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Plasmid-Mediated colistin resistance in *Escherichia coli* isolated from neonatal dairy calves without prior consumption of colistin: A threat to public health

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Abstract

Background: Colistin is the most significant last-line antibiotic for the treatment of multidrug-resistant infections caused by Gram-negative bacteria, especially the Enterobacteriaceae family. The emergence and rapid spread of the plasmid-mediated resistance gene, mcr-1 (mobilized colistin resistance), in some isolates of *Escherichia coli* in recent years provoked public health concerns since it has been shown that mcr-1 with other resistance genes, such as ESBLs (extended-spectrum beta-lactamases) and carbapenemases, could be carried on a single plasmid concurrently. The excessive consumption of colistin, particularly in the livestock industry, and the transmission of these resistant bacteria from livestock to humans may potentially increase the risk of the spread of resistance in humans. Therefore, this study aimed to detect the prevalence of mcr and carbapenem resistance genes among neonatal calves in Mashhad, Razavi Khorasan Province, Iran.

Methods: In the current study, 200 fecal samples from healthy and diarrheic neonatal calves (⩽35 days old) were collected in Mashhad (190 E. coli strains were isolated). Antibiotic susceptibility to ceftazidime, cefepime, cefixime, meropenem, colistin, and ciprofloxacin was examined. The double-disk diffusion method (ceftazidime + ceftazidime/clavulanic acid) was performed on Mueller-Hinton agar (MHA) media to phenotypically distinguish the ESBL producers. Afterward, the Multiplex polymerase chain reaction (PCR) method was used to detect colistin resistance genes (mcr-1, mcr-2, mcr-3, mcr-4, and mcr5), NDM-1 (New Delhi metallo-beta-lactamase 1), and OXA-48 as carbapenemases.

Results: The results of the resistance rate to antibiotics were cefepime, ceftazidime, cefixime, meropenem, and colistin. Based on the findings, 33.7% were phenotypically ESBL producers, 4.21% harbored mcr-1, and no NDM-1 or OXA-48 was detected. Among the mcr-1-positive isolates, 5 strains showed the ESBL phenotype.

Conclusion: The results highlight the need for continued monitoring of antibiotic resistance in livestock and the potential for transmission to humans. The findings also underscore the importance of responsible antibiotic use in both human and animal health to mitigate the spread of antibiotic resistance.

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Introduction

Antibiotic-resistant infections are responsible for more than 2.8 million illnesses in the United States each year, and more than 35,000 people die due to diseases (1, 2). The increasing prevalence of multidrug-resistant bacteria (MDR) is particularly alarming as infection with these microorganisms increases the use of broad-spectrum antibiotics, such as third and fourth-generation cephalosporins and carbapenems. Enzymes such as broad-spectrum beta-lactamases (ESBLs) and carbapenemases can inactivate these antibiotics (3, 4).

According to the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), Enterobacteriaceae carrying these enzymes are among the most serious threatening agents in terms of antibiotic resistance (1, 2). Meanwhile, the rapid increase in the population of carbapenemase-producing Enterobacteriaceae (CPE), which contain enzymes such as KpC-2 (*Klebsiella pneumoniae* carbapenemase 2), NDM-1 (New Delhi metallo-β-lactamase 1), and OXA-48, has been incrementally intensified (5, 6). While resistance to carbapenems is more widespread in humans, there have been reports of resistant bacteria to these antibiotics in food-producing animals (poultry, cattle, and pigs) and domestic animals (dogs, cats, and horses). This problem is especially worrying because carbapenems have not been consumed in animal treatment; besides, antimicrobial resistance (AMR) is not a problem only for pathogenic bacteria but also for commensal intestinal microbiota (7). Carbapenems, including imipenem, meropenem, ertapenem, and doripenem, are broad-spectrum antimicrobials against Gram-negative bacteria are a class of antibiotics with a beta-lactam ring that blocks transpeptidation by binding to the active sites of the penicillin-binding proteins (PBPs) irreversibly, thereby disrupting the bacterial cell wall synthesis and, finally, causing cell death (8).

