Original Article

Tetracycline and sulfonamide residues in commercial egg yolks in the north of Iran

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Abstract

Background: Excessive and anachronous antibiotics using in the food industry and the production of livestock products has resulted in multidrug resistance (MDR) in bacteria against various antibiotics. This study aim was to investigate tetracycline and sulfonamide residues in commercial egg yolks.

Methods: *Escherichia coli* was extracted from 500 egg yolk samples of 37 Iranian brands and tested by phenotypic isolation method and resistance tests for tetracycline, sulfonamide, and nitrofurantoin antibiotics. DNA was extracted from 12 identified multidrug-resistant strains to investigate the molecular mechanism of MDR by PCR with specific primers for *tetA, tetB, tetC, tetD, tetG, tetE, tetH, sul I, sul II, sul III, nfsA,* and *nfsB* genes compared to control DNA (ATCC25922). Strains were using the cluster analysis by average Euclidean distance with Jaccard coefficient in SPSS-22.

Results: Examination of 12 antibiotic-resistant *E. coli* by PCR indicated that there were a limited number of resistance genes in the strains. From the group of resistant genes, St2 and St12 strains contained the highest gene number (three genes) and tetracycline-resistant genes were absent in aTcc, St8, St4, and ST3 strains. A maximum of one gene from the sulfonamide-resistant group and one gene for nitrofurantoin-resistance were detected among the studied strains. The highest susceptibility belonged to atcc, St3, and ST8 strains, which were grouped compared to other strains (P-value ≤ 0.05).

Conclusion: Manufacturers and managers of the food industry should particularly consider the risk of increased bacterial resistance to antibiotics and implement programs for resistance monitoring to protect human and animal health.

Keywords: Commerce; Egg Yolk; Iran; Sulfonamides; Tetracyclines.

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Introduction

Eggs are an important commodity in commerce and contain unique nutrients for people of all ages; the ease of digestion of eggs makes them valuable in many therapeutic diets for people. It provides a significant part of essential nutrients for the growth of developing embryos, so the lipid requirement of egg yolk is significant from this point of view. Prevention and treatment of non-viral infectious diseases and, additionally, mass production of broilers and livestock result in the widespread use of antibiotics (1), and antimicrobial drugs

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are used in approx. 80% of poultry (2). The effects of antibiotics on eggs is a public health problem, and their illegal or accidental use can be a threat to the health of consumers, stimulate microbial resistance, and increase the incidence of infection with the entry of resistant pathogens into the food chain (3). After administration to laying hens, their residues are formed in the egg components (yolk and albumin) and intestinal absorption of the drug and its transfer to the bloodstream lead to the deposition of antibiotic residues in the egg (1).

In the study of Khannazer et al., in the investigation of antibiotic residues in poultry carcasses, which was carried out using 4 plates in slaughterhouses around Shiraz, the samples had sulfonamide residues (4). In a study by Cavaliere et al., analysis of sulfoquinoxaline-treated eggs showed that antibacterial agents were still present at 150 ppb levels up to one week after hatching (5). In Mohammadian et al., study on poultry carcasses using the 4-plate qualitative method to detect antibiotic residues in food, the carcasses were contaminated with antibiotic residues (6). Gaudin et al., in 2009 in France, Beck conducted a comparative study on three screening methods, two microbiology methods (premi test and explorer kit), and an ELISA kit to detect sulfonamide remaining in eggs. In this study, premi, explorer, and ELISA diagnostic kits were used. Of the 55 egg samples that were tested for the premi test, only one sample (1.82%) was a false positive, which was satisfactory. The Explorer and Premi tests can be used as general screening tests that allow identification of most microbial families (7). Due to improper quarantine in the distribution of eggs in Iran, which causes the spread of infection with pathogens, such as *Escherichia coli*, and the importance of public health, this study aimed to investigate the infection level of Iranian brand eggs.

Methods

500 egg yolk samples from 37 Iranian brands were used in this investigation to isolate Escherichia coli, which was then tested for its resistance to the antibiotic's tetracycline, sulfonamide, and nitrofurantoin. By using specific primers for the tetA, tetB, tetC, tetD, tetG, tetE, tetH, sul I, sul II, sul III, nfsA, and nfsB genes, DNA from 12 identified multidrug-resistant bacteria was extracted to examine the molecular mechanism of multidrug resistance (MDR).

Culture media

Using the MacConkey agar medium, 51 g of the powder were dissolved in 1 L of distilled water and autoclaved for 15 minutes at 121 °C. The culture medium was held in a plate with an 8-cm diameter.

Simmons citrate agar medium: To prepare this medium, 24.2 g of dry medium powder (Oxoid) was dissolved and boiled in 1 L of distilled water. Then, 5 cc of the solution was poured into test tubes (160 ×16 mm), which were sterilized and placed on a ramp to solidify in the same mode.

