Infection and Drug Resistance

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ORIGINAL RESEARCH

The first report of emerging mobilized colistin-resistance (*mcr*) genes and ERIC-PCR typing in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates in southwest Iran

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Background: The emergence of the plasmid-mediated *mcr* colistin-resistance gene in bacteria poses a potential threat for treatment of patients, especially when hospitalized. The aims of this study were to search for the presence of *mcr-1* and *mcr-2* genes among colistin-resistant *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) isolates from clinical specimens and to determine the fingerprint of strains by enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR) method.

Methods: In this study, 712 nonduplicate Enterobacteriaceae isolates from clinical specimens were examined. All of the isolates were subcultured on suitable media, and the isolated colonies were identified by standard biochemical tests. Antimicrobial susceptibility test on 7 antibiotics was performed by disk diffusion method, and minimal inhibitory concentration (MIC) of isolates to colistin was determined by the E-test method. These isolates were typed by ERIC-PCR method, and the presence of *mcr-1* and *mcr-2* genes was investigated by PCR method.

Results: Out of 712 nonduplicate Enterobacteriaceae, 470 isolates, including 351 (74.7%) *E. coli* and 119 (25.3%) *K. pneumoniae,* were detected. The results of antibiogram tests showed that most of the isolates (81.3%) were resistant to ceftazidime; however, the most susceptibility among of *E. coli* and *K. pneumoniae* isolates was observed (81.5%) to colistin. The typing results by ERIC-PCR method showed 36 and 23 fingerprint patterns for colistin-resistant *E. coli* and *K. pneumoniae* strains, respectively. Among 64 (13.6%) colistin-phenotypically-resistant Enterobacteriaceae, 8 isolates (1.7%) had *mcr-1* gene. These 8 isolates were attributed to *E. coli* and *K. pneumoniae* with 6 and 2 isolates, respectively. Whereas no isolates carrying the *mcr-2* gene was found. These colistin-resistant isolates displayed colistin MIC values >2 µg/ml in the antibiotic concentration by E-test method.

Conclusion: Spreading of Enterobacteriaceae strains harboring plasmid-mediated *mcr* could fail the colistin-included therapy regimen as the last line of treatment against multidrug-resistant bacterial infections.

Keywords: Enterobacteriaceae; colistin, *mcr-1*, *mcr-2*

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Introduction

Gram-negative rods, especially Enterobacteriaceae, are considered as the main organisms that cause nosocomial infections.[1,2] *E. coli* and *K. pneumoniae* are among the main strains associated with such infections. *E. coli* causes some major infections, including bacteremia, neonatal meningitis, urinary tract infections,

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intra-abdominal infections, sepsis and gastroenteritis in developing countries. *K. pneumoniae* causes early acquired lobar pneumonia, wound, soft tissue and urinary tract infections.[3]

Recently, the emergence of multidrug-resistant (MDR) phenotype as the consequence of antibiotics overconsumption in the treatment of human and animal diseases caused a global challenge to health systems. MDR was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories.[4–6]

Carbapenems are the choice drugs to treat such infections. However, with increasing global incidence of carbapenem resistance, colistin is now widely used as the last resort antibiotic for the treatment of carbapenem-resistant Enterobacteriaceae. Among carbapenem-resistant *K. pneumoniae* isolates, the rate of resistance to colistin has been reported to be 15% which is very worrying.[7]

Colistin uses lipopolysaccharide (LPS) as the bacterial target and disrupts the negative charge of the outer membrane.[8] Several studies proposed two main mechanisms for colistin resistance including LPS modifications and LPS removal. Other possible mechanisms are overexpression of efflux pumps, overproduction of capsule polysaccharide and production of colistinase.[8]

Resistance to colistin in gram-negative bacteria is attributed to a reduction in binding affinity to the outer membrane of LPS. Two-component systems (TCSs) including *PhoPQ* and *PmrAB*, as the regulatory systems, reduce the negative charge of lipid A and subsequently reduce the binding affinity of colistin to LPS.[9,10]

Recently, a plasmid transmitted resistance has been reported as the mobilized colistin resistance (*mcr*), and has been designated as *mcr-1*, which is the most prevalent *mcr* type.[11] The *mcr-1* gene is a phosphatidylethanolamine transferase, which adds this residue to lipid A component of the LPS, leading to a lower binding affinity of colistin to its target.[12] The *mcr-2* gene is a rare variant of *mcr*. The locations of *mcr-1* and *mcr-2* genes are on conjugative plasmid which may accelerate the transmission between strains and bacterial species, leading to breakdown of the therapeutic regimen.[13–16] The reports on the role of *mcr-1* and *mcr-2* genes indicated the increase in the resistance of *E. coli* and *K. pneumoniae* isolates.[7]

Several studies have reported the variants such as *mcr-3, mcr-4* and *mcr-5* in *E. coli* and *Salmonella* isolates.[17] Furthermore, a new plasmid-mediated colistin-resistance gene called *mcr-7.1* has been identified in *K. pneumoniae* in China.[18]

There are different methods to evaluate the clonality and genetic diversity of Enterobacteriaceae such as pulsed-field gel electrophoresis, multilocus sequence typing, multiple-locus variable-number tandem repeat analysis and enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR).[19]

In this study, we investigate the prevalence of *mcr-1* and *mcr-2* genes in colistin-resistant *E. coli* and *K. pneumoniae* clinical isolates. Also, the clonality of colistin-resistant E. coli and K. pneumoniae strains was evaluated by ERIC-PCR-based dendrogram. ERIC-PCR is cost-effective, easy to perform and a rapid method, which can be applied for comparison of clusters generated.[20]