A review study conducted in 2017 showed that the prevalence of carbapenem-resistant Enterobacteriaceae (CRE) among population-related samples is about 5.6% to 10.8% (9). The emergence of these carbapenemase-producing bacteria (CPs) narrowed the available antimicrobial choices for therapeutic interventions to tigecycline and colistin, either alone or in combination with other antimicrobials (10). Thus, the global spread of CPE has increased the use of colistin, even with the awareness of the probability of developing resistance to colistin (11, 12). This sensitive harmony in the clinical necessity of resistance prevention has already been jeopardized by humans using antibiotics in agriculture (livestock), for example, in the treatment of ruminant neonatal diarrhea syndrome; thus, some countries use colistin extensively in the livestock industry (13, 14). This rise in colistin use doubled concerns with the first report of the plasmid gene for the resistance to colistin mcr-1 (mobilized colistin resistance) in November 2015 in China (15). Furthermore, colistin belongs to the family of polymyxins, cationic polypeptides with broad-spectrum activity against Gram-negative bacteria, including many species of the Enterobacteriaceae family (11).

The 2 polymyxins currently in clinical use are polymyxin B and polymyxin E (colistin); a mere difference in an amino acid has led to a significant biological difference (15). The mechanism of resistance to polymyxins is induced by a change in lipid A, leading to a decrease in antibiotic binding. Additionally, the plasmid gene for colistin resistance encodes phosphoethanolamine transferases, which add a group of phosphoethanolamine to lipid A in lipopolysaccharide (LPS) (16, 17).

By the time the mcr gene was reported, the chromosome-related mechanisms of colistin resistance had been found; these mechanisms included the involvement of regulatory systems such as PmrA/B and PhoP/Q, which resulted in lipid A modification by fractions such as phosphoethanolamine or 4-amino-4-arabinose, or in rare instances, the loss of the entire LPS (18, 19). These days, mcr-producing bacteria have been reported in many parts of the world (20).

Numerous reports have shown that the concomitant presence of the mcr gene with other resistance genes, such as ESBL and CRE, in *E.coli* and *K. pneumoniae* is likely to lead to widespread drug resistance and increased treatment difficulty (21, 22). The presence of *E. coli* as a commensal infection in the gastrointestinal tract of livestock can make it a reservoir for the acquisition and transmission of resistance (7, 23). Several studies revealed the spread of antibiotic-resistant bacteria by animal manure to humans, based on which evidence of the prevalence of antibiotic resistance among farmworkers has been obtained (24, 25). According to the data released by the Ministry of Agriculture of Iran and other studies, antibiotic consumption in agriculture, especially in cattle and poultry farms, is higher than it is in the Organization for Economic Cooperation and Development (OECD) countries; as a result, the possibility of increasing the incidence of antibiotic resistance in the livestock sector in Iran is also expected (26, 27). Due to the lack of sufficient information about the incidence of colistin resistance in livestock, which is critical for human health, this study aimed to

investigate the prevalence of plasmid-mediated colistin resistance and the co-occurrence of NDM-1 and OXA-48 in *E. coli* isolates from neonatal calves (<35 days old) in Mashhad, Iran.

Methods

A total of 200 fecal samples were collected from 4 semi-industrial dairy farms from neonatal calves (<35 days old) using a rectal swab. Out of all samples, 10 were excluded from the study. Of the remaining samples 60% (total: 55 male and 59 female) were from calves with clinical signs of diarrhea, and 40% (total: 40 male and 36 female) were from normal cases. Ethics approval and consent to participate in the study (from the owner) were obtained according to the research and ethics guidelines and approval of local institutions (Ferdowsi University of Mashhad).

None of the calves had a history of receiving antibiotics, including colistin, near the time of sampling. Specimens were transferred to the Microbiology Laboratory of the School of Veterinary Medicine (Ferdowsi University of Mashhad, Iran) in a buffered peptone water medium (Merck, Germany), cultured on MacConkey agar and blood agar (Merck, Germany), and incubated for 18±2 hours at 37 °C. The suspect lactose-positive colonies were used for routine biochemical confirmation tests for *E. coli* detection (28). Finally, the isolates were stored at -20 °C in a Brain heart infusion broth (Merck, Germany) containing 15% glycerol for further analysis.

The antibiotic susceptibility test was performed for 6 commonly allowed antimicrobials in the treatment protocols of human enteric infections using the disk diffusion method based on the protocol recommended by the Clinical Laboratory Standard Institute (CLSI, 2020).