Triple Sugar Iron (TSI) agar medium: This is a differential medium and is used to observe the fermentation of sugars and the production of hydrogen sulfide (H_2S) and gas by bacteria. To prepare this medium, mix 65 grams of dry Oxoid powder in 1 liter of distilled water and boil until the powder dissolves completely. Next, 7 cc of the medium was sterilized in test tubes and placed on a ramp to solidify in the same mode.

SIM medium: It is a semi-solid medium that shows three properties of *Salmonella*, including H_2S, motion, and indole. Two-thirds of the depth of the SIM culture medium was pierced using a needle, and the tubes were covered with a loose cap and incubated at 37 °C for 24 h. Afterward, 3-4 drops of the Kovács reagent were added to the tube to examine the production of indole, and a red reaction color indicates a positive indole test.

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Lysogeny Broth (LB) medium: To collect the bacterial samples completely for a long time, 25 g of this powder was solved in 1 L of distilled water, distributed in suitable vessels, and autoclaved at 121 °C for 15 min. Samples were sterilized at 37 °C for 24 h, and then 700 µl of bacterial culture medium was replaced by 300 µl of glycerol (70%-30%) and frozen at −70 °C.

Mueller-Hinton agar medium: This medium was used to determine the sensitivity of microbial isolates. To prepare this medium, 38 g of the culture medium powder was dissolved in 1 L of distilled water, autoclaved at 121 °C for 15 min, and then distributed in sterile plates.

XLD culture medium: Xylose is fermented by *Salmonella* but consumed rapidly due to lysine carboxylation, making the culture medium alkaline which can mask the reaction. *Salmonella* colonies become darker due to iron sulfite deposits, and this is a common feature with *Edwardsiella*. This destructive phenomenon is absent in other species of Enterobacteria. Since the accumulation of acid is due to the high fermentation of lactose and sucrose, it prevents pH reversal and occurs by carboxylation and even the precipitation of iron sulfite in the first 24 h.

Preparation of TBE buffer: 50 ml of the buffer was dissolved in 950 ml of sterile distilled water and stored in the refrigerator until use. *Preparation of agarose gel:* samples were electrophoresed using 1% agarose gel. The volume required to prepare the gel was calculated with the percentage used, considering the length, width of the cast, and thickness of the gel, the amount of the requested powder was weighed with a scale. Preparation of agar gel with a concentration of 1% was used from 0.35 grams of agar powder in 35 ml. Then the powder was heated to dissolve. After the powder was dissolved, 5 microlambda of Red Safe Stain was added to the gel and poured into the gel cast.

Isolation and in vitro detection of E. coli isolates: From Iran's northern areas, samples of 500 eggs from 36 brands with designated expiration dates were taken. Before analysis, the yolks were removed from the albumin, mixed, and chilled.

Tetracycline and sulfonamide residues in samples were analyzed quantitatively by the ELISA method using a Ridascreen-RbioPharm kit in Laboratory and differential diagnostic t Diagnostic tests used to identify E. coli consisted of (Gram staining, H2S production in TSI medium, Movability in SIM culture medium, Growth in MacConkey agar medium, Growth in XLD medium, Gas production, Indole test, Simon Citrate culture medium).

Storage of samples: *E. coli* samples were stored in the LB medium containing 30% glycerol at −70 °C until further procedures.

Examination of drug resistance pattern

Determination of antibiotic resistance pattern using the disk diffusion method (DDM)

The DDM was used to evaluate the susceptibilities of 12 isolates in accordance with 2019 CLSI recommendations. A 24-hour bacterial culture was first established on solid media. A single colony of the pure culture was then dissolved using a loop in sterile normal saline. The turbidity of the suspension was determined using the half-McFarland turbidity standard. Bacteria were then cultivated on a Mueller-Hinton agar medium using a sterilised swab. The culture material obtained from the Padtan Teb Company was then covered with discs made of antibiotics. After 24 hours of incubation at 37 °C, the results of bacterial susceptibility or resistance were evaluated based on the aforementioned parameters. Antibiotic discs, including tetracycline, chlortetracycline, doxycycline, and oxytetracycline, are among the discs utilized in this investigation.

Preparation of samples for molecular analysis

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Using the boiling approach, 500 µlambda from LB medium cultivated with bacteria in 1.5 ml microtubes was boiled in a 96 °C water bath for 15 minutes to extract DNA from E. coli. They were immediately put on ice after emerging from the water bath, where they were frozen for three minutes. The lysed wall and contents released from the bacteria were separated by microcentrifugation at 13,000 rpm for 2 minutes.