Materials and methods

Bacterial isolates

A total of 470 isolates of *E. coli* and *K. pneumoniae* were detected among 712 Enterobacteriaceae isolates. These samples were isolated from different clinical specimens such as blood, urine, wound, etc. except stool. These isolates were collected from the laboratories of teaching hospitals collaborated with Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran) from January to June 2017. This study was approved by the Institutional Review Board (IRB) and Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences. The isolates were transferred to the Medical Microbiology Department and were subcultured on blood agar and eosin methylene blue agar (EMB Agar) media (Merck, Germany) for double checking and taking pure culture. After incubation of plates at 37°C for 18–24 hrs, the isolated colonies were identified by Gram staining and standard biochemical tests such as triple-sugar iron (TSI), Simmon's citrate, sulfide indole motility (SIM), urea, ornithine and lysine decarboxylase tests (Merck).[21]

Antimicrobial susceptibility testing (AST)

AST was performed using disk diffusion method (Kirby–Bauer) on Mueller–Hinton agar (Merck) plates according to the Clinical and Laboratory Standards Institute (CLSI) Guidelines.[22] The tested antibiotic disks were colistin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), imipenem (10 μg), ceftazidime (30 μg), azithromycin (15 μg) and amikacin (30 μg) (MAST Co., UK). The phenotype of Enterobacteriaceae was defined as MDR according to the International Expert proposal for Interim Standards Guidelines.[6]

Minimum inhibitory concentration (MIC)

Colistin MICs were measured by E-test strips (Liofilchem, Italy) and interpreted based on European Committee on Antimicrobial Susceptibility Testing (EUCAST, Ver. 6, 2016) guidelines. After measuring the MIC with E-test method, the *E. coli* and *K. pneumoniae* isolates with a MIC higher than 2 µg/ml were considered as "resistant".[23,24] *E. coli* ATCC 25,922 was used as a quality control for antimicrobial susceptibility testing.

DNA extraction and PCR amplification

DNA extraction was directly carried out by using the G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, South Korea) according to the manufacturer's instructions. The concentrations of extracted DNA from *E. coli* and *K. pneumoniae* isolates were measured by using a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

Detection of *mcr-1* and *mcr-2* genes by polymerase chain reaction (PCR)

DNA amplification was performed using a thermocycler (Eppendorf, Hamburg, Germany), and the set of primers as listed in Table 1.[25,26]

The master mix was prepared in a final volume of 25 µL containing 10× PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 10 µM of each primer, 5 U/µL of Taq DNA polymerase and 5 µL of extracted DNA as template. The DNA amplification was performed based on the following program: initial denaturation at 94°C for 5 mins, 25 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 mins. DNAs from *E. coli* KP81 and *E. coli* KP37 strains harboring *mcr-1* and *mcr-2*, respectively, were used as the positive controls. Genomic DNA from colistin-susceptible *E. coli* ATCC 25,922 was used as the negative control. The PCR products were separated by electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The bands were visualized under UV light using a gel documentation system (Protein Simple, Santa Clara, CA, USA).[23] PCR products were sequenced, checked with BLAST and annotated to GenBank.

Genbank accession number

The partial nucleotide sequences of *mcr-1* gene have been deposited in the GenBank database under accession number of MH627973 (*mcr-1* from *E. coli* strain EC9).

DNA fingerprinting

ERIC-PCR was applied using the ERIC-1 and ERIC-2 primers for colistin-resistant *E. coli* and *K. pneumoniae* isolates (Table 1).[27] The ERIC-PCR conditions were regulated based on the published report by Smith et al[28]. Analyses of the DNA fingerprints were performed by using GelCompar II; version 6.5 (Applied Maths, NV, Keistraat, Belgium). A cutoff value of 80% similarity was applied to define the clusters. The similarity between the profiles was evaluated with the band matching Dice coefficient, and dendrograms for each species were produced by the unweighted pair group method with arithmetic averages (UPGMA). Based on the UPGMA dendrograms, identical strains were defined as isolates with >97% similarity, closely related isolates with ≥95% similarity and isolates with <95% similarity as unrelated strains.

Statistical analysis

The results were analyzed by using SPSS version 22 software (IBM Armonk, North Castle, NY, USA). Statistical analyses were carried out by applying the Mann–Whitney, Chi-square and Kolmogorov–Smirnov tests, with a statistical significant *P*-value <0.05.

Table 1 Sequences of primers used for detection of *mcr-1* and *mcr-2* genes and ERIC-PCR

Target gene	Primer sequence (5′→ 3′)	Size (bp)	Annealing temperature (°C)	References
mcr1-F *mcr1*-R	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	309	51	25
mcr2-F *mcr2*-R	TGGTACAGCCCCTTTATT GCTTGAGATTGGGTTATGA	1,747	53	26
ERIC-F *ERIC*-R	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	Variable	51	27

Results

In the present study, among 712 examined Enterobacteriaceae isolates, 470 isolates were identified as *E. coli* (n=351, 74.7%) and *K. pneumoniae* (n=119, 25.3%). The remaining 242 isolates belonged to other Enterobacteriaceae species which were not in the scope of this study. These 470 isolates were collected from different clinical specimens and were screened for antibiotic resistance and presence of *mcr-1*and *mcr-2* genes. The distributions of *E. coli* and *K. pneumoniae* isolates in the specimens were as follows: urine, 87.4% (n=411); tracheal, 4.9% (n=23); blood, 3.4% (n=16); wound, 2.1% (n=10); discharge, 1.3% (n=6); and cord spinal fluid, 0.8% (n=4). It is important to mention that most isolates were collected from urine specimens.

Also, 55.95% (n=263) of isolates were from female, while 44.04% (n=207) of isolates were from male patients. The prevalence of *E. coli* and *K. pneumoniae* in different hospital wards is listed in Table 2. Outpatient clinic with 258 isolates (54.9%) and infectious ward with 2 isolates (0.4%) showed the highest and lowest rates, respectively.

