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Phenotypic and Genotypic Characterization of Colistin Resistance in Escherichia coli Isolated from Bovine Mastitis

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ABSTRACT

Mastitis is a global disease occurring in dairy cows, causing notable economic losses. Extensive use of antibiotics could allow the emergence of mobile antimicrobial resistance genes in mastitis-causing pathogens. This study aimed to investigate the prevalence and characterization of colistin resistance genes in *E. coli* recovered from bovine mastitic milk. A total of 74 *E. coli* isolates were investigated for antimicrobial resistance. The presence of *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* plasmid-mediated resistance genes, as the most crucial contributors to resistance to colistin, was examined by Multiplex PCR. Antimicrobial susceptibility patterns of all isolates to the seven most common antibiotics applied in dairy herds, including colistin, ceftriaxone, ampicillin, tetracycline, gentamicin, enrofloxacin, and trimethoprim-sulfamethoxazole were determined by the DD test. Among all samples, 70 isolates (94.6%) were resistant to colistin. In the MIC test, all isolates were also resistant to colistin, which was in agreement with the DD test. None of the *E. coli* isolates carried plasmid-mediated colistin resistance *mcr-1* to *5* genes in Multiplex PCR. Despite the important role of food-producing animals in the transfer of antibiotic resistance, mastitis-causing *E. coli* isolates were not the source of *mcr 1* to *5* genes in this study. The present research showed a high level of phenotypic resistance to colistin, while there was no agreement with their genotypic resistance. Consumption of polymyxins in dairy calves and the probable existence of other more effective resistance genes could be the reason for this high rate of phenotypic resistance.

Keywords

Bovine mastitis, Colistin resistance, Escherichia coli, mcr gene, MDR

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Abbreviations

E. coli: Escherichia coli
DD: Disk agar diffusion
MIC: Minimum inhibitory concentration
LPS: lipopolysaccharide
ESBL: Extended spectrum beta-lactamase
MDR: multi-drug resistant
CLSI: Clinical and Laboratory Standards Institute

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Introduction

Mastitis is considered a critical global condition in the dairy industry, causing notable economic losses due to various detriments, especially a significant reduction in milk production. The effects of mastitis on reproduction and product quality have been also documented [1]. Furthermore, clinical mastitis induces a vast range of symptoms from mild or moderate to severe with generalized signs, such as fever, anorexia, and pain, which are emergencies and should be instantly treated [2]. *E. coli* is one of the primary causative pathogens of mastitis, responsible for more than 80% of acute mastitis cases [3].

Colistin (also known as polymyxin E), which is a polypeptide with bactericidal activity against different species of Enterobacteriaceae, such as *E. coli*, targets the lipid A component of the LPS in the outer layer of Gram-negative bacteria [4, 5]. Oral formulations of colistin are usually used for intestinal disorders in calves. Intra-mammary colistin compounds also exist in the market to treat mastitis. Polymyxin resistance happens following changes in the lipid A moiety in the structure of LPS by either mutation in chromosomal genes or acquired resistance genes resulting in a more cationic LPS [3, 4]. Until 2016, chromosomal mechanisms leading to LPS modification, including augmented 4-amino-4-deoxy-L-arabinose (L-Ara4N), 2-aminoethanol, and phosphoethanolamine (PetN), or other approaches, such as capsule synthesis and efflux pump were considered the major reasons for colistin resistance attainment within Enterobacteriaceae [6]. The activation of PmrCAB and the two-constituent system PhoP/PhoQ due to mutation, inactivation, or mutation of the regulatory mgrB gene and consequent adverse feedback of the PhoP/PhoQ system causing lipid A modification in the LPS were recognized in animal E. coli isolates [3, 7]. The *mgrR* and *etk* encoding a tyrosine-kinase are other genes inducing colistin resistance in E. coli by altering the LPS charge [6].

