mcr-1* gene was present on ~ 60 kb IncI2 plasmid, which was successfully transconjugated.

Conclusion: The *mcr-1* gene's detection in diverse clonal types from MDR clinical isolates enforces priority basis large scale surveillance studies followed by corrective actions to prevent the spread of the mcr-1 gene in clinical, poultry, and environmental setting.

Background

Antibiotic resistance is a serious concern worldwide. The situation is escalating due to the misuse and overuse of antibiotics. Different antibiotic resistance mechanisms are developed by bacteria, among which the ESBL and MBL production are the most prominent. Most of the MDR bacteria show resistance to more than one drug [1]. Colistin, a cationic polypeptide antibiotic, was considered the last drug of choice against these superbugs [2]. Various chromosomal mediated colistin resistance such as the two-component system pmrAB, phoPQ, and regulator mgrB have to be known in Enterobacteriaceae that alter the lipopolysaccharide (LPS) of the bacterial cell wall, thus losing its affinity of attachment toward polymyxins [3]. However, it was not as high threatening due to their mode of vertical transmission [4]. In November 2015, Liu et al. for the first time discovered a plasmid-mediated mobilized colistin resistance gene from *E. coli* and *Klebsiella Pneumoniae*. The gene product belongs to phosphoethanolamine (PEtN) transferases enzymes that add PEtN to the phosphate group of lipid A. This results in the loss of Colistin binding ability to LPS as lipid A more cationic [5]. Later, the colistin resistance *mcr-1* gene was discovered in 47 countries [6]. It was initially discovered on IncI2 type of plasmid, but later on, it was found on other plasmid incompatibility types from different bacterial isolates collected from humans, animals, food, environment, insect, and water. Besides, other variants of mcr such as *mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8,* and *mcr-9* have been reported from different regions of the world [7].

In Pakistan, *mcr-1* harboring *E. coli* was detected in migratory birds and later on isolated from clinical samples and broiler in Faisalabad City of the Punjab Province of Pakistan [8-12]. In this study, we look for *mcr* genes (*mcr-1* to *mcr-5*) in 545 *E. coli* isolates from human samples collected in two major cities Islamabad and Peshawar of Pakistan. We have determined the Antibiotic resistance profile, ESBL genes, ST, plasmid Incompatibility type, and genetic context of *mcr-1* harboring isolates.

Methods

Sample collection, isolation, and species confirmation:

The present study was initiated with the screening of 545 *E. coli* isolates for colistin resistance. All isolates were collected from Microbiology laboratories of PIMS Islamabad (n= 260), HMC (n= 105), RMI (n=94), and KTH (n= 86) Peshawar - tertiary care hospitals in Pakistan from June 2018 to September 2019. The isolates obtained from human clinical samples included urine (n= 345), blood (n= 109), stool (n= 18), and pus (n= 73). The growth capacity of isolates was initially assessed on CLED and

MacConkey agar. A single colony from each Petri plate was stored in LB media for maintaining strains. Species confirmation of *E. coli* was performed with 16S rDNA PCR using specific primer mentioned in Table S1. The PCR products were sequenced, and species confirmation was performed using EZ Biocloud online software (https://www.ezbiocloud.net/identify).

Phenotypic and molecular detection of colistin resistance:

Phenotypic detection of colistin resistance was performed applying the broth microdilution method. The Minimum inhibitory concentration results were interpreted according to CLSI guidelines [13]. The genomic DNA from resistant isolates was extracted with the conventional boiling method [14]. The DNA samples were subjected to PCR using *mcr* genes-specific primers mentioned in Table S1. The expected size amplicons were visualized on 1% Agarose gel, which was sequenced and analysed with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Antimicrobial susceptibility testing:

The *mcr-1* harboring isolates were subjected to antimicrobial susceptibility testing using the broth microdilution method. The antibiotics used were Ampicillin, Cefotaxime, Chloramphenicol, Ciprofloxacin, Fosfomycin, Cefoxitin, Gentamycin, Aztreonam, Amikacin, Meropenem, and Tetracycline. ESBL and MBL activity of resistant isolates were also determined by the Double Desk Synergy Test (DDST) and combined desk test (CDT), respectively. The results were interpreted according to CLSI guidelines [13].