On the basis of the results of AST, most isolates (81.3%) showed high resistance to ceftazidime. However, the most susceptibility among *E. coli* and *K. pneumoniae* isolates was attributed to colistin (81.5%). Antibiotics resistance patterns of *E. coli* and *K. pneumoniae* isolates which have been presented in Figure 1 show that 68.5% of the isolates are MDR phenotype.

Among 470 isolates, 13.6% (n=64) were identified as colistin resistant by disk diffusion method. Among colistin-resistant isolates, 59.4% (n=38) were *E. coli* and 40.6% (n=26) were *K. pneumoniae*. These isolates were then examined by E-test to evaluate the MICs. By using the E-test, only 30 isolates were confirmed as colistin resistant. In this study, a significant difference was between MIC and disk diffusion methods (P=0.001).

Based on the PCR results, 1.7% (n=8) of *E. coli* and *K. pneumoniae* strains carried *mcr-1* gene (Figure 2); however, none of the isolates harbored *mcr-2* gene. All *mcr-1* positive isolates were collected from urine specimens. There was not any statistically significant relationship between the presence of *mcr* genes with the aforementioned hospital wards (P=0.13) as well as with the gender of the patients (P=0.92).

Table 2 Prevalence of *E. coli* and *K. pneumoniae* in different hospital wards

	Number of isolates (%)		
Wards	***E. coli***	***K. pneumoniae***	**Total (%)**
Outpatient clinic	205 (43.6)	53 (11.3)	258 (54.9)
Emergency	50 (10.6)	11 (2.3)	61 (13)
ICU	10 (2.1)	13 (2.8)	23 (4.9)
NICU	4 (0.8)	14 (3)	18 (3.8)
Urology	28 (5.9)	5 (1.1)	33 (7)
Nephrology	8 (1.7)	2 (0.4)	10 (2.1)
Internal medicine	4 (0.8)	3 (0.6)	7 (1.5)
Pediatrics	20 (4.2)	5 (1.1)	25 (5.3)
Neurology	7 (1.5)	4 (0.8)	11 (2.3)
Surgery	7 (1.5)	3 (0.6)	10 (2.1)
Infectious	2 (0.4)	0	2 (0.4)
Link section	6 (1.3)	6 (1.3)	12 (2.5)
Total	351 (74.7)	119 (25.3)	470 (100)

Abbreviations: ICU, intensive care unit; NICU, neonatal intensive care unit.

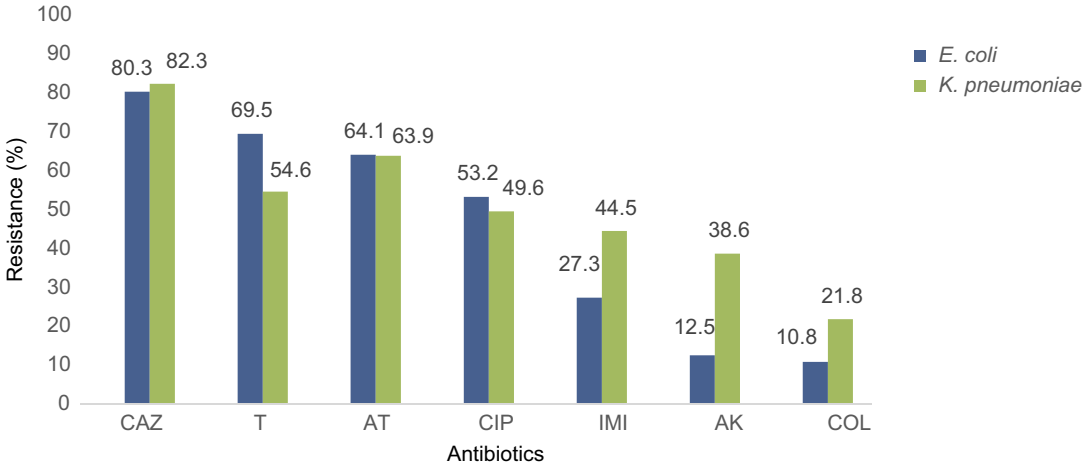


Figure 1 Antimicrobial resistance pattern of *E. coli and K. pneumoniae* isolates.
Abbreviations: CAZ, ceftazidime; T, tetracycline; AT, azithromycin; CIP, ciprofloxacin; IMI, imipenem; AK, amikacin; COL, colistin.