Briefly, bacterial suspension inoculum equal to 0.5 McFarland standard (approximately 10^8 CFU/mL) was provided and inoculated into the Mueller-Hinton agar (MHA) (Merck, Germany). After the disks were placed (Cypress Diagnostics, Belgium), incubation was performed at 35 °C for 18-20 hours. The diameter of the growth inhibition zone was measured and recorded according to the CLSI. The disks included ceftazidime (30 µg), cefixime (5 µg), cefepime (30 µg), meropenem (10 µg), ciprofloxacin (5 µg), and colistin (10 µg). There are no polymyxin breakpoints established for Enterobacteriaceae by the CLSI; therefore, initial screening of the susceptibility of colistin-resistant isolates to colistin was estimated using the CLSI-recommended susceptibility breakpoints for *Pseudomonas aeruginosa* (10-mg colistin disk; resistant ≤10 mm, susceptible ≥11 mm). The double-disk test (ceftazidime + ceftazidime/clavulanic acid) was carried out to discriminate the ESBL-producing isolates.

For further evaluation, the colistin MIC of the ESBL-producing isolates and those showing resistance to colistin disks was performed by E-test strips. The automated instrument antimicrobial susceptibility testing (AST) VITEK-2 was used to evaluate the antimicrobial activity of potentially mcr-positive isolates. Although the agar diffusion method is not recommended by the CLSI for this antibiotic, it is the most available and uncomplicated method for the determination of antibiograms that various companies still produce, such as disks and E-test strips.

Confirmed *E. coli* isolates were cultured on the LB agar medium and incubated at 37 °C for 18± 2 hours. Then, each colony was harvested with a sterile loop, transferred to 5 cc of the TSB medium, and incubated for 18± 2 hours. After incubation, 1 mL of the culture solution was transferred to 1.5 mL microtubes and centrifuged at 13000 Xg for 3 minutes. The supernatant was then discarded; 250 mL of sterile distilled water was added to the precipitate, vortexed, and centrifuged again for 3 minutes at 13000 Xg. Later, the supernatant was separated, and 200 mL of sterile distilled water was added to the sediment and vortexed for 30 seconds. The microtube was placed in a boiling water bath for 10 minutes and immediately cooled with ice. Subsequently, the microtubes were centrifuged at 11000 Xg for 5 minutes after cooling. Finally, 0.1 mL of the supernatant containing DNA (deoxyribonucleic acid) was removed and stored in a 0.5-mL microtube at -20 °C for molecular evaluation (29).

The multiplex polymerase chain reaction (PCR) method was employed to identify 5 genes of colistin resistance (mcr-1, mcr-2, mcr-3, mcr-4, mcr5). Each PCR reaction consisted of 12.5 µL of the Taq DNA Red PCR Master Mix (Ampliqon, Denmark), 2.5 µL of nuclease-free water, 0.75 µL of each primer (Macrogen, South Korea), and 2.5 µL of the crude DNA lysate. Running conditions were as follows: 1 cycle of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 75 sec, elongation at 72 °C for 75 sec, and a final cycle of elongation at 72 °C for 10 min. The amplification was visualized by electrophoresis using 1.5% agarose gel stained with Green Viewer at 100 V. Strain No. 2012-60-1176-27 was used as the mcr-1, and strain No. KP37 was used as the mcr-2 positive control (11) to ensure the accuracy of the test. Primer sequences and amplicon sizes are listed in (Table 1).

In addition to the mcr gene, for detecting NDM-1 as class B and OXA-48 as class D of the Ambler classification of carbapenemase enzymes, the primers listed in Table 1 were used under the following conditions: Each PCR reaction consisted of 10 µL of the Taq DNA Red PCR Master Mix (Ampliqon, Denmark), 6 µL of nuclease-free water, 0.5 µL of each primer (Sinaclon, Iran), and 2 µL of the crude DNA lysate. Running conditions were: 1 cycle of denaturation at 95 °C for 4 min, followed by 33 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 60 sec, elongation at 72 °C for 60 sec, and a final cycle of elongation at 72 °C for 5 min. The amplification was visualized by electrophoresis using 1.5% agarose gel stained with DNA Green Viewer at 100 V. Strain No. 8 *E. coli* carrying blaNDM-1 and blaOXA-48 in the microbial archive of the Shahid Chamran University of Ahvaz was used as the control strain. Primer sequences and amplicon sizes are shown in (Table 2).

