ORIGINAL ARTICLE

First detection of mobilized colistin resistance *mcr-1* gene in *Escherichia coli* isolated from livestock and sewage in Iran

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Abstract

Currently, few studies have investigated the mechanisms of resistance to colistin in Iran. The aim of this study was to investigate *mcr*-harbouring *Escherichia coli* dissemination in livestock and sewage in Iran. A total of 115 samples from cows (n = 38), chickens (n = 47) and urban sewage samples (n = 30) were collected. The presence of genes including *mcr1–6* and *ampC* β-lactamase (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, bla_{FOX}) for colistin-resistant isolates was investigated by multiplex PCR method. Genetic association of colistin-resistant strains was also evaluated by ERIC PCR. Sixty-five isolates were identified as *E. coli*. Meaningless were resistant to colistin. The highest (26.1%) and lowest (3.07%) resistance were shown to ampicillin and meropenem respectively. Among the three colistin-resistant isolates, 2 (66%) were multidrug resistant, with one of them being *mcr-1* positive and the other one positive for DHA *ampC* β-lactamase gene. No *mcr2–6* genes were found. Minimum inhibitory concentration of *mcr*-producing isolate was 4 mg/L by microbroth dilution. This study reports, first the detection of *mcr-1* in *E. coli* from farm animals in Iran, a finding that is indicative of a global distribution of this plasmidic element and threatning the use of colistin as a last resort antibiotic. No clonal relationship was observed between the colistin-resistant *E. coli* isolates by ERIC-PCR. Monitoring the presence of these strains in animal sources help as to controlling the spread of resistance genes from animal to human is vital.

Keywords: Colistin resistance, Escherichia coli, livestock, *mcr-1*
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Introduction

The increasing prevalence of antibiotic resistance is one of the global health threats in the 21st century [1]. *Escherichia coli* (*E. coli*) is recognized as one of the major causes of nosocomial infections [1,2], acting as a reservoir of antimicrobial resistance genes (AMRs). Polymyxins, including polymyxin B and colistin, are the latest agents for the treatment of infections related to multidrug resistant gram negative bacteria (MDR-GNB) [2]. These agents primarily bind to the bacterial surface and reduce its integrity, increase its permeability and ultimately lead to the death of bacteria [3]. However, the use of colistin has been limited for treatment considering its nephrotoxic and neurotoxic effects [4]. By 2015, mutations in two-component regulatory systems, including *PmrB, PmrA, PhoP, PhoQ* and *MgrB,* were the only resistance mechanisms to colistin [5]. The mobilized colistin resistance (*mcr*) gene, conferring plasmid-mediated resistance to colistin, was first detected in China [2,3]. So far, ten different plasmid-mediated colistin resistance genes have been reported in the *Enterobacteriaceae* family. *E. coli* studies have particularly demonstrated that poultry and livestock can potentially carry isolates containing *mcr* genes; therefore, they can transfer drug-resistant bacteria to humans. Colistin is widely used in veterinary medicine to treat gastroenteritis in food-producing animals, especially pigs and poultry [6].

Despite the increasing prevalence of *mcr* plasmid–mediated colistin resistance among clinical isolates and the risk of

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transmission among Gram-negative bacteria, few studies have focused on the prevalence of colistin-resistant bacteria in sewage and livestock in Iran. Therefore, considering the overuse of antibiotics, such as colistin in animal husbandry in Iran. This study aimed to determine the prevalence of colistin-resistant isolates as well as the prevalence of *mcr* genes in *E. coli* in Iran.

Materials and methods

Sample collection

A total of 115 samples (Ethics Approval Code: IR.QUMS.REC.1399.163), such as rectal stool swab samples from cows ($n = 38$), chickens ($n = 47$) and urban sewage samples ($n = 30$) from two veterinary clinics, were collected between February and August 2019 in Iran (Qazvin and Karaj). The livestock samples were subcultured on MacConkey agar (MAC; Merck, Germany), and the wastewater samples were cultured on MacConkey agar medium containing 4 μg/mL of colistin for initial screening. After 18 to 24 hours of incubation at 37°C, the isolates were identified using standard phenotypic microbiological tests and API 20E commercial strips (bioMérieux, France). Finally, they were stored at −20°C in a tryptic soy broth (Merck; TSB, Germany) containing 15% glycerol for further analysis.