Following the first record of the mcr-1 gene in 2016, many papers showed the presence of plasmid-mediated polymyxin resistance gene, which is coding mcr-1 phosphoethanolamine transferase on different plasmids in the isolates of animal, human, or environmental source in most countries [3, 4, 6, 8, 9]. Few retrospective studies have been conducted to separate mcr-1-positive isolates in the samples derived from chickens and calves in the 1980s and 2006, respectively. These studies revealed that the development of mcr-1-positive strains seemed to be a silent distribution of mcr genes during preceding decades rather than a current disaster [10, 11]. However, the growth of mcr-1 prevalence to 30% in 2014 from 5.2% in 2009 represented a striking raise in mcr-1 prevalence emphasized through the preceding years (11). The acquisition of the plasmid-mediated *mcr-1* gene currently has become the main reason for polymyxin resistance in *E. coli* as 98% of colistin-resistant *E. coli* can be described by the carriage of the plasmid-borne mcr-1 gene [12-14].

The announcement of a vast range of mcr-1-carrying plasmids in *E. coli* from various regions explains the potential of this gene to spread [13]. The mcr genes might spread quickly within important human pathogens due to the very high in vitro transfer of mcr-carrying plasmid among *E. coli* strains. The coexistence of *mcr-1* and genes encoding ESBLs and carbapenemases, namely CTX-M-55, CTX-M-15, and blaNDM, was observed in various sequences of *E. coli* isolates originated from several reservoirs [8, 11, 13,15]. Haenni et al. reported that 21% of recovered ESBL-producing *E. coli* samples had the mcr-1 gene with a higher frequency in veal calves [16].

The addition of transferrable *mcr-1* plasmid-mediated colistin resistance in carbapenem-resistant *E. coli* isolates, even in the absence of polymyxins' selective pressure, could be a global hazard of pan-drug resistant isolates development. However, the attainment of mcr-1 by *E. coli* could be a consequence of the substantial consumption of colistin in veterinary [3, 7, 14].

Co-occurrence of the *mcr-1* gene and resistance to various antibiotics, such as ampicillin, gentamicin, chloramphenicol, sulfonamides, trimethoprim, cephalosporins, and tetracyclines, has been reported [6, 11]. Extensive application of these antibiotics in veterinary medicine may have a role in distributing *mcr-1* and colistin resistance [11]. A 4- to 8-fold rise in the MICs of polymyxins may result from the presence of *mcr-1* in *E. coli* [4]. Considering the importance of colistin as the last-resort option for human infections caused by MDR bacteria and its broad consumption in veterinary medicine, the identification of mcr genes in food-producing animals is noteworthy in terms of public health concern that colistin resistance might be transmittable to humans [3, 4, 8].

Our study aimed to evaluate phenotypic resistance to colistin, the prevalence of plasmid-mediated colistin resistance genes (*mcr-1* to *5* genes), the relatedness of phenotypic and genotypic resistance, and also the agreement between two different phenotypic susceptibility tests in mastitis-causing *E. coli* isolates in a dairy farm.

Results

Antimicrobial susceptibility tests

A) Disk Diffusion: Based on the DD test, 70 *E. coli* isolates (94.6%) showed phenotypical resistance to colistin (zone diameter < 14 mm), which was the most prevalent resistance among all seven different antimicrobial agents. Enrofloxacin was the most effective agent compared to other antibiotics. Enrofloxacin inhibited bacterial growth in 62 isolates (83.8%) among all *E. coli* isolates. More details of phenotypical susceptibility to all seven antimicrobial agents are summarized in Table 1. Our study also revealed that 6 (8.1%) isolates were resistant to all seven antibiotics. Among all isolates (n = 74), 21 samples (28.4%) were known as MDR due to phenotypical resistance against at least three examined antibiotics other than colistin. Details of antibiotic susceptibility patterns to different antibacterial agents are summarized in Figure 1.

B) Minimum Inhibitory Concentration to colistin The MICs of isolates on cation-adjusted Mueller Hinton broth (Mueller Hinton broth 2) showed that all isolates were resistant to colistin (MIC > 8 µg/ml). Results also revealed that almost 42% of isolates (31 isolates) had MICs greater than 128 µg/ml.

Agreement between antimicrobial susceptibility tests

MICs demonstrated that all isolates were phenotypically resistant to colistin, while DD results showed that 70 isolates (94.6%) were colistin-resistant (Table 2). There was a significant agreement between the two phenotypical susceptibility tests. The details are shown in Figure 2.