Table 1. Plasmid-mediated colistin resistance primers and amplicon sizes

Primers	Sequence (5'→3')	Size	Reference
mcr-1_fw	AGTCCGTTTGTTCTTGTGGC	320 (bp)	(30)
mcr-1_rev	AGATCCTTGGTCTCGGCTTG		
mcr-2_fw	CAAGTGTGTTGGTCGCAGTT	715 (bp)	(30)
mcr-2_rev	TCTAGCCCGACAAGCATACC		
mcr-3_fw	AAATAAAAATTGTTCCGCTTATG	929 (bp)	(30)
mcr-3_rev	AATGGAGATCCCCGTTTTT		
mcr-4_fw	TCACTTTCATCACTGCGTTG	1,116 (bp)	(30)
mcr-4_rev	TTGGTCCATGACTACCAATG		
mcr-5_fw	ATGCGGTTGTCTGCATTTATC	1,644 (bp)	(31)
mcr-5_rev	TCATTGTGGTTGTCCTTTTCTG		

Table 2. Carbapenem resistance primers and amplicon sizes

Primers	Sequence (5'→3')	Size	Reference
bla_{ndm-1}_fw	GGCGGAATGGCTCATCACGA	286 (bp)	(32)
bla_{ndm-1}_rev	CGCAACACAGCCTGACTTTC		
bla_{oxa-48}_fw	TTGGTGGCATCGATTATCGC	744 (bp)	(33)
bla_{oxa-48}_rev	GAGCACTTCTTTTGTGATGGC		

Results

The antibiotic susceptibility test showed the most resistance rates against cefepime (96.8%) (total: 69 normal and 115 diarrhea cases) and ciprofloxacin (81.1%) (total: 63 normal and 91 diarrhea cases), followed by ceftazidime (total: 58 normal and 95 diarrhea cases), cefixime (total: 21 normal and 31 diarrhea cases) and meropenem (total: 16 normal and 8 diarrhea cases), which were respectively 80.5%, 27.4%, 12.6%. Finally, the most effective antibiotic was revealed to be colistin, with a resistance rate of 8.42% (total: 9 normal and 7 diarrhea cases), where 11 (68.75%) isolates had colistin MIC ⩾4 by the E-test method. According to the differential diameter of the ceftazidime with the ceftazidime/clavulanic acid disk zone, of 190 isolated E. coli strains, 64 cases (33.7%) were phenotypically potential ESBL-producers, 40 (62.5%) of them were diarrheic, and 24 (37.5%) were from normal cases. Among ESBL producers, 5 isolates (7.81%) demonstrated resistance to colistin disks, while 36 isolates (56.25%) revealed MIC⩾4 by the E-test. Among all the studied isolates, 8 isolates were mcr-1-positive, and none of the other 4 plasmid-mediated colistin resistance genes (mcr-2, mcr-3, mcr-4, and mcr-5) were detected. Among the mcr-1-positive strains, 5 were phenotypically ESBL-producers, and between those that were non-ESBL-producers, only 1 isolate belonged to a healthy calf.

The optimistic point in the results of antibiotic susceptibility testing of mcr-1-positive isolates by Vitek-2 was the susceptibility to imipenem, ertapenem, tigecycline, and piperacillin/tazobactam. These data also suggest that the possible presence of IRT and OXA beta-lactamases, as well as AAC (6)-resistant aminoglycosides for the 2 isolates, should be considered. Positive samples were sequenced by the Sanger sequencing method, and the resulting sequences were confirmed by GenBank, NCBI (National Center for Biotechnology Information), and NIH (National Institutes of Health), with accession number MW980938. Neither blaNDM-1 nor blaOXA-48 gene was detected.

Discussion

The routes of transmission of antibiotic-resistant bacteria to humans are very complex and unpredictable. They can generally be divided into the direct route (involving contact with food-producing animals or human carriers) and the indirect route (through the food chain or exposure to habitats of antimicrobial contaminants such as hospitals or animal manure) (34).

It is known that a significant amount of antibiotics used in agriculture and the treatment of humans and animals, have actively returned to the environment. Resistance genes similar to antibiotics, can be released into the environment (35, 36). Consequently, it is expected that this close relationship between the use of antibiotics in agriculture, animal husbandry, and human medicine should spread resistant bacteria. Estimations suggested that livestock-fed antibiotics excrete 75-90% of those antibiotics in a nonmetabolized form, and these drugs may then enter sewage systems and water sources (37, 38). The waste from livestock may contain antibiotic-resistant bacteria and active antibiotics that may contaminate the environment and then, foster the emergence of antibiotic resistance in bacteria other than those to be found in living livestock and the meat produced from it (38, 39).