Antimicrobial susceptibility testing

The disc diffusion method was performed according to the Clinical and Laboratory Standards Institute (CLSI) 2019 guideline to determine the susceptibility of *E. coli* isolates to ciprofloxacin (5 μg), meropenem (10 μg), ampicillin (10 μg), ceftazidime (30 μg), ceftazidime/clavulanic acid (30 μg), cefotaxime (30 μg), clavulanic acid/cefuroxime (30 μg), cefuroxime (30 μg), cefepime (30 μg), nitrofurantoin (300 μg), trimethoprim/sulfamethoxazole (25 μg) and cefoxitin (30 μg) (MAST Co, UK) [7]. Also, *E. coli* ATCC 25922 was used as the susceptibility test quality control strain. For colistin, the MIC was measured using the broth microdilution method according to the CLSI 2019 guideline. Also, Etest strips (Liofilchem, Italy) were used to determine the MICs of meropenem, ciprofloxacin, ampicillin, ceftazidime, cefotaxime, cefuroxime, cefepime, nitrofurantoin, trimethoprim/sulfamethoxazole and cefoxitin for the colistin-resistant isolates. Moreover, extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase–producing isolates were detected by clavulanic acid/ceftazidime and clavulanic/cefotaxime using the double-disc synergy test on Müller-Hinton agar and boronic acid test respectively [7]. Multidrug resistance (MDR) was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories. Extensive drug resistance (XDR) was defined as nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories [8].

Extraction of DNA and Polymerase chain reaction (PCR)

Genomic DNA was extracted by Total DNA Extraction Kit (iNtRON Biotechnology, South Korea). The presence of resistance genes, including *mcr1–6* and AmpC β-lactamase (*ampC*) were detected, using multiplex-PCR assay. All primers and their target segments are listed in Table 1. For detection of *mcr* genes, PCR amplification was performed in a thermocycler (Veriti Thermal Cycler; Thermo Fisher Scientific, Denmark) based on the following programme: initial denaturation at 94°C for 15 minutes, 25 cycles of denaturation at 94°C for 35 seconds, annealing at °C (Table 1) for 90 seconds, extension at 72°C for 60 seconds and a final extension at 72°C for 10 minutes. Also, for detection of *ampC* genes (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, bla_{FOX}), a multiplex PCR assay was performed, with an initial denaturation at 94°C for 3 minutes, 25 cycles of denaturation at 94°C for 35 seconds, annealing at 61°C for 35 seconds, extension at 72°C for 60 seconds and a final extension at 72°C for 10 minutes. *E. coli* KP81 harbouring *mcr-1* gene and *E. coli* ATCC 25922 were used as the positive and negative controls respectively. DNA sequences were analysed in the online BLAST search tool (http://blast.ncbi.nlm.nih.gov/BLAST.cgi).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR assay

The ERIC PCR assay was performed in a final volume of 25 μL, containing 0.5 μL of each primer, 12.5 μL of 2 × Master Mix RED (Amplicon, Denmark), 2.5 μL of template DNA and 9 μL of deionized water. The primers used to discriminate the isolated *E. coli* strains are listed in Table 1. The PCR conditions have been described previously Moosavian et al. [14]. Analyses of the DNA fingerprints were performed using CLC Genomics Workbench 6.5 (Qiagen, USA). The PCR products were electrophoresed on 1.2% agarose gel (Merck, Germany) at 80 V for 150 minutes and visualized under ultraviolet light (UV). Next, the gels were analysed visually for distinct DNA profiles to detect polymorphisms in the isolates. The similarity between the isolates (difference of up to two bands) indicated a similar group.

GenBank accession number

The nucleotide sequences of the *mcr-1* gene from *E. coli* were submitted to the GenBank database under accession number MN539105.

Statistic analysis

Data analysis was performed using SPSS version 23.0 (IBM Armonk, North Castle, NY, USA). Descriptive data are shown as frequency and mean.

