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-1-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 (blaMOX, blaCIT, blaDHA, blaACC, blaEBC, blaFOX) 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 threatening 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-meditated 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 [5].

Despite the increasing prevalence of mcr plasmid–mediated colistin resistance among clinical isolates and the risk of
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 (60°C to 24 hours of incubation at 37°C, the isolates were identified as resistant to at least one agent in all 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 [6].

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), cefazidime (30 μg), cefoxitin (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 used in 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, cefazidime, 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/cefazidime and clavulanic/cefotaxime using the double-disc synergy test on Mueller-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 [6].

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 mcr-1–6 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 (blaMOX, blaCP, blaDHA, blaACC, blaBEC, blaOXA), 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 KBP1 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 (Amblicon, 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 MNS39105.

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: AGTCCGGTTGTCTTGGC | 51 | [9] |
| mcr-2  | R: TACAGCGACTGGCCGCTGTAT | 56 | [9] |
| mcr-3  | F: AAATTACAAATGTGCGCTTATTG | 57 | [9] |
| mcr-4  | F: CTACCTTTCACTCGCTTTGC | 58 | [9] |
| mcr-5  | F: ATGGGCTGTGTCAGATTATC | 58 | [10] |
| mcr-6  | F: GTCCCCGTCATCCCTAGTCTG | 55 | [11] |
| ERIC   | F: AGTAAGCTCCTGGGGATTCAC | 51 | [12] |
| mcr-DHA| F: AAATTACACAGGTGGCTTGGT | 61 | [13] |
| mcr-CIT| F: CTACGGCATCAGTGCTTGGC | 61 | [13] |
| mcr-COX| F: AACATGGGGTGATCGGACATG | 61 | [13] |
| mcr-BAC| F: GCTGCTTCAGGACACAGGT | 61 | [13] |
| mcr-3  | R: CCTCCACTGGGGAATTGCCAAC | 51 | [12] |
| mcr-2  | R: AATGGAGATCCCCGTTTTT | 57 | [9] |

Abbreviations: F, forward; R, reverse.

Results

Bacterial strains and antibiotic resistance of isolates

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) 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.

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 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-harbouirng 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.
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-l 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 mcrB 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-l gene (47%) was reported to be high among colistin-resistant Escherichia coli isolates [19]. However, in our study, the prevalence of mcr-l-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 had three ERIC types (A1–A3). In the current study, the ERIC-PCR results showed that three colistin-resistant E. coli isolates had three ERIC types (A1–A3). The mcr-l 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-l 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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References

[1] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism mcr-l in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 2016;16:161–8.
[2] Control CfD, Prevention. Antibiotic resistance threats in the United States (2013) Centers for Disease Control and Prevention. Atlanta, GA: US Department of Health and Human Services; 2013. URL: https://www.cdc.gov/drugresistance/threat-report-2013/pdf/arthreats-2013-508.pdf.
[3] Wanty C, Anandan A, Piek S, Walshe J, Ganguly J, Carlson RW, et al. The structure of the nisselal lipoooligosaccharide phosphoethanolamine transferase A (LptA) required for resistance to polymyxins. J Mol Biol 2013;425(18):3389–402.
[4] Falagas ME, Rafaillidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Updat 2010;13(4-5):132.
[5] Fleury MA. Impact de traitements antibiotiques sur la flore digestive du porcelet: etude in vivo et developpement d'une approche en systeme de fermentation in vitro. PhD in Biology and Health Sciences; 2015. http://wwwtheses.fr/2015REN1B002. [Accessed 4 August 2016].
[6] Rhouma M, Beaudry F, Thérivalt W, Letellier A. Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and One Health perspectives. Front Microbiol 2016;7:1789.
[7] CLSI. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 4th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2019. CLSI Supplement.

[8] Majorako AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, 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):268–81.

[9] Rebele AR, Bortolai V, Kjeldsen JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, et al. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance purposes. Euro Surveill 2018;23(6):1700672.

[10] Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, AbuOun M, Stubber MJ. Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella enterica subsp. enterica serovar Paratyphi B. J Antimicrob Chemother 2017;72(12):3317–24.

[11] AbuOun M, Stubberfield EJ, Duggett NA, Kirchner M, Dormer L, Nunez-Garcia J, et al. Mcr-1 and mcr-2 variant gene identified in Moraxella species isolated from pigs in Great Britain from 2014 to 2015. J Antimicrob Chemother 2017;72(10):2745–9.

[12] Ranjar R, Ghazi FM. Antibiotic sensitivity patterns and molecular typing of Shigella sonnei strains using ERIC-PCR. Iran J Public Health 2013;42(10):1151.

[13] Gupta G, Tak V, Mathur P. Detection of AmpC β lactamases in Gram-negative bacteria. J Lab Physicians 2014;6(1):1.

[14] Moosavian M, Emam N. 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. Infect Drug Resist 2019;12:1001.

[15] Rhouma M, Beaudry F, Theriault W, Bergeron N, Beauchamp G, Laurent-Lewandowski S, et al. In vivo therapeutic efficacy and pharmacokinetics of colistin sulfate in an experimental model of enterotoxigenic Escherichia coli infection in weaned pigs. Vet Res 2016;47:58. https://doi.org/10.1186/s13567-016-0344-y.

[16] Aris P, Robatjazi S, Nikkhahi F, Amin Marashi SM. Molecular mechanisms and prevalence of colistin resistance of Klebsiella pneumoniae in the Middle East region: a review over five last years. J Glob Antimicrob Resist 2020;23(22):625–30.

[17] Ahmed S, Das T, Islam MZ, Herrero-Fresno A, Biswas PK, Olsen JE. High prevalence of mcr-1-encoded colistin resistance in commensal Escherichia coli from broiler chicken in Bangladesh. Sci Rep 2020;10(1):18637. https://doi.org/10.1038/s41598-020-75608-2.

[18] Lekunberri I, Balcázar JL, Borrego CM. Detection and quantification of the plasmid-mediated mcr-1 gene conferring colistin resistance in wastewater. Int J Antimicrob Agents 2017;50(6):734–6.

[19] Yamamoto Y, Calvopina M, Izurieta R, Villacres I, Kawahara R, Sasaki M, et al. Colistin-resistant Escherichia coli with mcr genes in the livestock of rural small-scale farms in Ecuador. BMC Res Notes 2019;12(1):121.

[20] Eiamphungporn W, Yainoy S, Jumderm C, Tan-Arsuwongkul R, Tiengrim S, Thamlikitkul V. Prevalence of the colistin resistance gene mcr-1 in colistin-resistant Escherichia coli and Klebsiella pneumoniae isolated from humans in Thailand. J Glob Antimicrob Resist 2018;15:32–5.

[21] Al-Kadmy IMS, Ibrahim SA, Al-Saryi N, Aziz SN, Besinis A, Hetta HF. Prevalence of genes involved in colistin resistance in Acinetobacter baumannii: first report from Iraq. Microb Drug Resist 2020;26(6):616–22.

[22] Rapoport M, Faccone D, Pastern F, Ceriana P, Albornoz E, Petrozzi A, et al. First description of mcr-1-mediated colistin resistance in human infections caused by Escherichia coli in Latin America. Antimicrob Agents Chemother 2016;60(7):4412–3.

[23] Liassine N, Assouvie L, Descombes MC, Tendon VD, Kieffer N, Poirol L, 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;51:4–9.

[24] Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, et al. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli. Belgium. Euro Surveill 2016;21(27):30280.