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Genetic Diversity, Antimicrobial Resistance, and Biofilm-Forming Potential of Equine Fecal Escherichia coli in Northern Iran

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ABSTRACT

This study was carried out to examine the biofilm-forming ability, antimicrobial resistance, frequency of biofilm, and resistance genes, as well as the phylogenic grouping of *Escherichia coli* isolates originating from equine samples. In total, 157 *E. coli* strains were isolated from fresh feces samples of healthy horses in northern Iran. The samples were examined in terms of biofilm formation and antimicrobial susceptibility using a microtiter plate and disc-diffusion test, respectively. PCR amplification was adopted to find the genes that confer biofilm formation and resistance to β-lactam, chloramphenicol, tetracyclines, aminoglycosides, quinolones, sulfamethoxazole, and trimethoprim, and for phylogenetic analysis. More than 50% of isolates showed MDR phenotype. The most significant level of resistance was detected for streptomycin (59.87%), followed by trimethoprim-sulfamethoxazole (29.93%) and oxytetracycline (28.66%). Imipenem and norfloxacin were the most potent antibiotics. Phylogenetic groups B1 (46.50%) and A (21.66%) were the most common groups in isolates, followed by C (6.37%), clade I (5.10%), E (4.46%), D (3.82%), and B2 (2.55%). All isolates in phylogroups B2 and D carried all biofilm-related genes. In addition, antimicrobial resistance genes were common in phylogroups B2, D, A, B1, and E. These findings demonstrate that in northern Iran, healthy horses harbor potential extraintestinal pathogenic and MDR *E. coli* isolates. These animals can be reservoirs for antibiotics-resistant isolates. The obtained data support the current interest regarding antimicrobial resistance, MDR shedding, and managing the use of antimicrobials in veterinary science.

Keywords

Equine, E. coli, Genetic diversity, Antimicrobial resistance, Biofilm

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Abbreviations

PCR: Polymerase Chain Reaction,
MDR: Multi-Drug Resistant
CLSI: Clinical and Laboratory Standard Institute
MBL: Metalo Beta-Lactamase
ESBL: Extended Spectrum Beta-Lactamase
ATCC: American Type Culture Collection, IMP:

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Introduction

The occurrence and expansion of antimicrobial resistance have turned into a major threat to the healthcare of human beings, animals, and the environment worldwide [1, 2]. Having direct or indirect contact with affected animals and contaminated environments can speed up the easy transfer of microorganisms between humans and animals [3]. Therefore, monitoring antimicrobial resistance in animal-originated bacteria, and finding enough data about the paths of the spread of antimicrobial resistance and associated genes and their transfer between different elements of the ecosystem is extremely important [4]. Nevertheless, despite the significant amount of information examining antimicrobial resistance in food-producing animals, a small number of studies have evaluated antimicrobial resistance in bacteria originating from equine [5]. Due to the recurrent use of identical antibiotics for curing human beings and horses, equine-origin antibiotic-resistant microorganisms not only affect their health and limit treatment options, but also endanger the health of people in contact with these animals [4, 6, 7]. *E. coli* is a predominant commensal microorganism that is found in the gastrointestinal microflora of human beings and animals. In comparison with other common bacteria, *E. coli* can easily gain antimicrobial resistance via genetic mutation or horizontal gene transfer through a variety of mobile genetic elements, such as self-transmissible plasmids, transposons, and integrons [8]. These mobile genetic elements may co-carry multi-antimicrobial resistance genes, including ESBL, aminoglycosides, sulfa-derivatives, trimethoprim, and quinolone resistance [9]. During the co-colonization of antibiotic-resistant bacteria along with non-resistant bacteria, resistance genes can quickly spread and exchange between bacterial isolates and may facilitate the appearance of MDR bacteria [8, 10].

One may find a considerable genetic substructure in E. coli species, which has been adopted as an easy and cheap method to assign an *E. coli* isolate to a phylogroup [11]. According to the revised Clermont PCR method for phylotyping *Escherichia coli,* the strains of *E. coli* species could be categorized as eight phylogenetic groups (A, B1, B2, C, D, F, and cryptic clade I) based on the presence or absence of chuA (a gene required for heme transport), yjaA (a gene having unknown performance), arpA, and trpA genes as well as the DNA fragment TspE4. C2 is identified as a putative lipase/esterase gene [12]. It was realized that phylogroup strains are different in terms of phenotypic and genotypic features, ecological niche, traits of life history, and capability to create a disease. For example, virulent extra-intestinal strains are the major members of group B2 and, to a lesser degree, group D, while the majority of commensal strains belong to group A or B1 and strains belonging to phylogroup F are more likely to be implicated as the extra-intestinal pathogens of companion animals, horses, cattle, and humans [11, 12].