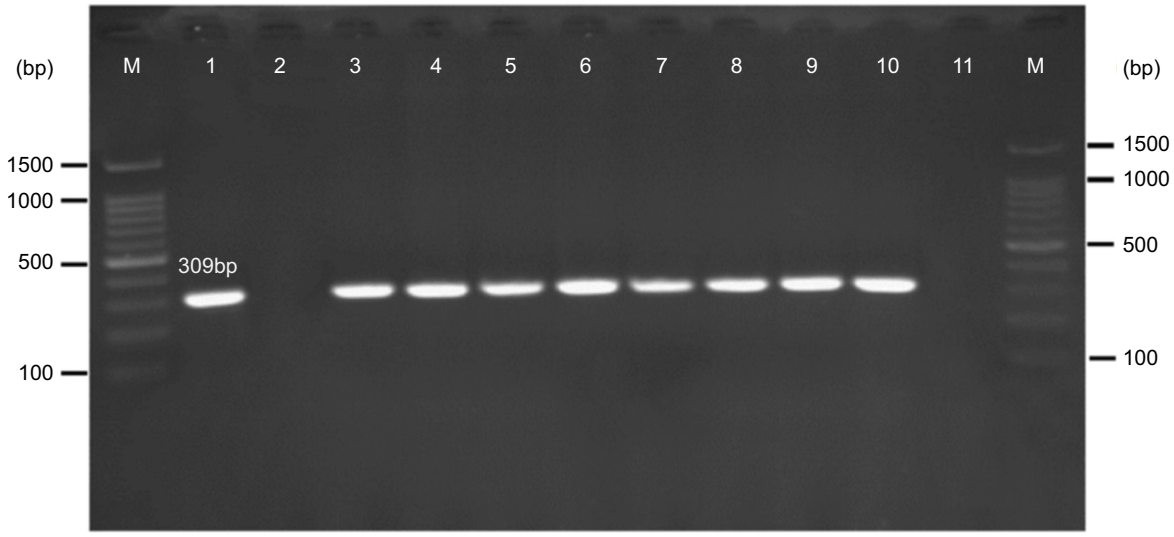


Figure 2 Gel electrophoresis of *mcr-1* gene (309 bp) encoding for colistin resistance. M: 100 bp DNA marker, line 1: positive control (*E. coli* KP81), line 2: negative control (*E. coli* 25,922), line 3: EC9, line 4: EC10, line 5: KP10, line 6: EC16, line 7: EC21, line 8: EC25, line 9: EC26, line 10: KP21, line 11: negative patient sample (*E. coli* isolates lack *mcr-1* gene).

The majority of *mcr-1*-positive *E. coli* and *K. pneumoniae* isolates were resistant to colistin, with MIC values in the rates of >2 µg/ml. Two *mcr-1*-positive isolates were reported to be colistin susceptible by the E-test method but were resistant to colistin by the disk diffusion method.

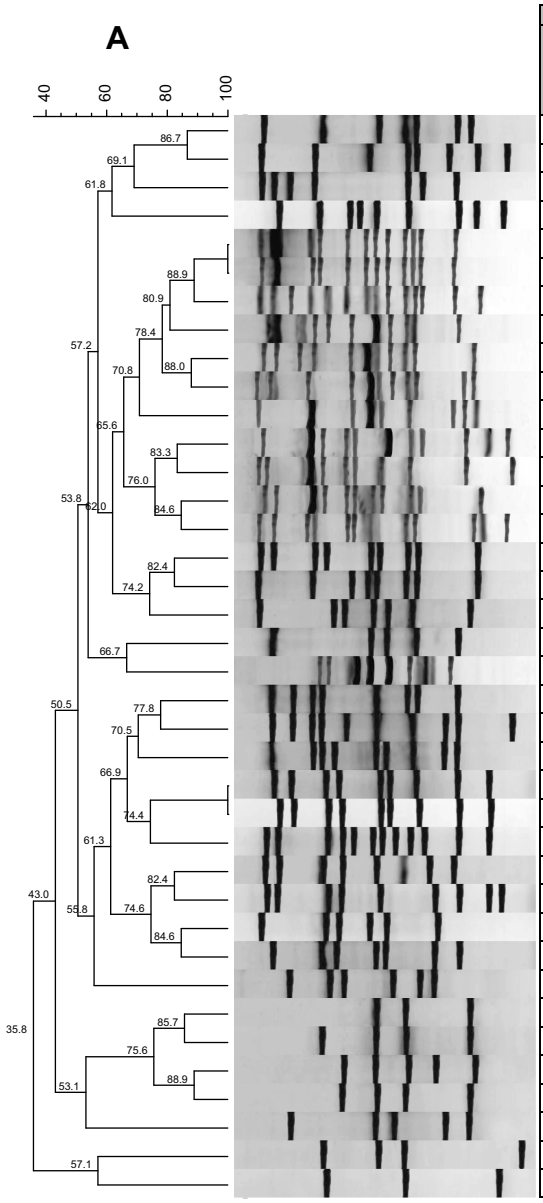
The determination of genomic diversity of 38 strains of phenotypic colistin-resistance *E. coli* and 26 strains of *K. pneumoniae* was carried out using the ERIC-PCR fingerprinting method. By analyzing the ERIC-PCR profiles, the 38 *E. coli* isolates were categorized into 36 ERIC-types, including 14 main clusters and 34 singletons. These 36 distinct types were then called A01 to A36. Moreover, 26 *K. pneumoniae* isolates were categorized into 23 ERIC types (called B01 to B23), including 11 main clusters and 20 singletons (Figure 3). The results of ERIC-PCR showed that the colistin-resistant strains in this study were categorized into different genotypes. However, 2 ERIC-types named ERIC-type A05 and ERIC-type A23 had the same genetic background. Complex fingerprint patterns were obtained for all the strains. Generally, the electrophoretic analysis of the PCR reaction products has revealed that the number of bands in particular electrophoretic paths ranged from 3 to 15. The sizes of the PCR products ranged from 100 bp to about 1,500 bp.

Discussion

The spread of antibiotics resistance to a wide variety of antibiotics such as beta-lactams, aminoglycosides and carbapenems is a global challenge to the health systems. Using colistin is regarded as the last resort for treating infections caused by MDR-gram negative rods, especially Enterobacteriaceae.[23,29] However, its nephrotoxicity and neurotoxicity impacts have reduced its application as a routine prescribed drug.[30]

In this study, ERIC-PCR typing method showed 36 patterns for 38 clinical *E. coli* isolates. 89.4% (n=34) of these isolates displayed a separate ERIC profile. The uniqueness of ERIC patterns suggests that they had a nonclonal transmission. However, 10.5% (n=4) of the isolates had identical patterns, indicating the similar origin of dissemination. In addition, ERIC-PCR typing method showed 23 patterns for 26 clinical *K. pneumoniae* isolates. Which 76.9% (n=20) of these isolates displayed a single ERIC profile, whereas, 23% (n=6) showed shared patterns. The isolates of the ERIC types, namely A05 and B08, had similar antibiotic resistance patterns. The ERIC type A23 dissemination with similar *mcr-1* gene could be causes anxiety in society. Genetic analysis using ERIC-PCR showed that majority of colistin-resistant isolates were clonally unrelated, suggesting that dissemination of these isolates was not due to a clonal outbreak.[31]