TABLE 1. Primers used in this study

Target	Sequence (5′–3′)	Annealing temperature (°C)	Reference
mcr-1	F: AGTCCGTTTGTTCTTGTGGC R: AGATCCTTGGTCTCGGCTTG	51	[9]
mcr-2	F: CAAGTGTGTTGGTCGCAGTT R: TCTAGCCCGACAAGCATACC	56	[9]
mcr-3	F: AAATAAAAATTGTTCCGCTTATG R: AATGGAGATCCCCGTTTTT	57	[9]
mcr-4	F: TCACTTTCATCACTGCGTTG R: TTGGTCCATGACTACCAATG	58	[9]
mcr-5	F: ATGCGGTTGTCTGCATTTATC R: TCATTGTGGTTGTCCTTTTCTG	58	[10]
mcr-6	F: GTCCGGTCAATCCCTATCTGT R: ATCACGGGATTGACATAGCTAC	55	[11]
ERIC	F: ATGTAAGCTCCTGGGGATTCAC R: AAGTAAGTGACTGGGGTGAGCG	51	[12]
bla_{DHA}	F: AACTTTCACAGGTGTGCTGGGT R: CCGTACGCATACTGGCTTTGC	61	[13]
bla_{CIT}	F: TGGCCAGAACTGACAGGCAAA R: TTTCTCCTGAACGTGGCTGGC	61	[13]
bla_{FOX}	F: AACATGGGGTATCAG GGAGATG R: CAAAGCGCGTAACCGGATTGG	61	[13]
bla_{MOX}	F: GCTGCTCAAGGAGCACAGGAT R: CACATTGACATAGGTGTGGTGC	61	[13]
bla_{EBC}	F: TCGGTAAAGCCGATGTTGCGG R: CTTCCACTGCGGCTGCCA GTT	61	[13]
bla_{ACC}	F: AACAGCCTCAGCAGCCGGTTA R: TTCGCCGCAATCATCCCTAGC	61	[13]

Abbreviations: F, forward; R, reverse.

Results

Bacterial strains and antibiotic resistance of islolates

In this study, a total of 65 isolates were identified as *E. coli* by phenotypic microbiological tests and API 20E commercial strips rectal stool swab samples from cows (n = 18) and chickens (n = 30) urban sewage (n = 17). These isolates were collected from different livestock and sewage samples. Resistance rates to ampicillin (26.1%), colistin (4.6%) and trimethoprim/sulfamethoxazole (13.8%) were observed in the isolates. However, only two (3.07%) isolates were resistant to meropenem. Eight isolates were recognized as ESBL by phenotypic detection. The antibiotic resistance patterns of *E. coli* isolates are presented in Table 2.

The antimicrobial susceptibility tests (MIC) showed that only 3 (4.6%) isolates were resistant to colistin (chicken, cow and sewage isolates), whereas the remaining 62 (95.3%) isolates were susceptible to colistin. All of the colistin-resistant isolates had MICs of 4 μg/mL. According to the PCR results, only one (33.3%) colistin-resistant isolate from cows harboured the *mcr-1* gene. From all the colistin-resistant isolates, only one isolate *mcr-1* positive, and it was MDR (Table 3). Out of 3 colistin-resistant isolates, 2 isolates were MDR. However, no *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* or *mcr-6* genes were detected. One isolate was positive for the bla_{DHA} gene.

TABLE 2. Antimicrobial susceptibility profiles of 65 *Escherichia coli* isolates in this study

Antimicrobial agent	Sensitive	Intermediate	Resistant
CTX	49 (75.5)	6 (9.2)	10 (15.3)
CTX	51 (78.4)	5 (7.6)	9 (13.8)
CIP	57 (87.6)	2 (3.07)	6 (9.2)
ME	42 (64.6)	21 (32.3)	2 (3.07)
AM	32 (49.2)	16 (24.6)	17 (26.1)
FM	56 (86.1)	7 (10.7)	2 (3.07)
FOX	55 (84.6)	2 (3.07)	8 (12.3)
CAZ	57 (87.6)	1 (1.5)	7 (10.7)
FEP	56 (86.1)	1 (1.5)	8 (12.3)
XM	44 (67.6)	16 (24.6)	5 (7.6)

Data are presented as n (%).

Molecular typing of *E. coli* strains

According to the genomic similarity analysis using the ERIC-PCR method, three colistin-resistant *E. coli* isolates were categorized into three ERIC types that were classified as type A1 to A3. Assessment of the genomic diversity of colistin-resistant strains showed that the isolates contained different genotypes. After electrophoresis, six to eight bands were observed on agarose gel; the size of these bands ranged from 190 to 2600 bp.