There is limited data about antimicrobial resistance and phylogroup distribution in the equine population in northern Iran. *E. coli* is occasionally adopted as a sentinel strain for examining antimicrobial resistance in fecal bacteria [13]. Therefore, the present study was conducted to determine the antimicrobial resistance patterns, biofilm-forming ability, and distribution of resistance and biofilm-associated genes in different phylogroups of fecal *E. coli* isolates in healthy horses in Guilan province, north of Iran.

Results

Bacterial Isolates and Phylogenetic Typing

In this study, a total of 157 *E. coli* strains were isolated. Analysis of PCR results for determining phylogenetic groups among these isolates showed that phylogenetic groups B1 (46.50%) and A (21.66%) were the most common followed by C (6.37%), clade I (5.10%), E (4.46%), D (3.82%), and B2 (2.55%). In 9.55% of isolates, the phylogenetic group was unknown.

Antimicrobial Susceptibility Testing

We observed that 26 isolates were sensitive to all 16 antibiotics used in the assay and 131 isolates (83.4%) were resistant to at least one antibiotic. More than 50% of isolates (83 isolates) showed MDR phenotype (resistant to three classes of antibiotics), three horses (1.9%) were colonized by ESBL-positive isolates, and one isolate (0.63%) indicated an imipenem-resistant phenotype.

The outcomes of testing the antibacterial susceptibility of *E. coli* isolates to common antibiotics have been shown in Table 1 and Figure 1. Overall, 85 diverse patterns of antibiotic resistance have been found in 157 isolates (Supplementary data). The most considerable level of resistance was observed for streptomycin (59.87%) followed by trimethoprim-sulfamethoxazole (29.93%) and oxytetracycline (28.66%). Imipenem and norfloxacin were the most potent antibiotics. All isolates belonging to phylogenetic groups B2, D, and E were MDR. The other MDR isolates were