The results of this study showed a low rate of resistance against colistin for *E. coli* and *K. pneumoniae* (10.8% and 21.8%, respectively). The isolates were generally susceptible to amikacin; however, most of them were resistant to ceftazidime and tetracycline. In addition to colistin, most of the *mcr-1*-positive isolates were



Strains characterizations						
Strain No.	ERIC-types	Specimens	Colistin MIC (μg/ml)	mcr-1	mcr-2	Resistance profile(Kirby-Bauer)
EC20	A01	Urin	1.5	-	-	T, CIP, IMI, CAZ
EC4	A02	Urin	0.5	-	-	-
EC2	A03	Urin	1.5	-	-	AT
EC26	A04	Urin	>256	+	-	AT, T, CAZ, IMI, CIP
EC28	A05	Discharge	1	-	-	T, CIP, CAZ
EC29	A05	Urin	2.5	-	-	T, CIP, CAZ
EC27	A06	Urin	1.5	-	-	CAZ
EC32	A07	Urin	6	-	-	T, CAZ
EC30	A08	Urin	4	-	-	AT, IMI, CIP
EC33	A09	Urin	0.75	-	-	AT, T, CAZ
EC34	A10	Urin	1	-	-	AT, CIP
EC36	A11	Urin	0.5	-	-	T, CIP, CAZ
EC38	A12	Urin	2	-	-	AT, CIP
EC37	A13	Urin	12	-	-	AK, CIP, T, CAZ, IMI, AT
EC31	A14	Urin	2.5	-	-	T, CIP, IMI, CAZ
EC3	A15	Urin	6	-	-	CAZ
EC6	A16	Urin	0.25	-	-	AK, CIP, T, CAZ, AT
EC14	A17	Urin	1	-	-	T, AK, CIP, CAZ
EC5	A18	Urin	0.75	-	-	AK, CIP, T, CAZ, AT
EC35	A19	Urin	1.5	-	-	AT
EC7	A20	Urin	0.75	-	-	T, AT, CAZ
EC9	A21	Urin	2.5	+	-	T, AT, CAZ
EC8	A22	Urin	0.75	-	-	CAZ
EC21	A23	Urin	32	+	-	CAZ, IMI
EC25	A23	Urin	2	+	-	T, CIP, CAZ, IMI
EC24	A24	Urin	1	-	-	T, AT, CIP, CAZ, IMI
EC18	A25	Blood	>256	-	-	T, AT, CAZ, IMI
EC23	A26	Urin	>256	-	-	CAZ, IMI
EC1	A27	Urin	1.5	-	-	T, CIP, CAZ
EC10	A28	Urin	4	+	-	T, CAZ
EC11	A29	Urin	>256	-	-	AK, CIP, T, CAZ, IMI, AT
EC15	A30	Urin	8	-	-	AT, T, CAZ, IMI
EC17	A31	Urin	>256	-	-	CAZ, IMI
EC12	A32	Urin	>256	-	-	AK, CIP, T, CAZ, AT
EC16	A33	Urin	>256	+	-	AT, T, CAZ, IMI
EC13	A34	Urin	1.5	-	-	AT, CIP, CAZ
EC19	A35	Urin	1.5	-	-	T, AT, CAZ, IMI
EC22	A36	Urin	3	-	-	AK, T, CAZ, IMI

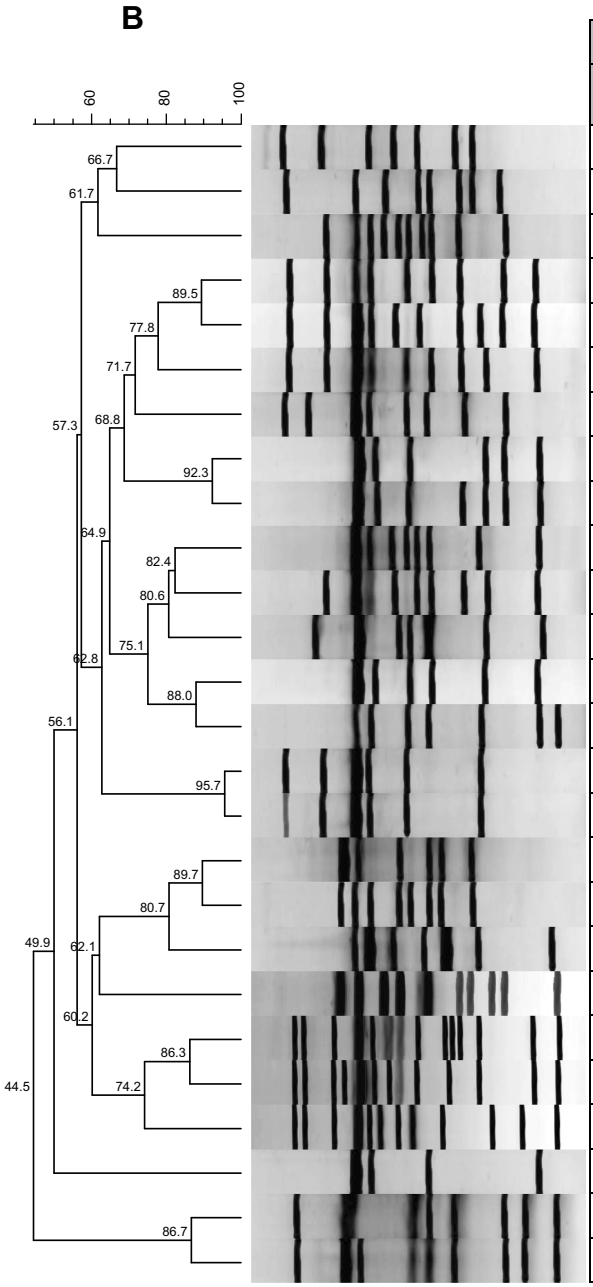
Figure 3 ERIC-PCR-based dendrogram. The analysis of ERIC-PCR profiles was performed using the Dice coefficient with optimization set at 0.5% and tolerance at 0.5%. (**A**) ERIC-PCR profiles from 38 phenotypic colistin-resistance *E. coli* isolates (**B**) and 26 *K. pneumoniae* isolates.