Discussion

Although colistin plays an essential role in the treatment of multidrug-resistant *Enterobacteriaceae*-associated infections, resistance to this antimicrobial agent has increased due to not only its overprescription in clinical settings but also its inappropriate use in veterinary medicine. Although so far no study has clearly shown the relationship between the use of colistin and improving animal growth, but today due to the cheapness of the drug and to improve animal growth conditions, the use of colistin in veterinary medicine has developed in Iran. In a study by Rhouma et al., it was shown that the oral use of colistin for the treatment of pigs in an experimental model was associated with selective pressure on *E. coli* population [15]. Therefore, the use of colistin as the first treatment of choice in intestinal infections in pigs should be avoided.

For decades, colistin has been used in veterinary medicine on all continents. Interestingly, the use of colistin in broilers and pigs has been shown to lead to the emergence of colistin-resistant *E. coli* isolates whereas initially, those isolates were sensitive to colistin [5,16]. Colistin-resistant isolates (positive *mcr-1*) are more likely to be found in samples of animal origin rather than human origin [17]. However, their prevalence in sewage treatment plants is yet mainly unknown [18]. In this study, we evaluated the presence of a plasmid-mediated colistin resistance gene in *E. coli* isolates from livestock and sewage samples. Significantly, this is the first study detecting *mcr-1* gene-harbouring *E. coli* isolates from livestock in Iran. Overall, the increasing rate of colistin resistance is a serious concern in the healthcare system of Iran. First time *mcr* gene was detected from colistin resistant urine strains within west of Iran but until

TABLE 3. Profile of colistin-resistant *Escherichia coli* isolates

Isolate	ERIC type	Specimen source	ESBL phenotype	MIC microbroth colistin (μ/mL)	MDR or XDR	*mcr-1*	*mcr-2, -6*	*ampC* genes	Resistance profile (Kirby-Bauer)
E.c1	A1	Sewage	+	4	MDR	—	—	+	CP, FOX, CTX
E.c2	A2	Chicken	+	4	—	—	—	—	CTX, AM, CAZ, FEP, CIP
E.c3	A3	Cow	+	4	MDR	+	—	—	XM, CTX, SXT, CP, AM, FM, FOX, CAZ, FEP

Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; ESBL, extended-spectrum β-lactamase; MRD, multidrug resistant; XDR, extensively drug resistant.

now, *mcr* gene was not reported in any animal study in Iran [14].

In the present study, 4.6% of isolates were resistant to colistin. Only one colistin-resistant isolate harboured *mcr-1* gene and the remaining colistin-resistant isolates lacked this gene. Therefore, other mechanisms may contribute to the resistance of these isolates to colistin, such as mutations in two-component regulatory systems and *mgrB* gene or inactivation of genes involved in lipopolysaccharide biosynthesis. The mechanism of colistin resistance could be also one of the *mcr* genes not screened for in this study [14]. In a recent study on 66 isolates from cow and pig samples in Ecuador, the prevalence of *mcr-1* gene (47%) was reported to be high among colistin-resistant *E. coli* isolates [19]. However, in our study, the prevalence of *mcr-1*–producing isolates was 4.6%, which is contradictory to studies from Thailand, Latin America, Switzerland and China [1,19–23]. This difference may be attributed to the source, number and geographical location of the samples being tested. Also, we did not detect *mcr-2* to *mcr-6* genes in our study, similar to the study by Moosavian et al., whereas in a study by Xavier et al. (2016) on 53 colistin-resistant samples, 11 isolates harbored *mcr-2* gene [24]. In the current study, the ERIC-PCR results showed that three colistin-resistant *E. coli* isolates had three ERIC types (A1–A3). The *mcr-1* strain, classified as ERIC type A3, contained an MDR resistance phenotype and showed resistance to colistin, cefuroxime, cefotaxime, trimethoprim/sulfamethoxazole, ciprofloxacin, ampicillin, nitrofurantoin, cefepime and cefoxitin. Also, this isolate showed intermediate sensitivity to meropenem; nevertheless, the Etest method showed its sensitivity to meropenem. Based on this finding, if these strains are transmitted from animals to humans, treatment will be challenging.

Conclusion

In conclusion, to this study is first report the *mcr-1* gene in a colistin-resistant *E. coli* isolate collected from animal samples in Iran. According to the present results, the spread of this gene in domestic animals and its possible transmission to humans raise public health concerns. To prevent the transmission of this gene to humans, it is necessary to the colistin-resistant strains. Proper administration of colistin and subsequently decreasing the selective pressure caused prevent the spread of antibiotic resistance.

Conflict of interest

None declared.

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