Abbreviations-Cont'd

Imipenem
CFU: Colony Forming Unit
OD: Optical Density

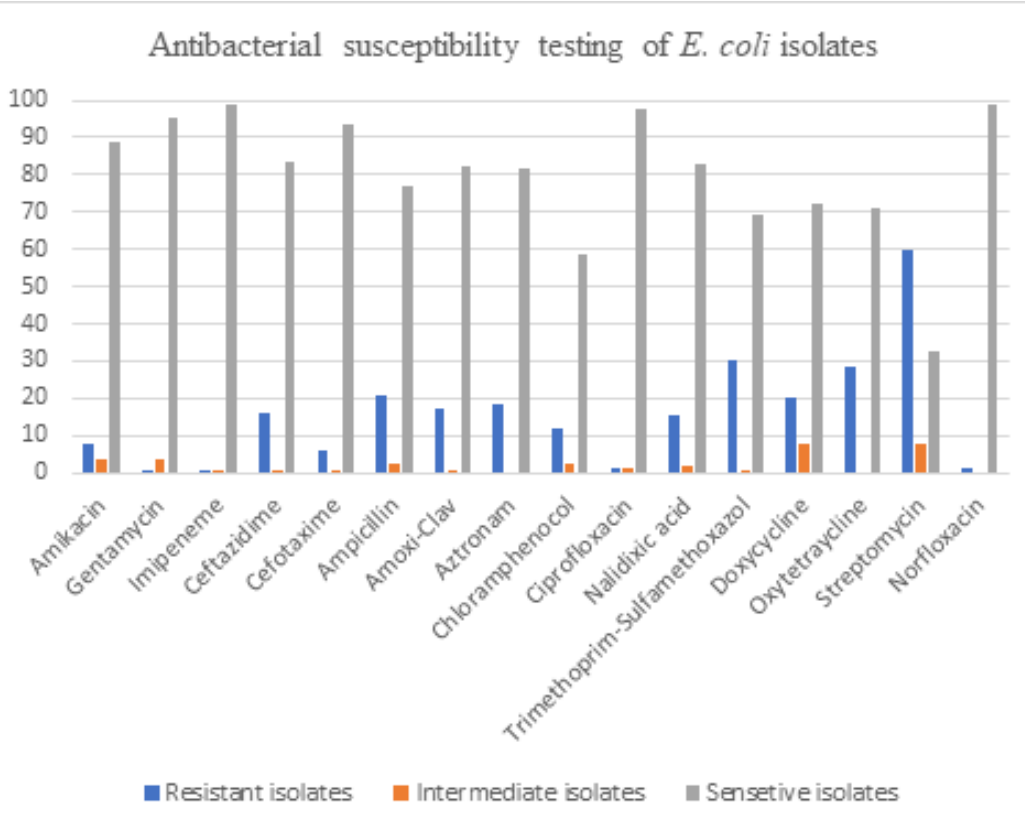

Figure 1.
Antibacterial susceptibility testing of E. coli isolates

Table 1.
Antibacterial susceptibility testing of E. coli isolates

Antibiotic	Resistant isolates	Intermediate isolates	Sensetive isolates
Amikacin	12 (7.64%)	6 (3.82%)	139 (88.53%)
Gentamycin	1 (0.63%)	6 (3.82%)	150 (95.5%)
Imipeneme	1 (0.63%)	1 (0.63%)	155 (98.72%)
Ceftazidime	25 (15.92%)	1 (0.63%)	131 (83.43%)
Cefotaxime	9 (5.73%)	1 (0.63%)	147 (93.63%)
Ampicillin	32 (20.83%)	4 (2.54%)	121 (77.07%)
Amoxi-Clav	27 (17.19%)	1 (0.63%)	129 (82.16%)
Aztronam	29 (18.47%)	0 (0%)	128 (81.52%)
Chloramphenocol	19 (12.10%)	4 (2.52%)	134 (85.35%)
Ciprofloxacin	2 (1.27%)	2 (1.27%)	153 (97.45%)
Nalidixic acid	24 (15.28%)	3 (1.91%)	130 (82.80%)
Trimethoprim-Sulfamethoxazol	47 (29.93%)	1 (0.63%)	109 (69.42%)
Doxycycline	32 (20.38%)	12 (7.64%)	113 (71.97%)
Oxytetraycline	45 (28.66%)	0 (0%)	112 (71.33%)
Streptomycin	94 (59.87%)	12 (7.64%)	51 (32.48%)
Norfloxacin	**2 (1.27%)**	**0 (0%)**	**155 (98.72%)**

in the phylogroups A (20/34), B1 (39/73), C (4/10), and unknown (3/15).

Biofilm-Forming Assay

Of 157 evaluated isolates, 81 (51.6%) were positive in terms of biofilm-forming ability. All MDR isolates were capable of forming a biofilm. The frequency of biofilm-related genes fimH, papC, csgA, and fliC in biofilm former E. coli isolates was 100%, 100%, 77.77%, and 65.43%, respectively. In addition, resistance to all examined antibiotics in biofilm-former strains was higher than biofilm-negative ones (data not shown).

Dissemination of Biofilm-Related Genes in Various Phylogroups

Dissemination of different phylogenetic groups in biofilm-former and biofilm-non-former strains of E. coli is presented in Table 2 and Figure 2. All isolates in phylogroups B2 and D carried all biofilm-related genes. More than half of the isolates in the E and A phylogroups were biofilm-formers and carried variable biofilm-related genes. The allocation of biofilm-related genes in various phylogroups is presented in Supplementary Table 2. There was no significant association between different phylogroups and bio-

film-forming ability among test bacteria ($p < 0.05$).

PCR Screening for Antimicrobial Resistance Genes

Streptomycin resistance-associated genes were more common in test isolates, followed by *tet* and *dfr1* genes as the most frequent ones. The *blaTEM* and/or *blaSHV* genes were detected in three ESBL-positive isolates. Most of the resistance genes were more frequent in biofilm-forming isolates ($p < 0.05$). Antimicrobial resistance genes were common in phylogroups B2, D, A, B1, and E ($p < 0.05$). Each isolate in phylogroups B2 and D carried at least three investigated drug-resistance genes. The distribution of antimicrobial resistance genes in biofilm-former, biofilm-non-former, and different phylogroups of *E. coli* isolates is shown in Table 3.