resistant to ceftazidime (87.5%) and were variably resistant to tetracycline (62.5%), azithromycin (62.5%), imipenem (50%) and ciprofloxacin (25%). Consistently, high resistance to ceftazidime and tetracycline was reported in previous studies.[32] The susceptibility rates of these isolates to amikacin were 100%.[33]

In our study, 53.1% (n=34) of the colistin-resistant isolates tested by disk diffusion were susceptible when examined by E-test. This observation suggested that the specificity and sensitivity of the E-test were higher than the disk diffusion method. In the E-test method, 10% of the *K. pneumoniae* strains were resistant to colistin, which was consistent with the study conducted by Singh et al (10.5%).[34]

The recent reports have shown plasmid harboring novel colistin-resistance genes, *mcr-1* and *mcr-2*, in some isolates such as *E. coli* and *K. pneumoniae*.[25,26] An *E. coli* isolate containing *mcr-1* gene which was sensitive to colistin has been before reported by Liassine et al.[35] Before Liassine et al, Pham et al reported the first *mcr-1*-positive but colistin-susceptible isolate, a *Shigella sonnei*.[36]

Our PCR results showed that among 470 *E. coli* and *K. pneumoniae* isolates, 6 *E. coli* isolates (1.2%) and 2 *K.*



Strains characterizations						
Strain No.	ERIC -types	Specimens	Colistin MIC (μg/ml)	mcr-1	mcr-2	Resistance profile(Kirby-Bauer)
KP18	B01	Urin	1.5	-	-	T, CAZ
KP9	B02	Urin	12	-	-	AK, CIP, T, CAZ, IMI, AT
KP12	B03	Blood	1	-	-	AK, CIP, T, CAZ, IMI, AT
KP21	B04	Urin	1	+	-	AT
KP22	B05	Urin	0.75	-	-	AT, CIP
KP20	B06	Urin	1	-	-	AT, CAZ
KP13	B07	Tracheal	2	-	-	AK, CIP, T, CAZ, IMI, AT
KP2	B08	Urin	1	-	-	T, CIP, IMI, CAZ
KP3	B08	Urin	4	-	-	T, CIP, IMI, CAZ
KP16	B09	Urin	>256	-	-	T, AT, AK, CAZ
KP19	B10	Urin	3	-	-	AK, CIP, CAZ, IMI, AT
KP7	B11	Tracheal	1	-	-	AK, CIP, T, CAZ, IMI, AT
KP1	B12	Urin	3	-	-	T, CAZ, CIP, IMI
KP11	B13	Tracheal	1.5	-	-	AT, AK, T, CAZ, CIP
KP14	B14	Tracheal	1.5	-	-	CAZ
KP15	B14	Blood	1	-	-	T, CIP, CAZ, AK
KP6	B15	Urin	0.75	-	-	T, CAZ, IMI
KP8	B15	Urin	1	-	-	T, AT, CAZ
KP17	B16	Urin	1.5	-	-	T, CIP, IMI
KP23	B17	Tracheal	16	-	-	T, CAZ
KP25	B18	Tracheal	8	-	-	AT, CIP
KP26	B19	Urin	32	-	-	T, CIP, CAZ
KP24	B20	Urin	8	-	-	AT, CAZ
KP10	B21	Urin	2.5	+	-	AT, CAZ
KP4	B22	CSF	12	-	-	AK, CIP, T, CAZ, IMI, AT
KP5	B23	Urin	24	-	-	CAZ

Figure 3 (Continued).

pneumoniae isolates (0.4%) had *mcr-1* gene. Consistently, Quan et al[29]. reported that among 1,494 *E. coli* and 571 *K. pneumoniae* isolates, only 20 (1%) isolates and 1 (<1%) isolate were *mcr-1* positive, respectively. The *mcr-2* gene was not detected in our study, consistent with other studies.[37–39]

The results of previous studies have shown the presence of *mcr-1* gene in 7 countries in Southeast Asia (China, Thailand, Laos, Japan, Vietnam, Cambodia and Malaysia).[40] In the studies conducted in Asia, the isolation rate of *mcr-1* gene is about 1%, which is consistent with our results (1.7%) (Table 3).[25,29,41]

In this study, among 8 isolates of positive *mcr-1* gene, 2 isolates were colistin sensitive (25%), which was in agreement with the Newton-Foot et al study (26%).[23] In both the studies, PCR was used as the molecular method which does not examine the gene expression. The identification of a colistin-susceptible/*mcr-1*-positive isolate in this study indicates that the silent spread of this gene might happen. The gene expression as well as the expression of

Table 3 Comparison of the previous studies with the current study

Authors	Isolates		References
	E. coli harbored *mcr-1* gene	*K. pneumoniae* harbored *mcr-1* gene	
	Number (%)	Number (%)	
Liu et al, 2015	13 (1.4)	3 (0.7)	25
Shen et al, 2016	104 (6.45)	–	43
Quan et al, 2017	20 (1)	1 (<1)	29
He et al, 2017	4 (0.6)	–	41
Moosavian and Emam	6 (1.2)	2 (0.4)	(Present study)

Note: (–) = Not accessible.

the gene by real-time PCR, in samples with higher MIC, could demonstrate the inactivation of *mcr-1* gene in some strains.[23] These studies show the importance of approving phenotypic methods by molecular methods.