Occurrence of mcr genes in E. coli isolates

In order to investigate the existence of mcr-1 to 5 resistance genes, Multiplex PCR was carried out, and the obtained results revealed that among all mastitis-causing E. coli isolates, none of the isolates carried *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* plasmid-mediated resistance genes.

Discussion

The phenotypic colistin resistance of *E. coli* isolates in this research was 94.6% and 100% in DD and MIC, respectively. These two phenotypically colistin resistance tests had great concordance in our study. However, most studies showed that the DD test is unreliable and introduced standard broth microdilution as the golden standard for colistin resistance detection [17, 18]. There is a wide range of colistin resistance

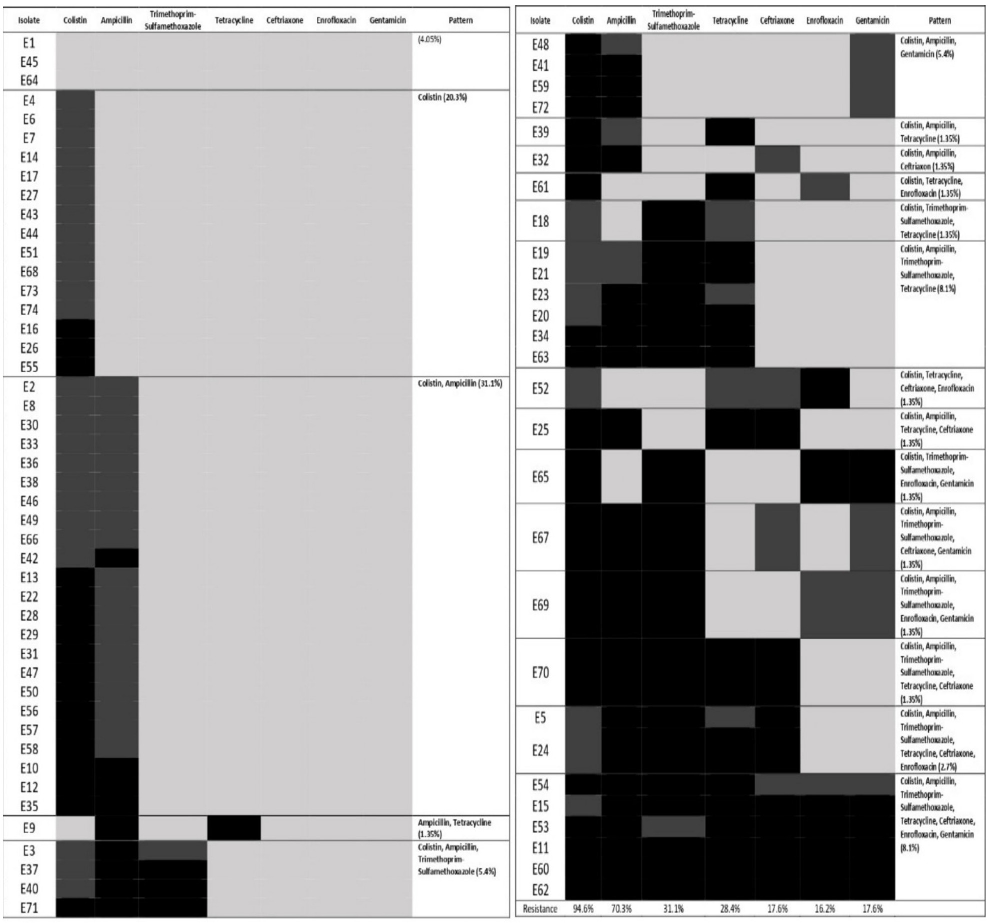

Figure 1. Antibiotic Susceptibility Patterns of E.coli Clinical Isolates

Table 1.
Comparison of phenotypic E. coli isolates susceptibility to 7 different antibiotics.