PCR detection of ESBL genes:

ESBL genes *bla*TEM, *bla*SHV, *bla*CTX-M, and *bla*OXA variants were amplified from the genomic DNA of colistin-resistant isolates with ESBL genes-specific primers mentioned in Table S1. The expected sizes were visualized on 1% Agarose gel. For further confirmation, PCR products of ESBL genes were sequenced and blast via the NCBI blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Molecular typing:

The multilocus sequence typing of four *mcr-1* harboring isolates was carried out. The sequence result of 7 alleles, *adk, fumC, gyrB, icd, mdh, purA*, and *recA*, were determined from the MLST database (http://enterobase.warwick.ac.uk/species/index/ecoli). To further determine the genetic link among mcr-1 harboring isolates, the Xba I PFGE was performed according to PulseNet PFGE protocol [15]. DNA fingerprint analysis and sketch drawing were performed by BioNumerics v.8.0 (Applied Maths, Sint-Martens-Latem, Belgium). Clusters were examined by the unweight pair-group method with arithmetic mean (UPGMA) analysis. The Dice similarity coefficient was calculated with a position tolerance of 1.5%, and a dendrogram was created based on the UPGMA.

Transconjugation and PCR based replicon typing:

In order to detect the transferability of four colistin-resistant isolates, trans-conjugation experiments were performed. These isolates were selected as the donor, and *E. coli* EC 600 (Nal^R, Rif^R) were taken as recipients. The experiment was performed, as described previously [16]. The transconjugants were analysed using antibiotic susceptibility testing and *mcr-1* specific PCR, as described earlier. The conjugation transfer rates were calculated by dividing the number of transconjugants by the number of donors.

To further investigate the plasmid incompatibility type responsible for *mcr-1*, the plasmid DNA of transconjugants was extracted by the alkaline lysis method [17]. The plasmid type was determined by using the PBRT 2.0 kit (MBK0078, Diatheva, Italy). The PCR products of eight multiplex PCR were visualized on 2% Agarose gel, and the results were analysed according to the manufactured instructions.

S1 PFGE and southern blotting:

S1 PFGE and southern hybridization of the successfully trans-conjugant *E. coli* EC600 were performed to determine the plasmid location. For S1 PFGE, the bacterial isolates were embedded into 1% low melting agarose plugs. Following cell lysis and washing, the plugs were pre-incubated in 200µl of 1x S1 buffer at 37° c for 30 minutes. S1 digestion was performed by ten units of S1 enzymes (Thermo scientific) in 200 µl of 1x S1 buffer for 10 min at 37° c. The digestion was stopped by removing enzymes and adding 200 µl of 0.5M EDTA and left for 10 min at room temperature. Before running on the gel, the plug was incubated in 200 µl of TE buffer for 30 minutes. The products were electrophoresed on the CHEF mapper PFGE system (Bio-Rad USA) for 21 h at 6 V/cm with initial switch time 2.5 sec and final switch time 60 sec. The plasmids from the gel were transferred onto the nylon membrane via the capillary transfer technique [18]. Southern hybridization of plasmid DNA with a digoxin-labelled *mcr-1*-specific probe was performed according to kit instructions (Roche Diagnostics, Mannheim, Germany).

PCR mapping of *mcr-1* genetic context:

To explore the genetic context of *mcr-1*, whether pHNSHP45[5] like key genetic component surrounds it, we designed seven pairs of primers targeting *ParA, nikB, tnpA, mcr-1, hp, pilN*, and *virD4* genes. The plasmid DNA for PCR mapping was extracted by the alkaline lysis method and quantified by one drop [17]. The primers used for mapping are mentioned in Table S1. The PCR products were visualized on 1 % Agarose gel and subsequently sequenced and blasted via the NCBI blast tool.

Results

Bacterial isolation and colistin resistance screening:

Among the 545 *E. coli* isolates, four isolates (PKE051, PKE141, PKE196, and PKE211) showed resistance to Colistin on the broth microdilution method. Upon molecular confirmation through PCR, all the four isolates harbored *mcr-1* genes, while the other *mcr* genes were absent. Data about the isolates, location, patients, demography, samples, source, and colistin MIC values are presented in Table 1.

Table 1
samples sources, patient demography, and colistin MIC values.