Determination of DNA concentration using a NanoDrop: The concentration of isolated bacterial DNA was measured using a NanoDrop system (ThermoNanoDrop 2000c).

Identification of tetA, tetB, tetC, tetD, tetG, tetE, tetH, sul I, sul II, sul III, nfsA, and nfsB genes by PCR: DNA of the samples was introduced to a pre-prepared PCR Master Mix and pipetted well using a sampler. In each series of the PCR test, PCR should be performed for positive and negative control DNA samples along with unknown samples (to ensure no contamination in the test). The amounts (Dose (µl)) of each component to prepare the PCR complex were listed (Master mix:12.5, Primer forward:1, Primer reverse:1, Water D.D:9.5, DNA template:1).

PCR mixtures containing unknown and control DNAs (ATCC25922) were prepared and placed in a thermocycler. The primers used in PCR are described in Table 1.

Table 1. The sequence of primers used for PCR

Primers	Primer sequence
F-tetA	GCGCCGATCTGGTTCACTCG
R-tetA	AGTCGACAGTAGCGCCCGC
F-tetB	TACGTGAATTTATTGCTTCGG
R-tetB	ATACAGCATCCAAAGCGCAC
F-tetC	GCGGGATATCGTCCATTCCG
R-tetC	GCGTAGAGGATCCACAGGACG
F-tetD	GGAATATGTCCCGGAAGCGG
R-tetD	CACATTGGACAGTGCCAGCAG
F-tetG	GCAGAGCAGGTCGCTGG
R-tetG	CCYGCAAGAGAAGCCAGAAG
F-tetH	CAGTGAAAATTCACTGGCAAC
R-tetH	ATCCAAAGTGTGGTTGAGAAT
F-tetE	GTTATTACGGGAGTTTGTTGG
R-tetE	AATACAACACCCACACTACGC
F-sulI	5'-TGA GAT CAG ACG TAT TGC GC-3'
R-sulI	5'-TTG AAG GTT CGA CAG CAC GT-3'
F-sulII	5'-GCG CTC AAG GCA GAT GGC ATT-3'
R-sulII	5'-GCG TTT GAT ACC GGC ACC CGT-3'
F-sulIII	5'-GAGCAAGATTTTTGGAATCG-3'
R-sulIII	5'-CATCTGCAGCTAACCTAGGGCTTTGGA-3'

All primers were confirmed in BLAST and their quality was checked by OligoAnalyzer software. The temperature and time of each reaction are described in Table 2.

Table 2. Temperatures used for PCR

Factor	Temperature(°C)			Time		
Gene Step	tetA,B,C,D,G	tetE,H	nfsA,B	tetA,B,C,D,G	tetE,H	nfsA,B
Initial denaturation	95	95	95	5 Min	5 Min	5 Min
Denaturation	95	95	95	20 Secs	20 Secs	20 Secs
Anealing	55	53	58	20 Secs	20 Secs	20 Secs
Extension	72	72	72	20 Secs	20 Secs	20 Secs
Final extension	72	72	72	5 Min	5 Min	5 Min
Cycle	40	40	40			
Factor	Temperature(°C)			Time		
Gene Step	sulI	sulII	sulIII	sulI	sulII	sulIII
Initial denaturation	95	95	95	5 Min	5 Min	5 Min
Denaturation	95	95	95	20 Secs	20 Secs	20 Secs
Anealing	56	62	51	20 Secs	20 Secs	20 Secs
Extension	72	72	72	20 Secs	20 Secs	20 Secs
Final extension	72	72	72	5 Min	5 Min	5 Min
Cycle	40	40	40			

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Figure 1. *E. coli* Gram-negative bacilli resulting from Gram staining (left) and *E. coli* grown on XLD medium(right)

Electrophoresis: The required amount of 5 XTBE buffer was poured into the electrophoresis tank, and the tray containing agarose gel was placed in the tank. Six µlambda of the prepared PCR sample was slowly run into the wells. The tank lid was then closed and the power supply was set to the appropriate voltage. After electrophoresis, the gel was placed in a Gel-Doc system. The gel image was adjusted in the device, followed by taking photos of genes. Based on these data and using genes obtained from positive control strains, the presence or absence of the target genes can be determined in each of the experimental samples.

Data were analyzed using descriptive statistics. Strains were grouped using the cluster analysis by average Euclidean distance with Jaccard coefficient in SPSS ver. 22.

Results

Examination of 12 samples of antibiotic-resistant *E. coli* for resistance genes by PCR revealed that each strain contained a limited number of resistance genes. From the group of resistant genes, St2 and St12 strains contained the highest gene number (three genes). From the same group, TRGs were absent in aTcc, St8, St4, and ST3 strains. A maximum of one gene from the sulfonamide-resistant group and one gene for nitrofurantoin-resistance were detected among the studied strains. The highest susceptibility belonged to atcc, St3, and ST8 strains, which were grouped compared to other strains ($P\text{-value} \leq 0.05$).