Antibiotic Name	Susceptible (S)	Intermediate (I)	Resistant (R)
Tetracycline (TE) (30 µg)	53 (71.6%)	4 (5.4%)	17 (23%)
Trimethoprim-Sulfamethoxazole (SXT) (1.25+23.75 µg)	51 (68.9%)	2 (2.7%)	21 (28.4%)
Colistin (CL) (10 µg)	4 (5.4%)	34 (45.9%)	36 (48.7%)
Gentamicin (GM) (10 µg)	61 (82.4%)	7 (9.5%)	6 (8.1%)
Enrofloxacin (NFX) (5 µg)	62 (83.8%)	3 (4%)	9 (12.2%)
Ampicillin (AM) (10 µg)	22 (29.7%)	23 (31.1%)	29 (39.2%)
Ceftriaxone (CRO) (30 µg)	61 (82.4%)	4 (5.4%)	9 (12.2%)

Table 2.
Comparison of two phenotypically colistin resistance tests (Disk diffusion and MIC).

			Disk diffusion results			Total
			susceptible	Intermediate	Resistant	
MIC (µg/ml)	8	Count	1	0	0	1
		% of Total	1.4%	.0%	.0%	1.4%
	16	Count	0	2	2	4
		% of Total	.0%	2.7%	2.7%	5.4%
	32	Count	1	11	6	18
		% of Total	1.4%	14.9%	8.1%	24.3%
	68	Count	0	4	9	13
		% of Total	.0%	5.4%	12.2%	17.6%
	128	Count	0	5	2	7
		% of Total	.0%	6.8%	2.7%	9.5%
	> 128	Count	2	12	17	31
		% of Total	2.7%	16.2%	23.0%	41.9%
Total		**Count**	**4**	**34**	**36**	**74**
		% of Total	**5.4%**	**45.9%**	**48.6%**	**100.0%**

reported from different animals in various origins and locations, including five continents and forty countries [4, 8, 13, 15, 19]. High resistance to colistin (> 50%) was reported from piglets in Thailand. It was observed that 4.6% of avian E. coli isolates were colistin-resistant in Morocco. Resistance to colistin (3%-5%) in *E. coli* isolates from small animals was low in Sweden. Colistin resistance in bovine samples in Europe was 2% [8, 20, 21]. In Vietnam, 11% of the MDR *E. coli* strains derived from food animals were resistant to colistin [9]. The high rate of resistance to colistin in the current study compared to other investigations might result from orally administrated compounds of polymyxins to treat calves' intestinal disorders.

The screening of *E. coli* isolates from various animal species during 2000-2014 revealed that 1% of samples were classified as colistin-resistant cases. Generally, 0.4% of *E. coli* from several regions of the world were colistin-resistant cases (MIC ≥ 4 mg/L), whereas 32.2% of colistin-resistant isolates (overall prevalence: 0.1%) had *mcr-1* gene in 2014 and 2015 (13). A survey during 2010-2015 in Germany showed that 3.8% of *E. coli* isolates from various origins were resistant to colistin, while 79.8% of them had the *mcr-*