Isolates ID	Microbiology Laboratory	patient		Sample source	Colistin MIC	mcr-1
		gender	Age			
PKE051	PIMS Islamabad	female	41y	urine	8mg/l	+ve
PKE141	PIMS Islamabad	female	32y	urine	16mg/l	+ve
PKE196	HMC Peshawar	female	25y	urine	4mg/l	+ve
PKE211	RMI Peshawar	male	28y	pus	4mg/l	+ve

Footnote: PIMS= Pakistan Institute of Medical Sciences, HMC= Hayatabad Medical Complex, RMI= Rehman Medical Institute. MIC= Minimum Inhibitory Concentration, +ve= positive

Antibiotic susceptibility testing and resistant genes:

All colistin-resistant isolates were MDR but were susceptible to meropenem and were MBL negative. Three isolates showed susceptibility to Chloramphenicol, Ciprofloxacin, and Fosfomycin, while one among them was susceptible to Tetracycline. All of the isolates were ESBL positive via DDST. The *bla*CTXM-15 gene was found in all of the isolates, while three isolates had the *bla*TEM-1 gene. The complete antibiogram data are presented in Table 2.

Table 2
Antibiogram of mcr-1 positive Isolates.

Isolates ID	MIC of Antibiotics in mg/L											ESBL	ESBL genes	MBL
	AMP	CTX	CAP	CIP	FOX	FOM	GEN	AZT	MEM	AN	TET	DDST		CDT
PKE051	>256	128	1	0.064	64	8	32	256	0.032	256	128	+ve	CTXM-15 TEM-1	-ve
PKE141	256	64	2	0.032	32	4	16	128	0.032	128	256	+ve	CTXM-15	-ve
PKE196	>256	64	2	1	32	128	64	128	0.032	128	128	+ve	CTXM-15 TEM-1	-ve
PKE211	256	256	8	4	16	32	4	128	0.064	64	1	+ve	CTXM-15/TEM-1	-ve

Footnote: ID= identification, MIC= Minimum Inhibitory Concentration, ESBL= extended spectrum β lactamases, MBL= Metallo β lactamases, AMP= Ampicillin, CTX= Cefotaxime, CAP= chloramphenicol, CIP= ciprofloxacin, fox= cefoxitin, FOM=fosfomycin, GEN= gentamycin, AZT=Aztreonam, An= Amikacin, TET= tetracycline, 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. Gray shaded cell= Light grey shaded cell= moderate resistant Not fill cell= susceptible

Molecular typing:

Our four *mcr-1* positive isolates were assigned to ST 405 (n=1), ST 156 (n=2) and ST 117 (n=1) according to Warwick MLST Database (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). The results sre presented in Table S2. The Xba I PFGE patterns of four *mcr-1* positive isolates are shown in figure 2. The PFGE pattern of two isolates from ST 156 shows resemblance to each other while the two other isolates from ST 405 and ST 117 show a different pattern.

Transconjugation and PBRT:

Assuming that the *mcr-1* gene is present on the plasmid, a transconjugation experiment was performed. All of the strains were successfully conjugated with *E. coli* EC 600. The conjugation rates are presented in Table 3. Single colony from the selected plates were analysed for antimicrobial susceptibility testing and resistant genes. The transconjugants showed resistance to Colistin, and the presence of the *mcr-1* gene was confirmed via PCR, while the ESBL genes were not detected in transconjugants. To further determine the Incompatibility type of *mcr-1* harboring plasmid, the PBRT was performed from the transconjugants. The results confirmed that all of the four isolates had Incl2 type of plasmid, as shown in figure 3.

Table 3
Conjugation rate of mcr-1 positive isolates to *E. coli* EC600.

Isolate ID	Transferability	Trans conjugants	Donor	Conjugation rate
PKE051	Yes	2.1 x 10⁻	4.5×10^{2}	$2.1 \times 10^{-}$
PKE141	Yes	1.8 x 10⁻	7.8×10^{3}	4.3×10^{-4}
PKE196	Yes	2.3 x 10⁻	3.9×10^{2}	1.6×10^{-4}
PKE211	Yes	2.7 x 10⁻	4.3×10^{2}	1.5×10^{-4}

S1 PFGE and southern blotting:

The S1 PFGE results of transconjugants indicate the transfer of a plasmid of size near to 60kb from donor strains. The presence of the *mcr-1* gene on the plasmid was confirmed from Southern hybridization, as shown in figure 4.