Table 2.
Distribution of different phylogenetic group in biofilm former and biofilm non former *E. coli* strains

Phylo-group	No. of isolates	
	Biofilm positive	Biofilm negative
A, (34)	18	16
B1, (73)	34	39
B2, (4)	4	-
C, (10)	6	4
D, (6)	6	-
E, (7)	5	2
clade I, (8)	3	5
Unknown, (15)	6	9

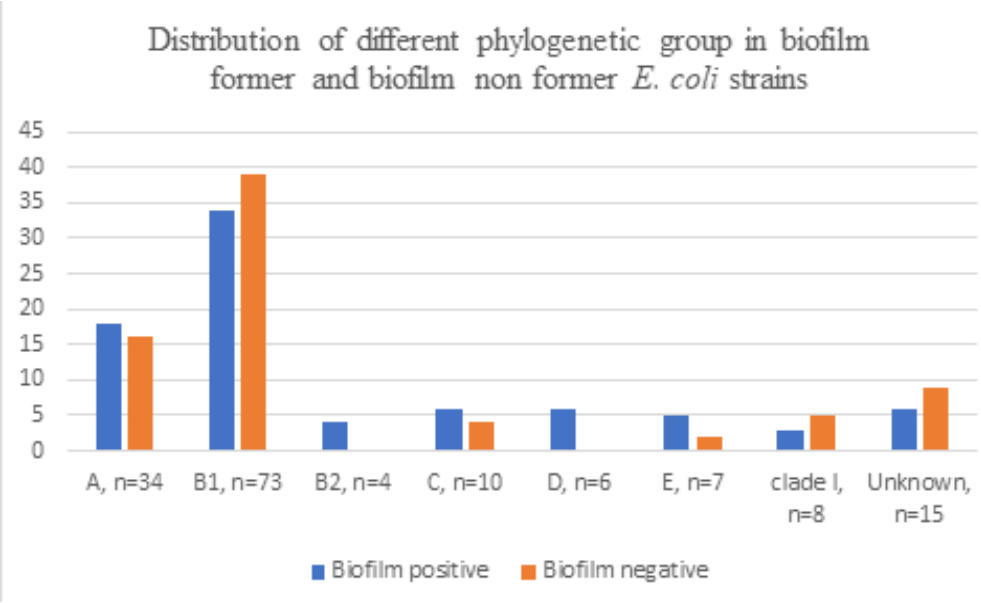

Figure 2.
Distribution of different phylogenetic group in biofilm former and biofilm non former *E. coli* strains

Discussion

Commensal *E. coli* resistance is of special importance to maintaining consumer health as it includes a source of resistance genes. This resistance can be transferred to any other bacteria, such as pathogenic bacteria, through horizontal gene transfer [9, 14]. Veterinary healthcare settings in recent decades have been introduced as the origin of the outbreaks of different multidrug-resistant microorganisms [10, 15, 16]. Injection of antimicrobial agents into horses in both hospital and community contexts is accompanied by a higher risk of the fecal shedding of antimicrobial-resistant *E. coli* [5]. In this study, 85 diverse patterns of antibiotic resistance were detected in 157 isolates, and 131 isolates (83.4%) were resistant to at least one antibiotic. Of 157 *E. coli* isolates examined in terms of antibiotic sensitivity, more than 50% (83 isolates) showed MDR phenotype. Compared to other studies, frequency of MDR in equine fecal *E. coli* was high in the present study. However, our results were comparable with that of Lagarde et al. (2019), which reported 46.3% MDR in *E. coli* isolated from healthy horses [17]. A high level of AMR in domesticated animals is related to the use of antimicrobial agents and is also related to direct exposure to the bacteria responsible for carrying these genes [18].