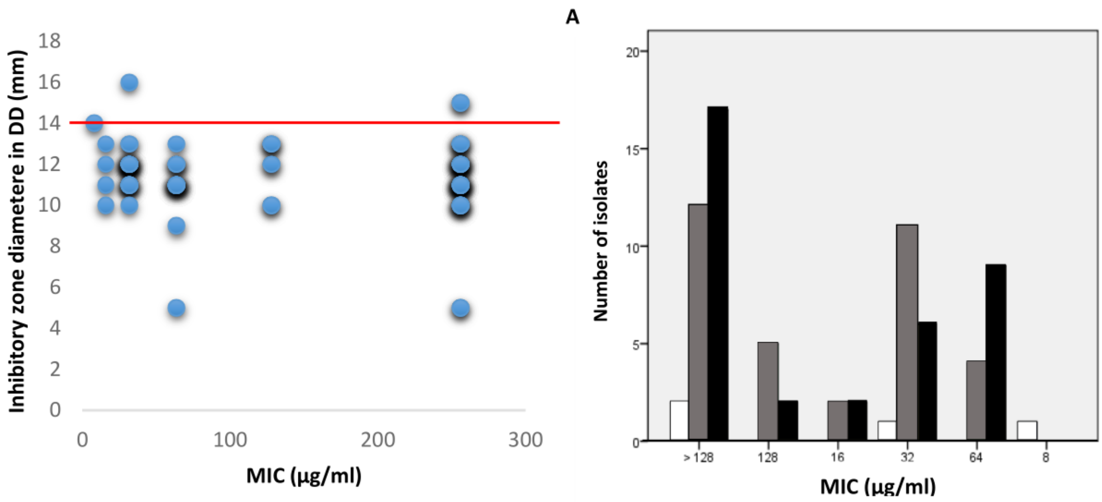

Figure 2.
Agreement between antimicrobial susceptibility tests

Table 3.
Primers and PCR conditions for molecular confirmation of isolates and mcr 1 to 5 detection.

Genetic target	Primer	Primer sequence (5'-3')	Annealing temp. (° C)	Amplicon size (bp)
23S rRNA	Eco223-F	ATCAACCGAGATTCCCCCAGT	64	232
	Eco455-R	TCACTATCGGTCAGTCAGGAG		
mcr-1 gene	mcr1-fw	AGTCCGTTTGTTCTTGTGGC	54	320
	mcr1-rev	AGATCCTTGGTCTCGGCTTG		
mcr-2 gene	mcr2-fw	CAAGTGTGTTGGTCGCAGTT		715
	mcr2-rev	TCTAGCCCGACAAGCATACC		
mcr-3 gene	mcr3-fw	AAATAAAAATTGTTCCGCTTATG		929
	mcr3-rev	AATGGAGATCCCCGTTTTT		
mcr-4 gene	mcr4-fw	TCACTTTCATCACTGCGTTG		1116
	mcr4-rev	TTGGTCCATGACTACCAATG		
mcr-5 gene	mcr4-fw	ATGCGGTTGTCTGCATTTATC		1644
	mcr4-rev	TCATTGTGGTTGTCCTTTTCTG		

1 gene. The *mcr-1* was also observed in 73.68% (14/19) of *E. coli* isolates collected from dairy cows in China.

The role of food-producing animals in spreading the mcr genes, even in healthy calves, was revealed in different countries [22]. In Belgium, the *mcr-1* to *mcr-5* genes were detected in healthy cattle, pigs, and poultry with the highest frequency of 77.5% (31 from 40 isolates) found for the *mcr-1* gene, 27 (67.5%) of which were carried out from cattle in 40 phenotypically colistin-resistant samples. In our study, although most isolates phenotypically were resistant to colistin, none of them had *mcr-1* to *5* genes [22, 23].

Amongst different sources, the prevalence of mcr genes in veal calves was low, and *mcr-1* was not detected in beef cattle, which is in agreement with our results [12]. The absence of mcr-positive isolates in our research was similar to other reports in human and animal specimens, such as bovine isolates, where these genes were not detected or the detection rate was low [8, 13, 24]. The mcr genes were reported the highest in the porcine and poultry collected isolates [25-27]. In contrast, the Islamic countries had no *mcr* genes isolation or very low prevalence of mcr genes due to the lack of pig industry [28, 29]. In Saudi Arabia, mcr genes were not detected until the first report in 2016 [29]. In Iran, the first *mcr-1* gene detection from an animal source was in 2021 from a cow rectal swab, whereas no *mcr-2* to *mcr-6* genes were detected [30]. Ilbeigi et al. did not detect *mcr-1* and *mcr-2* genes in 36 bovine mastitis-causing and other 571 *E. coli* isolates of animal origin in Iran [28]. However, the presence of *mcr-1* in *E. coli* isolates recovered from cattle mastitis was reported in Egypt, Japan, and currently Greece [31-33]. Phenotypic resistance to colistin (with MIC ≥ 4 µg/mL) has been reported at 4% in bovine mastitis-causing Pseudomonas aeruginosa, and the *mcr-2* gene was also detected in two colistin-resistant isolates in Iran [34]. The prevalence of mcr-1-harboring *E. coli* isolated from bovine mastitic milk in China was 2% [24]. The first report of mastitis caused by ESBL-producing, *mcr-1*-harboring E. coli was recently in Greece, where the *mcr-1* gene was detected in 1.5% of isolates, while 22.25% of milk samples were phenotypically resistant to colistin [19, 31]. The high rate of phenotypic colistin resistance in our study could be related to its use in calves' digestive disorders treatment. Colistin constant consumption as well as other antibiotics, namely cephalosporins, cause the transfer of other resistance genes [7, 8, 11].