PCR mapping of *mcr-1* context:

In all colistin-resistant isolates, six genes in the nearby region of *mcr-1* were amplified and sequenced. However, the *tnpA* gene, in terms of amplicon size, showed an unexpected result. The uncertainty was cleared using the other pair of primers for *tnpA* loci (absence of *IsApl1*). This time the expected bands were visualized, and the sequencing result showed missing *IsApl1*. The genetic contexts of four *mcr-1* isolates in comparison with pHNSHP45 are shown in figure 5.

Discussion

This study identified, for the first time, *mcr-1* harboring *E. coli* from Khyber Pakhtunkhwa province and the capital city of Islamabad, Pakistan. The prevalence of mcr-1 was 0.73%, while three out of four isolates were found in urine samples. To date, only a single case of *mcr-1* harboring *E. coli* out of 29 ESBL positive clinical isolates from the Punjab province of Pakistan has been reported [11]. In another study, the *mcr-1* gene in *E. coli* was reported from migratory birds and broiler in the Faisalabad region [9 12]. The *mcr-1* gene has also been found in *Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* isolated from clinical samples [19 20].

The four isolates we report here were MDR and ESBL positive and have *the bla*CTXM-15 gene, while three of them also harbor the *bla*TEM-1 gene. The co-existence of *mcr-1* with ESBL has been frequently reported [21–23]. A recent study suggests that since 1980, there is an evolutionary link between ESBL and *mcr-1*; however, this statement needs further confirmation by tracing the *mcr-1* gene in archived ESBL isolates, which might provide clues regarding the kinetics over time between ESBL and *mcr-1*[24]. One study suggests that the co-occurrence of *mcr-1* and *bla*CTXM-15 is perhaps due to intricate genetic actions taken under antibiotic pressure [25].

In the present study, the clonal assortment of *E. coli* is diverse, that is, two of the isolates belong to ST 156, while two belonged to ST405 and ST 117, altogether different sequence types. The diversity in ST proposes that the distribution of plasmid mediated colistin resistance from 2018 to 2019 is multiclonal. In this study, the ST 405 and ST 156 having *mcr-1* are reported for the first time from Pakistan. The ST 405 and ST 156 have been previously reported in association with carbapenem-resistant *bla*NDM-5 from clinical isolates and chicken meat, respectively [26 27]. In other regions of the world, the ST 405 and ST156 harboring *mcr-1* have been reported, such as ST 405 in the United States and Algeria and ST 156 in China and Brazil [28–31]. ST 405 and ST156 belong to extraintestinal pathogenic *E. coil*, and avian pathogenic *E. coli* lineage, respectively. They are mainly involved in the global spread of *bla*CTXM-15 type extended-spectrum β lactamases [28 30]. A recent study from Pakistan reported *mcr-1* harboring ST117 in the broiler [32]. ST117 belongs to avian pathogenic *E. coli* lineage and may form a reservoir for human extraintestinal pathogenic *E coli* and antimicrobial resistance[33]. The detection of *mcr-1* in avian pathogenic and extraintestinal pathogenic *E. coli* in human clinical isolates is threatening because it supports the statement of *mcr-1* transmission via the food chain [34].

All the four isolates have been successfully trans-conjugated to *E. coli* EC600. The S1 PFGE, southern blot, and PBRT results revealed that the *mcr-1* gene was present on 60 Kb Incl2 plasmid. The Antibiotic susceptibility profiles of transconjugants showed that only the *mcr-1* antibiotic-resistant gene was present on Incl2 plasmid. The similar incl2 plasmid harboring only *mcr-1* antibiotic resistance gene had been reported in ST 155 isolated from a healthy broiler in Pakistan. The insertion sequence *IsApl1* of *mcr-1* in ST155 incl2 was missing, while the remaining genetic context was similar to pHNSHP45 [9]. The PCR based mapping of *mcr-1* genetic context in our isolates revealed that almost all amplified genes were similar to pHNSHP45, except the truncated tnpA gene, missing *IsApl1.* Globally, plasmid analysis of various Incompatibility types has shown that *IsApl1* is usually absent in IncX4 type plasmid while in Incl2 type plasmid, it is either absent or present [6]. The missing of *IsApl1* might be due to the co-evolution via the acquisition of *IsApl1* for speedy relocation into other plasmids [35]. Alternatively, it might reinforce the constancy of *mcr-1* on the plasmid [36]

The missing of *IsApl1* strengthens our statement of having the same plasmid in ST155 (detected earlier from healthy broiler) and in our isolates [9]. This finding of *mcr-1* on ST117 and similar plasmid profiles with a previously detected plasmid from a healthy broiler suggests that both horizontal transfer and transfer via food chain might be responsible for disseminating colistin resistance *mcr-1* gene in Pakistan.