In the present study, three ESBL-producing isolates harboring *blaTEM* (three isolates) and *blaSHV* (two isolates) genes were identified. Kaspar et al. (2019) detected 9 (4.0%) ESBL-producing *E. coli* positive in

Table3.
Distribution of drug resistance genes among biofilm former and biofilm non former and different phylo-groups of *E. coli* strains

Gene (No.)	Biofilm formation		Phylo-group
	Positive, (81)	Negative, (76)	
TEM, (2)	3 (100%)	-	B2 (2), D (1)
SHV, (1)	2 (100%)	-	B2 (1)
qnr A, (0)	-	-	-
qnr B, (3)	3 (100%)	-	B1 (1), B2 (2)
tetA, (39)	28 (71.79)	11 (28.21)	A (12), B1 (16), B2 (4), D (4), E (3)
tetB, (48)	42 (87.5%)	6 (12.5%)	A (14), B1 (17), B2 (4), D (5), C (2), E (5), clade I (1)
strA-strB, (54)	42 (77.77)	12 (33.33)	A (19), B1 (23), B2 (3), C (2), D (4), E (1), unknown (2)
aadA, (5)	5 (100%)	-	B2 (3), D (2)
aadB, (8)	8 (100%)	-	B1 (2), B2 (3), D (2), E (1)
Cat I, (11)	8 (72.72)	3 (27.28)	A (2), B1 (1), B2 (4), D (2), E (1), unknown (1)
Cat II, (7)	5 (71.42)	2 (27.58)	A (1), B2 (3), D (2), E (1)
Sul 1, (23)	21 (91.3)	2 (8.7)	A (4), B1 (7), B2 (3), D (4), E (2), Clade I (2), unknown (1)
Sul 2, (17)	17 (100%)	-	A (3), B1 (5), B2 (3), D (2), E (1), Clade I (2), unknown (1)
Dfr1, (36)	26 (72.22)	10 (27.28)	A (7), B1 (12), B2 (4), C (2), D (5), E (5), unknown (1)

terms of *blaCTX-M* and/or *blaTEM* in non-hospitalized horses in Northwest Germany [10]. In another research conducted in the United Kingdom, 6.3% of 650 fecal samples from non-hospitalized horses were reported to be positive regarding ESBL-*E. coli* [19]. In Canada, ESBL/AmpC genes were found in *E. coli* obtained from 7.3% of healthy horses [17].

In a higher frequency, Johns et al. (2012) reported 138/228 (60.5%) MDR and 17/228 isolates positive for ESBL production in fecal *E. coli* isolates of horses treated with antimicrobial agents, among which 12/17 isolates had *blaTEM* gene and 4/17 had *blaSHV* [5]. Moreover, examinations of equine patients in a veterinary clinical context revealed even higher percentages (10.7% and 34.2%) of animals that carry ESBL [20, 21]. These findings confirm previous descriptions based on which feces from hospitalized horses and horses cured with antibiotics harbored more antimicrobial-resistant *E. coli* isolates compared to untreated horses and are more likely to harbor MDR [22, 23].

Present results showed the highest frequency was for streptomycin resistance (59.87%) and the most prevalent resistance gene (54/94) was related to this medication. This result is consistent with those obtained by Sato et al. (2020) indicating the highest (30.9%) streptomycin resistance among *E. coli* strains obtained from healthy racehorses in Japan. Furthermore, in accordance with the present results, they reported that almost all *E. coli* isolates have been susceptible to kanamycin and gentamycin (98.8% and 100%, respectively) [24]. In equine medicine, streptomycin is frequently used to cure gram-negative bacterial infections [8]. These can explain the high resistance rate of equine microbiota against this antibiotic. According to the phenotypic assay in this study, *aadA* and *aadB* were detected in five and eight aminoglycoside-resistant isolates, respectively, and *strA-strB* (streptomycin-resistance genes), as the most common resistant genes, were identified in 54 isolates.

In addition, possibly as a result of extensive application of trimethoprim-sulfamethoxazole to cure infections induced by gram-positive and gram-negative bacteria among horses [24], resistance to this antibacterial combination was relatively high in the tested isolates (47/157). However, in South Korea and Portugal, *STX* resistance was found to be 9.8% (5/51) and 2.8% (2/71), respectively [8, 25]. In a study in Japan, 15.8% of *E. coli* isolates were found to be resistant to trimethoprim [24]. Among trimethoprim-sulfamethoxazol-resistant isolates, we detected dfr1, sul1, and sul2 in 76.6%, 48.9%, and 36.2%, respectively. Ahmed et al. (2010) reported that 93% of the trimethoprim-resistant equine fecal *E. coli* isolates were positive regarding at least one dfr gene [22]. These genes are often encod-

ed on mobile genetic elements, leading to this wide dissemination of *dfr* genes.