Among 30 colistin-resistant isolates tested by E-test, only 26.6% (n=8) were harboring the plasmid-mediated colistin-resistance *mcr-1* gene. Therefore, other possible resistance mechanisms should be considered. These mechanisms are as follows: 1) inactivation of genes encoding proteins involved in the LPS biosynthesis (*lpxA, lpxC, lpxD*), 2) LPS modification by the regulatory role of the *PhoPQ* and *PmrAB* TCSs and 3) *mgrB* gene alterations.[42]

Continuous monitoring is very important for determining the exact frequency of *mcr-1* gene among gram-negative bacteria in both human and veterinary medicine. Reevaluation of polymyxins application in animals and implementation of large regular screening of animal isolates for *mcr* genes would be an important step in preventing the spread of these genes to the human isolates.[25]

Conclusion

Spreading of Enterobacteriaceae strains harboring *mcr* containing plasmids could fail the colistin-included therapy regimen which is used as a last line of treatment against MDR gram-negative bacterial infections. Increased surveillance of colistin-resistance mechanisms for monitoring their acquisition and spread is vital. Persistent efforts to ensure the judicious use of colistin (and indeed all antibiotics) both in agriculture and in health-care systems are welcome.

Ethics approval and informed consent

The study was approved by the research committee of the Ahvaz Jundishapur University of Medical Sciences (No: IR.AJUMS.REC.1396.331), Ahvaz, Iran. As the bacterial isolates were collected as a part of routine patient care investigation in the hospital, ethical approval was not required.

Consent for publication

All authors have consent for the manuscript publication.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Gu DX, Huang YL, Ma JH, et al. Detection of colistin resistance gene *mcr-1* in hypervirulent *Klebsiella pneumoniae* and *Escherichia coli* isolates from an infant with diarrhea in China. *ACS Infect Dis*. 2016;60 (8):5099–5100.

2. Yao X, Doi Y, Zeng L, Lv L, Liu JH. Carbapenem-resistant and colistin-resistant *Escherichia coli* co-producing NDM-9 and *MCR-1*. *Lancet Infect Dis*. 2016;16(3):288–289. doi:10.1016/S1473-3099(16)00057-8
3. Al-Agha AG. Extraction and purification of lipopolysaccharide of *Klebsiella pneumoniae* isolates. *Int J Curr Microbiol Appl Sci*. 2017;6(8):90–100. doi:10.20546/ijcmas.2017.608.012
4. Cannatelli A, Giani T, Antonelli A, Principe L, Luzzaro F, Rossolini GM. First detection of the *mcr-1* colistin resistance gene in *Escherichia coli* in Italy. *ACS Infect Dis*. 2016;60(5):3257–3258.
5. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis*. 2016;16(3):281. doi:10.1016/S1473-3099(16)30197-9
6. Magiorakos AP, Srinivasan A, Carey RB, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 2012;18(3):268–281. doi:10.1111/j.1469-0691.2011.03570.x
7. Di Pilato V, Arena F, Tascini C, et al. *Mcr-1.2*, a new *mcr* variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing *Klebsiella pneumoniae* strain of sequence type 512. *Antimicrob Agents Chemother*. 2016;60(9):5612–5615. doi:10.1128/AAC.01075-16
8. Kim Y, Bae IK, Lee H, Jeong SH, Yong D, Lee K. In vivo emergence of colistin resistance in *Acinetobacter baumannii* clinical isolates of sequence type 357 during colistin treatment. *Diagn Microbiol Infect Dis*. 2014;79(3):362–366. doi:10.1016/j.diagmicrobio.2014.03.027
9. Hankins JV, Madsen JA, Giles DK, Brodbelt JS, Trent MS. Amino acid addition to *Vibrio cholerae* LPS establishes a link between surface remodeling in gram-positive and gram-negative bacteria. *Proc Natl Acad Sci U S A*. 2012;109(22):8722–8727. doi:10.1073/pnas.1201313109
10. Petrou VI, Herrera CM, Schultz KM, et al. Structures of aminoarabinose transferase ArnT suggest a molecular basis for lipid A glycosylation. *Science*. 2016;351(6273):608–612. doi:10.1126/science.aad1172
11. Kline KE. Investigation of first identified *mcr-1* Gene in an isolate from a US patient—pennsylvania, 2016. *MMWR Surveill Summ*. 2016;65(36):977–978.
12. Hinchliffe P, Yang QE, Portal E, et al. Insights into the mechanistic basis of plasmid-mediated colistin resistance from crystal structures of the catalytic domain of *MCR-1*. *Sci Rep*. 2017;7:39392. doi:10.1038/srep39392
13. Falgenhauer L, Waezsada SE, Yao Y, et al. Colistin resistance gene *mcr-1* in extended-spectrum β-lactamase-producing and carbapenemase-producing gram-negative bacteria in Germany. *Lancet Infect Dis*. 2016;16(3):282–283. doi:10.1016/S1473-3099(16)00009-8
14. Bontron S, Poirel L, Nordmann P. Real-time PCR for detection of plasmid-mediated polymyxin resistance (*mcr-1*) from cultured bacteria and stools. *J Antimicrob Chemother*. 2016;71(8):2318–2320. doi:10.1093/jac/dkw139
15. Du H, Chen L, Tang YW, Kreiswirth BN. Emergence of the *mcr-1* colistin resistance gene in carbapenem-resistant *Enterobacteriaceae*. *Lancet Infect Dis*. 2016;16(3):287–288. doi:10.1016/S1473-3099(16)00056-6
16. 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):e01191–16. doi:10.1128/mBio.01191-16
17. Sun J, Zhang H, Liu YH, Feng Y. Towards understanding *MCR*-like colistin resistance. *Trends Microbiol*. 2018;26:794–808. doi:10.1016/j.tim.2018.02.006
18. Yang YQ, Li YX, Lei CW, Zhang AY, Wang HN. Novel plasmid-mediated colistin resistance gene *mcr-7.1* in *Klebsiella pneumoniae*. *J Antimicrob Chemother*. 2018;73:1791–1795. doi:10.1093/jac/dky111
19. Ross IL, Heuzenroeder MW. A comparison of three molecular typing methods for the discrimination of *Salmonella enterica* serovar Infantis. *FEMS Immunol Med Microbiol*. 2008;53(3):375–384. doi:10.1111/j.1574-695X.2008.00435.x
20. Adzitey F, Huda N, Ali GR. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. *3 Biotech*. 2013;3(2):97–107. doi:10.1007/s13205-012-0074-4
21. Till PM. *Bailey & Scott's Diagnostic Microbiology*. 14th ed. Missouri: Elsevier; 2016.
22. Wayne P. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. CLSI document M100-S25; 2015.
23. Newton-Foot M, Snyman Y, Maloba MR, Whitelaw AC. Plasmid-mediated *mcr-1* colistin resistance in *Escherichia coli* and *Klebsiella spp*. clinical isolates from the Western Cape region of South Africa. *Antimicrob Resist Infect Control*. 2017;6(1):78. doi:10.1186/s13756-017-0234-8
24. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0, 2016. Available from: http://www.eucast.org. Accessed March 30, 2016.
25. 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
26. Xavier BB, Lammens C, Ruhal R, et al. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill*. 2016;21(27):30280. doi:10.2807/1560-7917.ES.2016.21.27.30280
27. Ranjbar R, Ghazi FM. Antibiotic sensitivity patterns and molecular typing of *Shigella sonnei* strains using ERIC-PCR. *Iran J Public Health*. 2013;42(10):1151.
28. Smith JL, Drum DJ, Dai Y, et al. Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonizing broiler chickens. *Appl Environ Microbiol*. 2007;73(5):1404–1414. doi:10.1128/AEM.01193-06
29. 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
30. Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Updat*. 2010;13(4–5):132–138. doi:10.1016/j.drup.2010.05.002
31. Peymani A, Farivar TN, Sanikhani R, Javadi A, Najafipour R. Emergence of TEM, SHV, and CTX-M-extended spectrum β-lactamases and class 1 integron among *Enterobacter cloacae* isolates collected from hospitals of Tehran and Qazvin, Iran. *Microb Drug Resist*. 2014;20(5):424–430. doi:10.1089/mdr.2013.0191
32. Sonnevend Á, Ghazawi A, Alqahtani M, et al. Plasmid-mediated colistin resistance in *Escherichia coli* from the Arabian Peninsula. *Int J Infect Dis*. 2016;50:85–90. doi:10.1016/j.ijid.2016.07.007
33. Castanheira M, Griffin MA, Deshpande LM, Mendes RE, Jones RN, Flamm RK. Detection of *mcr-1* among *Escherichia coli* clinical isolates collected worldwide as part of the SENTRY antimicrobial surveillance program in 2014 and 2015. *ACS Infect Dis*. 2016;60(9):5623–5624.
34. Singh S, Pathak A, Kumar A, et al. Emergence of chromosome-borne colistin resistance gene *mcr-1* in clinical isolates of *Klebsiella pneumoniae* from India. *Antimicrob Agents Chemother*. 2018;62(2):e01885–17. doi:10.1128/AAC.01885-17
35. Liassine N, Assouvie L, Descombes MC, et al. Very low prevalence of *MCR-1/MCR-2* plasmid-mediated colistin resistance in urinary tract *Enterobacteriaceae* in Switzerland. *Int J Infect Dis*. 2016;51:4–5. doi:10.1016/j.ijid.2016.08.008