Conclusion

We reported the *mcr-1* gene in human clinical isolates belonging to avian pathogenic *E. coli* lineage. The plasmid analysis and sequence typing indicate *mcr-1* spread via the food chain and horizontal transfer. It might have severe consequences in the community. Our data stresses the need for *mcr-1* prevention in humans, food chains, veterinary, and the environment by involving health care professionals, researchers, and government authorities to take concrete steps to prevent colistin resistance among the population.

Abbreviations

bla = Gene encoding β-lactamase

CLSI= Clinical and Laboratory Standards Institute

CMH= Combined Militry Hospital

CTX-M= Cefotaximase Munich, β-lactamase of ESBL-A type

HMC= Hayatabad Medical Complex

KTH= Khyber Teaching Hospital

LB= Luria-Bertani

mcr = mobilized colistin resistant gene

MLST= Multi locus sequence type

PBRT= PCR base replicon typing

PetN= Phosphoethanolamine

PFGE=Pulse Field Gel Electrophoresis

PIMS= Pakistan Institute of Medical Sciences

RMI= Rehman Medical Institute

ST= Sequence Type

TEM= Temoneira, β -lactamase of ESBL-A type.

Declarations

Ethics approval and consent to participate:

Not Applicable

Consent for publication:

Not Applicable

Availability of Data:

The data supporting the findings of this study are included within the manuscript and its supporting information.

Competing interests:

The authors declare that they have no competing interests.

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Author contributions:

Study concept and design: HB and XY, Acquisition of data: HB. FH, and MAK , Analysis and interpretation of data: HB, ZGH, JH and XY, Drafting of the manuscript: HB, XY, ZGH and JH, Critical revision of the manuscript for important intellectual content: XY, TR and MAK., Administrative, technical, material support and institutional study supervision: XY. All the authors read and approved the final version of the manuscript.