Among 19 chloramphenicol-resistant isolates identified in this study, *catI* and *catII* were detected in 11 and 7 isolates, respectively. *catI* gene was previously accountable for most of the plasmid-mediated resistance to chloramphenicol [22]. In addition, this article detected 51/157 isolates to be resistant to oxytetracycline and/or doxycycline, among which *tetA* and *tetB* genes were determined in 39 and 48 isolates, respectively. Tetracycline resistance in *E. coli* most often happens among animals, such as horses, and the efflux genes *tetA* and *tetB* are generally disseminated in gram-negative bacteria [22, 26]. This pattern of prevalence was also shown in equine fecal *E. coli* strains from hospitalized horses in Northwest England in which the *tetB* gene showed to be the most common (71%) resistant to tetracycline, followed by tetA (18%), while no other tet gene was detected [17].

In the current study, 17.19% of E. coli isolates were not susceptible to the first generation of quinolones (nalidixic acid) but norfloxacin and ciprofloxacin were potent antibacterial agents (1.27% resistance). Compared to our results, Lagarde et al. reported that 54.1% of isolates from healthy horses were not susceptible to nalidixic acid, and 20.3% of isolates were not susceptible to ciprofloxacin, which is a fluoroquinolone in this collection [17].

Furthermore, the present assay detected biofilm-forming ability in 81/157 (51.6%) of fecal *E. coli* isolates. Resistance to every examined antibiotic in biofilm-former strains was significantly higher than in biofilm-negative antibiotics. The frequency of biofilm-associated genes *fimH, papC, csgA*, and *fliC* in biofilm-former *E. coli* isolates was 100%, 100%, 77.77%, and 65.43%, respectively. However, less data is available on biofilm formation and the distribution of its associated genes in microorganisms isolated from horses. In the USA, the prevalence of fimH and papC genes among 25 horse *E. coli* isolates was 92% and 4%, respectively [27]. Also, P fimbriae structural subunits-encoding genes, including papC, were the most commonly identified (75/164, 45.7%) virulence genes in *E. coli* from companion animals, including horses in the UK [28].

The present article also investigated the phylogenetic group of *E. coli* obtained from horses. In the present assay, commensal strains were predominant, and 68.2% of isolates were related to the phylogroups B1 and A. Among the remaining isolates, 10 (6.4%) were potential extraintestinal pathogens (B2 and D). In accordance with the present assay, in a study conducted in Iran, phylotype B1 was detected as the most common phylotype (76.92 %) among *E. coli* isolated from healthy riding horses in Kerman [29].

In the present study, every isolate in phylogroups B2 and D carried all biofilm-related genes. These findings are in line with the results of Olowe et al. (2019) who reported that phylogroups B2 and D contained 100% of each of the biofilm-related genes [30]. Biofilm-related genes *fimH* and *papC* were found in 100% of biofilm-positive isolates and were prevalent in all phylogroups. The fimH gene was determined to be common in every phylogroup of *E. coli* according to various studies [30-32].

Moreover, several studies have shown that phylogroups B2 and D have more biofilm-related gene characteristics compared to phylogroups B1 and A [30, 33-35]. Our results showed that all isolates belonging to B2, D, and E phylogenetic groups were MDR. However, the isolates in the other phylogroups were also MDR, and several studies have shown that the strains of group B2 are mainly MDR [30, 36, 37].

Conclusion

Our findings demonstrated that in Guilan province, Northern Iran, non-hospitalized, healthy horses harbor potential extra-intestinal pathogenic and MDR *E. coli* isolates. These animals can be reservoirs for ESBL-producing and noteworthy human and veterinary medicine-used antibiotics-resistant isolates. The obtained data emphasized the growing concern regarding antimicrobial resistance, MDR shedding, and management of antimicrobial applications in veterinary.

Materials & Methods

Equine Study Population and Demographic Data

During May-August 2021, fresh feces samples were collected from 23 farms of 157 healthy and non-hospitalized horses that lived on private farms around Rasht, Northern Iran. The studied horses aged 3 months to 20 years, 72 were female, and 85 were male without receiving antibiotic therapy during the past six months.