36. Pham TD, Thanh TH, Nguyen TNT, et al. Inducible colistin resistance via a disrupted plasmid-borne *mcr-1* gene in a 2008 Vietnamese *Shigella sonnei* isolate. *J Antimicrob Chemother*. 2016;71(8): 2314–2317.
37. Zurfluh K, Stephan R, Widmer A, et al. Screening for fecal carriage of *MCR*-producing *Enterobacteriaceae* in healthy humans and primary care patients. *Antimicrob Resist Infect Control*. 2017;6(1):28. doi:10.1186/s13756-017-0186-z
38. Terveer EM, Nijhuis RH, Crobach MJ, et al. Prevalence of colistin resistance gene (*mcr-1*) containing *Enterobacteriaceae* in feces of patients attending a tertiary care hospital and detection of a *mcr-1* containing, colistin susceptible *E. coli*. *PLoS One*. 2017;12(6):e0178598.
39. Schrauwen EJ, Huizinga P, van Spreuwel N, Verhulst C, Kluytmans-van den Bergh MF, Kluytmans JA. High prevalence of the *mcr-1* gene in retail chicken meat in the Netherlands in 2015. *Antimicrob Resist Infect Control*. 2017;6(1):83. doi:10.1186/s13756-017-0242-8
40. Ye H, Li Y, Li Z, et al. Diversified *mcr-1*-harbouring plasmid reservoirs confer resistance to colistin in human gut microbiota. *MBio*. 2016;7(2):e00177–16. doi:10.1128/mBio.00177-16
41. He QW, Xu XH, Lan FJ, et al. Molecular characteristic of *mcr-1* producing *Escherichia coli* in a Chinese university hospital. *Ann Clin Microbiol Antimicrob*. 2017;16(1):32. doi:10.1186/s12941-017-0207-z
42. Pragasam AK, Shankar C, Veeraraghavan B, et al. Molecular mechanisms of colistin resistance in *Klebsiella pneumoniae* causing bacteremia from India—a first report. *Front Microbiol*. 2017;7:2135. doi:10.3389/fmicb.2016.02140
43. Shen Z, Wang Y, Shen Y, Shen J, Wu C. Early emergence of *mcr-1* in *Escherichia coli* from food-producing animals. *Lancet Infect Dis*. 2016;16(3):293. doi:10.1016/S1473-3099(16)30197-9