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Figures

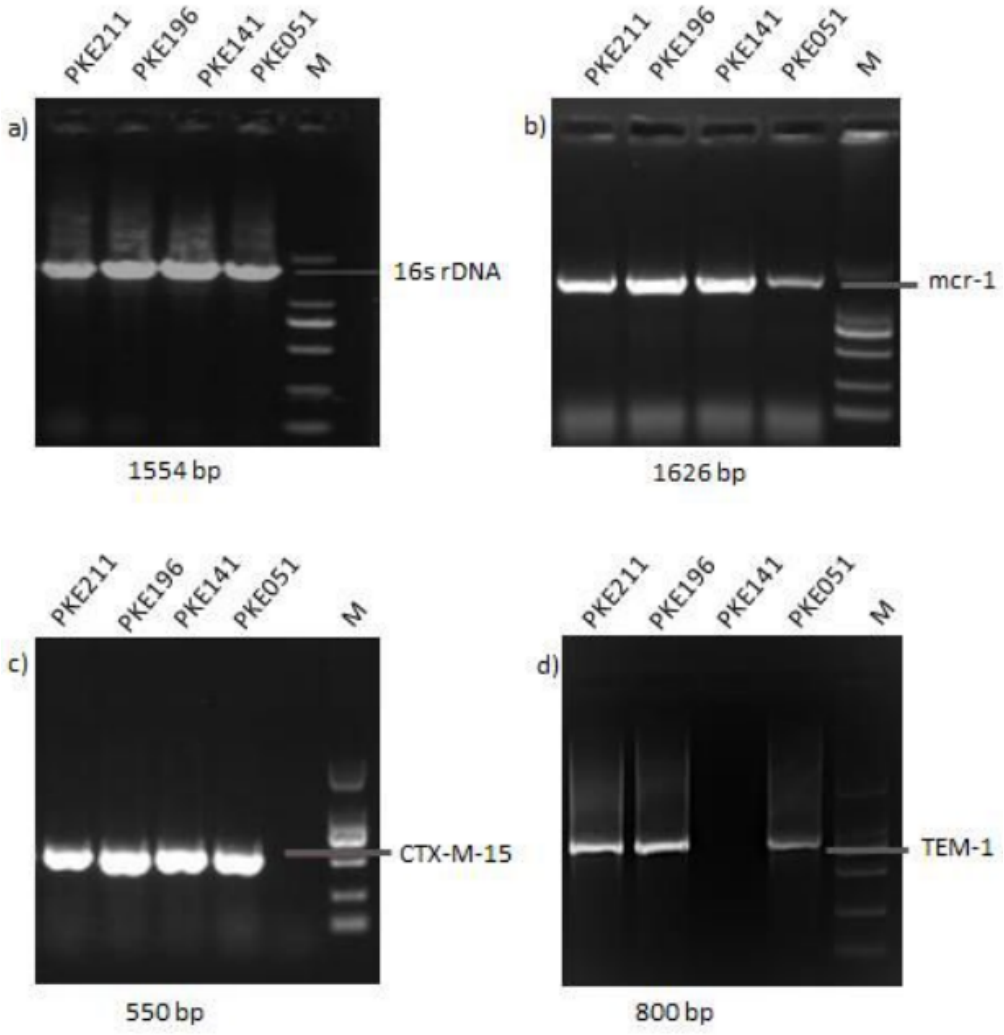

Figure 1

PCR results; a) 16s rDNA (1554bp), b) mcr-1 (1626bp), c) blaCTX-M-15 (550bp) and d) blaTEM-1 (800bp) on 1% Agarose gel, PKE051, PKE141, PKE196 and PKE211 are Isolates ID and M is for 2000pb marker.

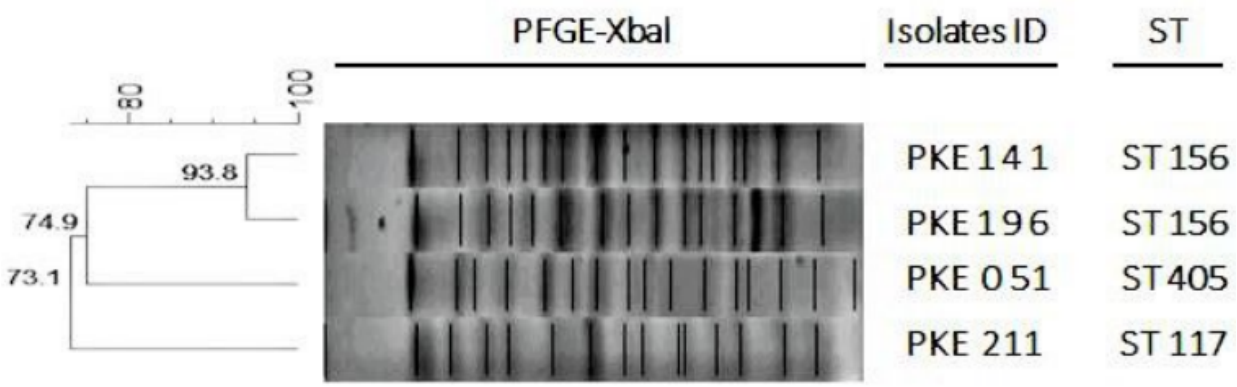

Figure 2

XbaI PFGE result of PKE141 (ST 156), PKE196 (ST 156), PKE051 (ST 405), PKE211 (ST 117), cluster analysis, and dendrogram is constructed by BioNumerics V 8.0, ST is abbreviated for sequence type.