E. coli Isolation and Antimicrobial Susceptibility Testing

Every single sample was transferred to the laboratory on the ice in 4 h. *E. coli* was isolated on MacConkey (MC) agar and EMB agar media and was evaluated based on standard bacteriology and biochemistry methods and one *E. coli* isolate was selected per sample for further investigation. The disk diffusion assay was adopted to determine the antimicrobial susceptibility pattern for each isolate of *E. coli* based on the Clinical and Laboratory Standards Institute suggestion [38].

The antimicrobials used in these assays were selected based on their use and importance for human and equine medicine as follow: beta-lactams [amoxicillin+clavulanic acid, ampicillin, ceftiofur, cefotaxime, and cefquinome ampicillin (AM; 10 µg), amoxicillin/clavulanic acid (AMC; 20/10 µg), ceftazidime (CAZ; 30 µg),

Table 4.
Nucleotid sequences of primers used in this study

Gene	Primer Sequence (5"-3")	Amplicon size (bp)	Annealing tem. (°C)	Ref.
blaTEM	F-CTAGTATGACGTCTGTCGC	1080	58	[15]
	R-GACAGTTACCAATGCTTAATC			
blaSHV	F-TTATCTCCCTGTTAGCCACC	795	58	[15]
	R-GATTTGCTGATTTCGCTCGG			
qnr A	F-ATTTCTCACGCCAGGATTTG	516	53	[15]
	R-GATCGGCAAAGGTTAGGTCA			
qnr B	F-GATCGTGAAAGCCAGAAAGG	469	54	[15]
	R-ACGATGCCTGGTAGTTGTCC			
tetA	F-CCTCAATTTCCTGACGGGCT	712	55	[15]
	R-GGCAGAGCAGGGAAAGGAAT			
tetB	F-ACCACCTCAGCTTCTCAACG	586	55	[15]
	R-GTAAAGCGATCCCACCACCA			
strA-strB	F- ATG GTG GAC CCT AAA ACT CT	893	60	[16]
	R- CGT CTA GGA TCG AGA CAA AG			
aadA	F- GTG GAT GGC GGC CTG AAG CC	525	60	[16]
	R- AAT GCC CAG TCG GCA GCG			
aadB	F-GAG GAG TTG GAC TAT GGA TT	208	53	[16]
	R- CTT CAT CGG CAT AGT AAA A			
cat I	F-AGTTGCTCAATGTACCTATAACC	585		[17]
	R-TTGTAATTCATTAAGCATTCTGCC			
cat II	F-ACACTTTGCCCTTTATCGTC			[17]
	R-TGAAAGCCATCACATACTGC			
sul 1	F- CGG CGT GGG CTA CCT GAA CG	433	66	[16]
	R-GCC GAT CGC GTG AAG TTC CG			
sul 2	F-CGG CAT CGT CAA CAT AAC CT	721	66	[16]
	R-TGT GCG GAT GAA GTC AGC TC			
dfr1	F-ACGGATCCTGGCTGTTGGTTGGACGC	254	55	[17]
	R-CGGAATTCACCTTCCGGCTCGATGTC			
fimH	F-TGCAGAACGGATAAGCCGTGG	506		[18]
	R- GCAGTCACCTGCCCTCCGGTA			
csgA	F-GCAATCGTATTCTCCGGTAG	418		[18]
	R -GATGAGCGGTCGCGTTGTTA			
papC	F-TGATATCACGCAGTCAGTAGC	501		[18]
	R- CCGGCCATATTCACATAAC			
fliC	F-ATGGCACAAGTCATTAATACCCAAC	1491		[18]
	R- CTAACCCTGCAGCAGAGACA			

cefotetan (CTT; 30 µg), imipenem (IMP; 10 µg)], aminoglycosides [amikacin (AN; 30 µg), streptomycin (S; 10µg) and gentamicin (GM; 10 µg)], tetracyclines (oxytetracycline and doxycycline), sulphonamides/potentiated sulphonamides (sulphamethoxazole and trimethoprim-sulphamethoxazole), fluoroquinolones [nalidixic acid (NA; 30 µg), ciprofloxacin and enrofloxacin], carbapenems (imipenem), and chloramphenicol (C; 30µg), aztreonam (ATM; 30 µg). Plates were incubated at 37°C for 18-20 h. An isolate resistant to at least one antimicrobial agent in at least three antimicrobial classes was considered MDR. *E. coli* ATCC 25922 was included as quality control for the applied antimicrobial agents.