Although the detection of colistin-resistant E. coli isolates from ruminants or their products was not witnessed in some research, in 2014 the percentage of colistin resistance was estimated to be less than 2.5% for isolates from calves following cattle mastitis. The latter finding is contrary to the results of this study that only 5.4% of isolates were susceptible to colistin. In mastitis-causing Klebsiella pneumoniae strains, resistance to colistin was also reported by 1% in France [35].

Extensive use of cephalosporins, sulfonamides, and tetracyclines in veterinary medicine may also play a part in colistin resistance cases and even the distribution of mcr genes. Moreover, the co-occurrence of *mcr* genes with tetracyclines and sulfonamides resistance encoding genes was recorded [16]. Porcine *mcr-1*-harboring colistin-resistant *E. coli* isolates which simultaneously were resistant to ampicillin, gentamicin, sulfonamides, chloramphenicol, trimethoprim, tetracycline, or cefotaxime have also been reported [6]. Emerging colistin and carbapenems resistance in bovine mastitis-causing Pseudomonas aeruginosa was also recorded in Iran [34]. In our study, the higher percentage of MDR and pan-drug resistant isolates also confirmed resistance to colistin and other antibiotics. High rates of colistin resistance have been also noted among the strains of K. pneumoniae producing carbapenemase in Brazil and Italy but they lack mcr genes, which is in line with our results that phenotypic and genotypic resistance patterns were not compatible [36]. This is evidence of PCR limitation in which a negative result in PCR does not indicate susceptibility to colistin. PCR cannot exclude the chromosomal mechanisms of resistance, such as mutations, or even novel mcr genes not possessed in the test. Therefore, a negative PCR result for mcr genes would have insufficient predictive value for a colistin-susceptible phenotype [36].

Conclusion

The current study indicated high phenotypic resistance to colistin in *E. coli* isolates from bovine mastitic milk and the significant concordance between two phenotypically colistin susceptibility tests MIC and DD. However, phenotypic and genotypic resistance patterns were not compatible. The high rate of colistin resistance may result from colistin use in dairy calves and its potential to induce resistance in mastitis-causing pathogens, such as *E. coli*. Despite the frequent usage of colistin in farm animals, the lack of mcr genes revealed that these genes were not widespread in veterinary and human clinical isolates in Iran, consistent with previous studies. Further investigations are also needed to understand the role of other colistin-resistance genes. The selection pressure of polymyxins in the dairy industry, even in calves, could provide a source of colistin resistance. Consequently, the possibility of other colistin-resistance genes' presence and their ability to spread to humans could be a global risk for public health. Hence, it should be noted that significant interruptions are required to lessen the spread of resistance to colistin in food animals.

Materials & Methods

Sample collection

The current retrospective cohort study was planned to investigate the prevalence of mcr-positive isolates among E. coli (n = 74) samples from mastitic cows in a dairy farm collected from October 2018 to February 2019. The severity status of all cases had been evaluated and recorded during sample collection. All isolates were collected based on National Mastitis Council guidelines. All milk samples were quickly transported on ice to the laboratory for microbiological culture.

Isolation and identification of E. coli

Conventional bacteriological culture was performed based on the National Mastitis Council (1999). To this aim, 0.01 ml of milk was primarily overlaid on McConkey and Blood agar and incubated aerobically for 24 and 48 h at 37°C, respectively. A milk sample was described as positive if at least two colonies of any pathogen were observed on the plate. Plates with more than two different colony types were reported as contaminated samples. After morphological analysis of colonies, isolates were investigated by Gram staining. Supplementary metabolic and biochemical evaluations were performed as

needed applying particular microbiological analyses.
McConkey-positive samples were then subcultured on eosin methylene blue agar. In addition, sucrose and glucose fermentation, citrate, gas and H_2S production, indole, and motility tests were performed to screen the samples for the existence of *E. coli*. Seventy-four *E. coli* confirmed isolates were finally selected to be included in the study.