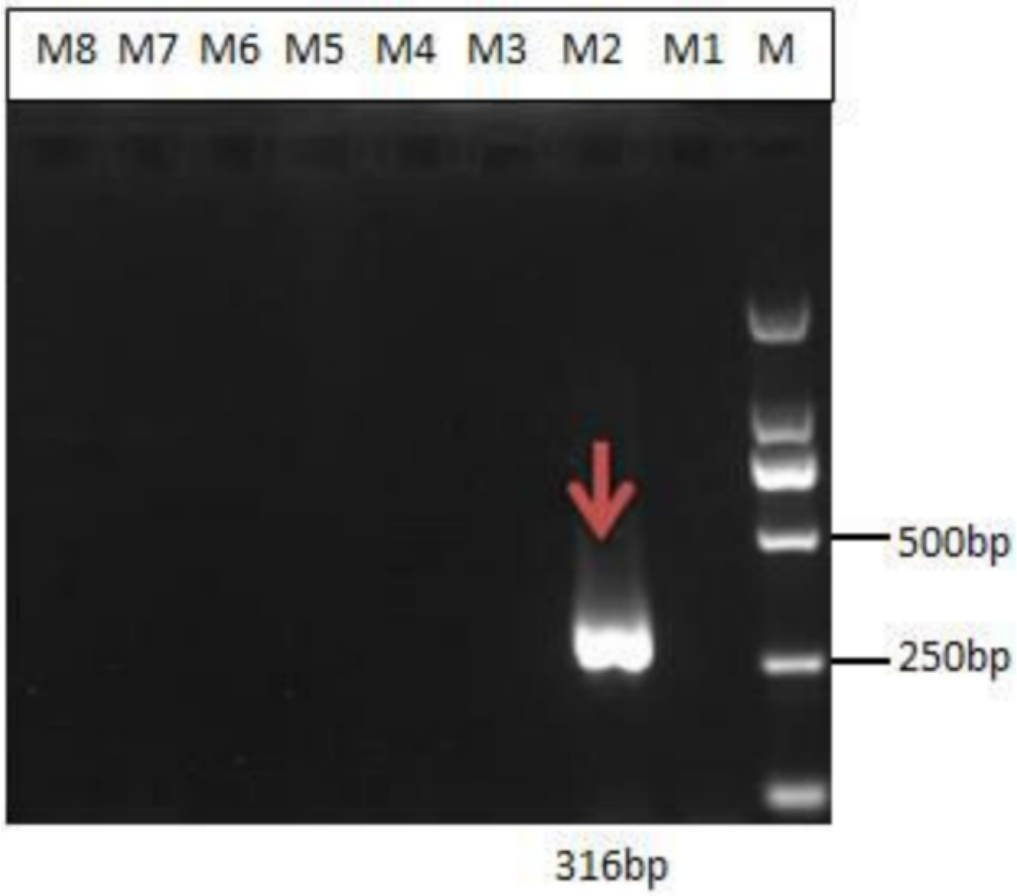

Figure 3

PBRT of transconjugants on 2% Agarose gel. M1 to M8 are the eight multiplex PCR, M is 2000bp marker, only M2 shows a band size of 316pb, indicating Incl2 type plasmid.