Extended-Spectrum Beta-Lactamase (ESBL) Production

ESBL production was detected by double disk diffusion technique with ceftazidime (30 µg) and cefotaxime (30 µg) disks alone and combined with clavulanic acid (10µg) on Muller Hinton agar. The positive test result was determined as ≥5 mm elevation in the diameter of the zone compared to a disk free of clavulanic acid. Moreover, an imipenem-EDTA double disk synergy assay was adopted for evaluating MBL enzyme production. Improvements in the diameter of the inhibition zone in IMP+EDTA in comparison with IMP-only disks were determined as MBL producers.

Biofilm Formation Assay

This test was carried out on a microtiter plate. Standard overnight cultures (1.5×108 CFU/ml) were diluted 100 times in PBS. A volume of 200 µl of each culture dilution was transferred to separate wells of a 96-well, flat-bottomed polystyrene plate, and was incubat¬ed overnight at 37°C. After the process of incubation, the planktonic bacteria were discarded, the wells were delicately washed three times using sterile physiological saline and were fixed using methanol for 20 min. Then, each well was stained using crystal violet and washed. Biofilm-related crystal violet was destained in 1 ml ethanol-acetone (95:5, vol/vol). Afterwards, the optical density of the mixed solution was found to be 600 nm. Isolates with OD > 0.625 were categorized to be biofilm-positive [39].

Extraction of DNA

A colony of each bacterial strain was purified and bacterial genomic DNA was extracted by a GenElute Bacterial Genomic Kit (Sigma-Aldrich, St Louis, MO).

Phylogenetic Typing of E. coli Isolates

Bacterial phylogenetic groups were determined using the previously described method of quadruplex phylogroup assignment [12]. The isolates were categorized in phylogenetic groups A, B1, B2, C, D, and F via scoring the presence or absence of genes in arpA/chuA/yjaA/TspE4.C2 order according to the following criteria: A: (+---); B1: (+--+); B2: (-++-/-+++/-+-+); F: (-+--); A or C: (+-+-); D or E: (++--/++-+); E or clade I: (+++-). The three last ones were screened using C or E-specific primers.

PCR Screening for Biofilm-Associated Genes

The frequency of four hypothetical biofilm-related genes in biofilm-former *E. coli* isolates has been investigated by Olowe et al. (2019). Table 1 shows the utilized primers. Extracted nucleic acid was adopted as template DNA for PCR. PCR was carried out based on a total volume of 25 mL, including 0.5 ml dNTPs (10 mM), 5 mL enzyme buffer (10X), 3 ml forward/reverse primers (10 pmol), 2 ml template DNA (2 mg), 0.5 mL enzyme (2.5 U), and 14 ml deionized water. Each gene was individually expanded and PCR products were assessed by electrophoresis on 1% agarose gel. Afterwards, PCR products were sequenced to affirm the identity of the amplicon sequence (Bioneer, South Korea).

PCR Screening for Antimicrobial Resistance Genes

Based on the patterns of antimicrobial susceptibility, PCR was performed for evaluating the corresponding antimicrobial resistance genes. Isolates showing resistance to investigated antimicrobial classes were screened regarding the existence of 14 diverse resistance genes by PCR as described before (Table 4). Tetracycline-resistance genes (*tetA* and *tetB*), sulfamethoxazole-resistance genes (sul1, sul2), TMP-resistance genes (dfr1), streptomycin-resistance genes (*strA-strB*), *blaTEM*, and *blaSHV* were screened. Furthermore, the existence of plasmid-mediated quinolone-resistance genes (*qnrA, qnrB*), and genes encoding aminoglycoside-modifying enzymes (*aadA* and *aadB*) were investigated. Table 2 demonstrates the sequences of oligonucleotide primers adopted in this evaluation. The conditions of PCR reactions were as mentioned above.

Statistical analysis

Correlations between the phylogenetic group, biofilm-forming ability, and frequency of resistance-related genes in examined bacteria were assessed by SPSS Statistics and the Chi-square test. For all tests, $p < 0.05$ was considered significant.

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Competing Interests

There is no any conflict of interests.

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