The results of a study confirmed that 133 mg of antibiotic substances was used per kg of milk, meat, and egg produced in 2010 in Iran. Besides, the information presented and the data from the Agriculture Ministry of Iran in 2010 revealed that over 1 806 tons of antibiotic-active substances were consumed in

livestock and poultry farms in Iran, of which 66.4% were used in cattle farms, which can lead to the emergence of AMR (40). Pishnian et al. also confirmed this high consumption of colistin in Iran (41).

In a study conducted by Tiseo et al., the global consumption of antibiotics in the poultry, cattle, and pig industries was about 93,000 tons in 2017 and is estimated to experience a rise of 11.5% by 2030 to 104 079 tons. The study also noted that China, followed by Brazil, the US, Thailand, India, and Iran (with 45%, 7.9%, 7.0%, 4.2%, 2.2%, and 1.9%, respectively) were the top 6 veterinary antimicrobial consumers in 2017 (27). Therefore, this excessive use of antibiotics in Iran may have increased the emergence of antibiotic resistance, which is also confirmed in the present study. The study of Ilbeigi et al. in Iran reported that among 607 *E. coli* strains isolated from different animals from 2008 to 2016, no mcr-1 and mcr-2 were reported (11). Moreover, the results of the study by Nikkhahi et al. revealed that in Iran, 4.6% of the isolates were resistant to colistin, and 33.3% of them harbored mcr-1; meanwhile, in our study, the resistance rate to colistin was 8.4%, and 8 mcr-1-positive isolates were detected (42). Based on the study by Filioussis et al. in Greece on 400 mastitis milk samples, 89 *E. coli* isolates were detected, of which 6 isolates had an ESBL phenotype, and all of them were also mcr-1-positive; however, in our study, of 8 mcr-1-positive isolates, only 5 isolates had the ESBL phenotype (43). In this context, Zhang et al., in a study of 651 dairy cows' fecal samples, found that of 290 containing ESBL-producing strains, 3 were mcr-1-positive (44). According to an examination performed in Nigeria, only 1 of 36 cattle fecal samples carried the mcr-1 gene, which was also localized on an IncX4 plasmid (45). An earlier study conducted on 51 animal manure samples indicated that 31% of the specimens were mcr-1 carriers (46). Among 150 *E. coli* strains isolated in Greece, just 20 were colistin-resistant, and only 1 of them was mcr-1-positive (47). According to the European Union summary report on AMR in zoonotic and indicator bacteria (*E. coli*) from humans, animals, and food in 2017/2018, resistance to third-generation cephalosporins in *E. coli* was rare, and the number of ESBL producer isolates among livestock was low (48). In the present study, the resistance rate to ceftazidime and cefixime as the third and to cefepime as the fourth generation of cephalosporins was 80.5%, 27.4%, and 96.8%, respectively. Therefore, the resistances detected in the current study, especially in the case of third and fourth-generation cephalosporins used in human medicine, are probably related to the results of the mentioned studies. Consequently, the findings of this study reveal a comparatively higher rising trend in cephalosporins resistance in *E. coli* strains isolated from calves.

Conclusion

The current study demonstrated the presence of the colistin resistance gene in neonatal dairy calves that had not previously received colistin. Furthermore, the concurrent presence of ESBLs, mcr-1-positive strains, and resistance to antibiotics used in human medicine, such as cephalosporins, indicates a major threat to public health. The excretion of resistant bacteria by livestock and poultry strengthens the transmission cycle and develops resistance to antimicrobial drugs, whether poultry litter is used for animal feed or manure for human agriculture. Furthermore, the co-existence of other resistance genes, such as ESBLs and carbapenemases, along with mcr, on transmissible genetic elements requires further studies to identify the dispersion and sequencing of the plasmids.

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Ethical statement

The study was conducted according to research and ethics guidelines and the approval of local institutions (Ferdowsi University of Mashhad). The samples of animals were collected upon the owner's consent. This research was conducted under the auspices of Ferdowsi University of Mashhad with the project number 48968.

Conflicts of interest

All the authors declare that they have no conflict of interest.

Author contributions

Arvin Shajeie carried out the experiment.
Arvin Shajeie wrote the manuscript with support from Kamran Sharifi
Mehrnaz Rad and Gholamreza Hashemi Tabar helped supervise the project.
Arvin Shajeie and Mahdi Askari designed the model and the computational framework and analysed the data.

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