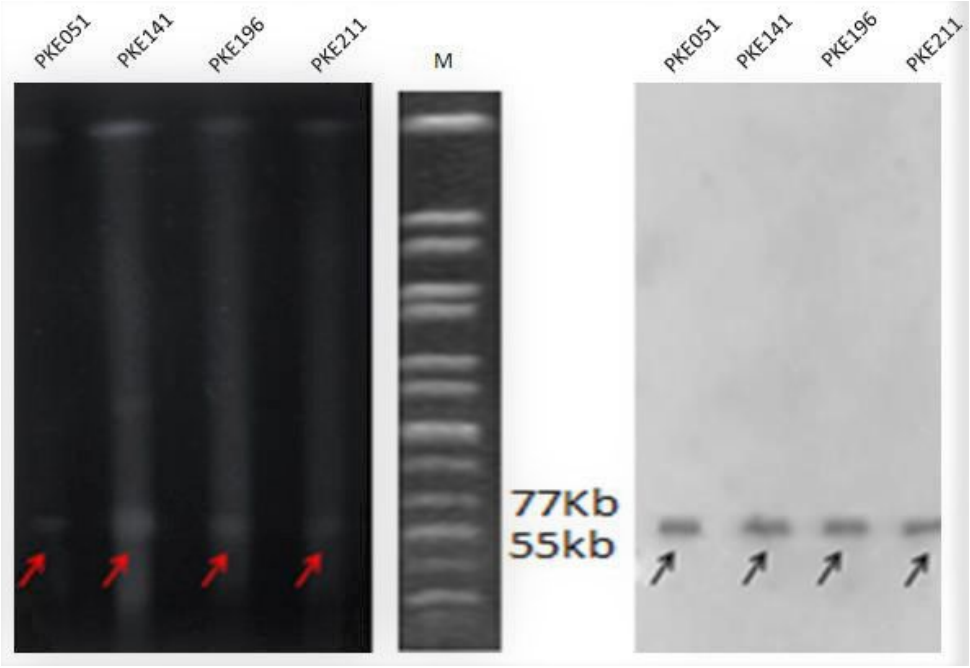

Figure 4

S1 PFGE and Southern blot, PKE051, PKE14, PKE196, and PKE211 are the isolates; M is the H9812 molecular size marker (1135 to 20.5kb) digested with Xbal enzymes. All strains show to 60kb plasmid on S1 PFGE gel and southern blot.

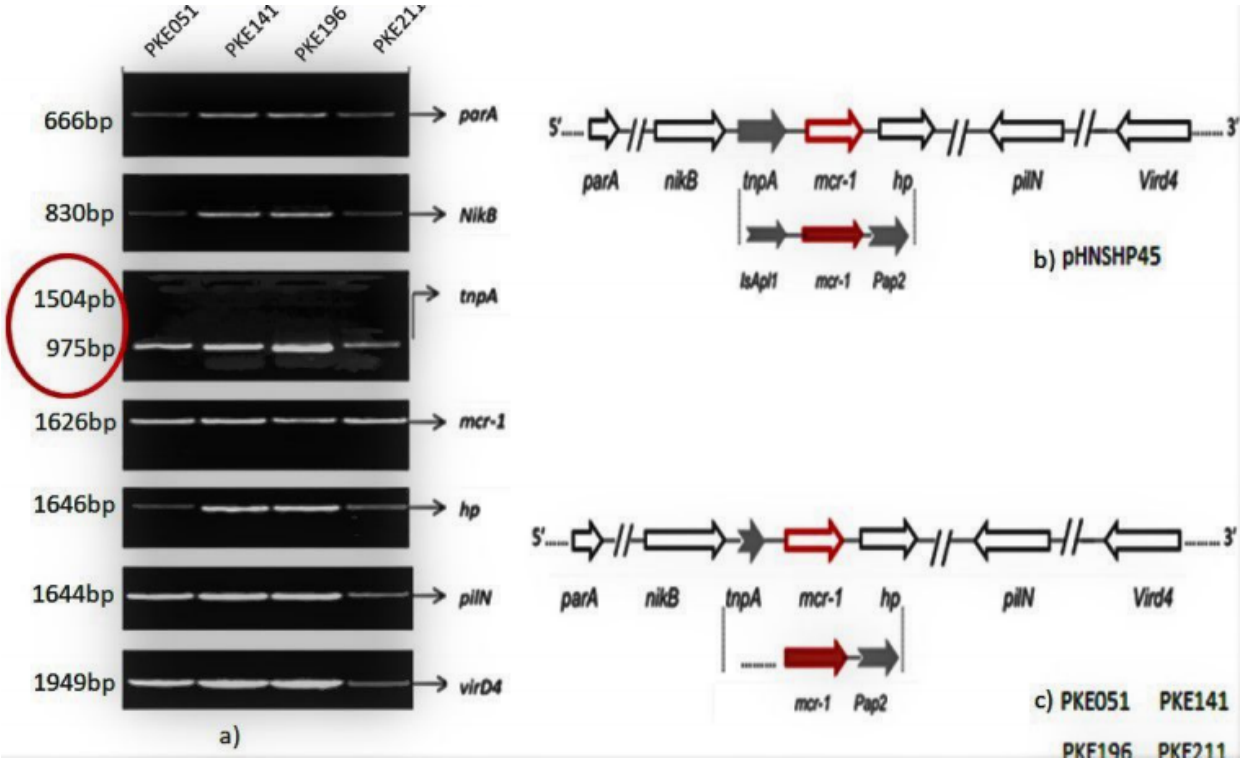

Figure 5

PCR based analysis of mcr-1 genetic context. a) PCR assay of six genes in the surrounding of mcr-1, red circle indicates the truncated band size of tnpA. b) Map of reference pHNSPH45 showing IsApl1 on upstream and Pap2 on downstream of mcr-1. c) Map of mcr-1 genetic context in our isolates showing missing of IsApl1 due to truncated tnpA.

Supplementary Files

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