Molecular confirmation of *E. coli* isolates

To confirm the presence of *E. coli*, biochemically-positive samples were reanalyzed by PCR. For DNA extraction, 250 µl lysis buffer (0.2 M NaOH, 1% SDS, pH=8) and 250 µl Tris-EDTA buffer (100 mM Tris, 10 mM EDTA, pH=8) was first added to 200 µl of milk samples. Next, 550 µl phenol was added to the mixture. The supernatant was rinsed twice with phenol after 5 min centrifugation at 6000 rpm. Following the addition of 0.1 of 3.0 M sodium acetate (pH = 5.2), DNA was precipitated by ethanol and redissolved in distilled water after drying. Afterwards, 1 µg of extracted DNA was used to perform PCR. The primers were synthesized according to Riffon et al. (37). The details are given in Table 3.

Antimicrobial susceptibility testing

A) Disk Diffusion Test: A total of 74 E. coli samples confirmed by bacteriological tests were selected to evaluate antibiotic susceptibility status to ceftriaxone (30 µg), colistin (10 µg), ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), enrofloxacin (5 µg), and trimethoprim-sulfamethoxazole (1.25+23.75 µg) disks by DD method. The diluted samples were equivalent to a 0.5 McFarland standard cultured on Mueller Hinton agar media. After overnight incubation at 37°C, the inhibitory zone was measured, and the susceptibility of samples was recorded by comparing to the standards of the CLSI and García-Meniño et al. study for colistin (17). However, the CLSI recommended the broth microdilution method as the gold standard for colistin susceptibility testing. We included the DD test in our with the cut-off value of ≤ 13 mm suggested by García-Meniño et al. to evaluate agreement with the gold standard method. Using a cut-off value of ≤ 13 mm, as inhibition zone diameter,have increased the sensitivity to 100% with a specificity of 98.7% (17). Isolates with antimicrobial resistance against at least three examined antibiotics other than colistin were considered MDR.

B) Evaluation of minimum inhibitory concentrations of isolates: To determine the MIC of isolates, the broth microdilution method was performed based on ISO standards for coliforms. Pure colistin sulfate powder (Sigma-Aldrich, Merck KGaA, Germany) was dissolved in distilled water and then kept at -80°C until the test, at the final dose of 1024 µg/ml. Cation-adjusted Mueller Hinton broth culture medium was poured into polyester pellets after preparation. For each of the tested isolates, eight serial concentrations of colistin from 1 to 128 µg/ml were added to the media. After overnight incubation, 50 µl of each sample equal to the standard concentration of 0.5 McFarland was added to each well and then incubated for 16-20 h at a temperature of 35°C ± 2°C. The MIC value was calculated based on the lowest concentration that completely inhibited bacterial growth.

Molecular detection of mcr-1 to 5 resistance genes

The presence of *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* genes in all the isolates were analyzed by Multiplex PCR to evaluate the plasmid-mediated colistin resistance genes. All reactions were accomplished in a final volume of 25 µl. Multiplex PCR screened the existence of mcr-1 to 5 in isolates with the primers synthesized based on Rebelo et al. study (38). A volume of 1 µl of extracted DNA templates was added to 12.5 µl of master mix buffer solution, 10 ρmol of each 10 forward and reverse primers, and 9.5 µl of distilled water in a 0.5 ml microfuge tube. After applying a pre-PCR step at 94°C for 15 min, 25 cycles were run under the following condition: denaturation at 94°C for 30 sec, annealing at 58°C for 90 sec, and extension at 72°C for 60 sec. To finalize the reaction, the preparation was held at 72°C for 10 min following the last cycle. The details of the PCR protocol are summarized in Table 3. A 1.7% agarose gel stained with 0.5 mg of ethidium bromide/ml was used, and the agarose gel was finally visualized under UV light.

Authors' Contributions

M.Z., S.H., H.M., M.A., and B.KH. conceived and planned the experiments. M.Z., S.H., and B.KH. carried out the experiments. M.Z. and K.I contributed to sample preparation. M.Z., M.A., and B.KH. contributed to the interpretation of the results. M.Z. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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