Phenotypic tests to identify the genus and species of E. coli

Phenotypic tests: The isolated Gram-stained bacteria were observed as Gram-negative bacilli Figure 1(left).

E. coli isolates are mobile bacteria, as demonstrated in Table 3. Figure 1 (right) shows worm-like colonies of the majority of isolates on the XLD medium.

Table 3. Differential tests to identify *E. coli*

Diagnostic tests	Result
Gram staining	Gram
TSI	+
Movability in SIM culture medium	+
Growth in McConkey agar medium	+
Urease test	-
VP	-
Growth in XLD medium	+
MR	+
Simon Citrate	+
Indole	+

All E. coli isolates were able to develop and turn the medium from green to blue in the TSI medium of Figure 2 (left). Figure 2 (center). E. coli was urease-negative and methyl red-positive, as seen in Figure 2 (right), and adding the reagent to the growth

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medium caused the medium to turn red cherry in colour. 2-4 Figures.

Figure 2. *E. coli* cultured on TSI medium(left), Culture of *E. coli* on Simon Citrate medium(middle)and Urease test(right)

Figure 3. MR positive test(left), VP negative test, and Indole positive test(right)

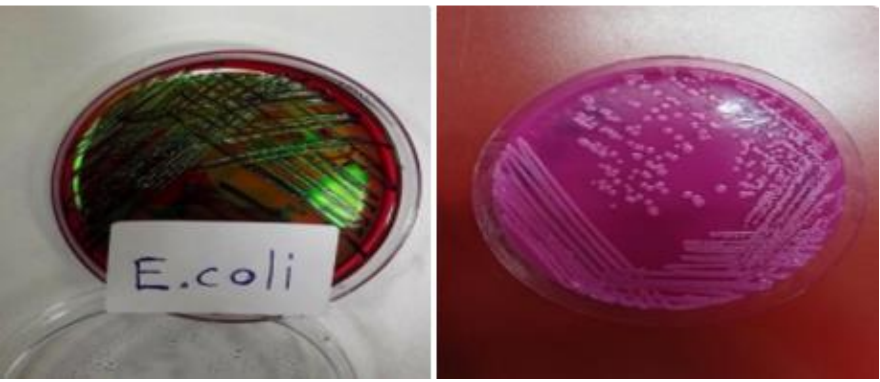

Figure 4. Growth of *E. coli* on EMB medium(left)Growth of *E. coli* on MacConkey agar medium(right)

Results of the antibiogram using the DDM

According to the results obtained from the DDM, 10 out of 12 bacterial samples showed resistance to the antibiotics used here in Table 4.

Examination of the percentage of inhibition (the halo diameter) under antibiotic treatments revealed that the highest inhibition was seen in multidrug-resistant strains in chlortetracycline treatment. This means that 60% of the strains were created

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by this antibiotic, and the other antibiotics inhibited 50% of the strains (P-value ≤ 0.05). Examination of strains under antibiotic treatments showed that strains 1, 2, 3, 3, 5, and 8 were resistant to all four types of antibiotics (oxytetracycline, tetracycline, chlorocycline, and doxycycline), but strain 3ST was the most resistant to all four antibiotics, and 8ST was the most inhibited strain. St2 showed more than 20% and St5 and St6 strains had up to 15% sensitivity to antibiotics. Other strains 4, 7, 9, and 12 were not inhibited under the above-mentioned four antibiotics and showed complete resistance. The standard strain presented the highest sensitivity to four types of antibiotics.

Table 4. Results of antibiotic resistance of *E. coli* strains from diffusion disc

Antibiotic	Oxytetracycline	Tetracycline	Chlortetracycline	Doxycycline
Resistance (%)	50 %	50 %	60 %	50 %
str	oxytet	Tet	clrotet	Doxytet
atcc25922	100	100	100	100
st1	21.74	21.74	23.81	31.25
st2	21.74	21.74	23.81	31.25
st3	100	100	100	100
st4	0	0	0	0
st5	8.7	8.7	9.52	12.5
st7	0	0	0	0
st8	91.3	91.3	100	100.25
st10	0	0	0	0
st11	0	0	0	0
st12	0	0	0	0

Results of PCR

Analysis of the *tetA* gene: Based on the results obtained from the PCR and electrophoresis of samples, the *tetA* gene was found in four of 12 samples Figure 5(left up), which is confirmed by the length of the PCR product. The presence of two bands in the sample indicates the possible presence of different copy numbers in different positions in the plasmids. Analysis of the *tetB* gene: Based on the PCR and electrophoresis results of samples, the *tetB* gene was found in seven of 12 samples according to the length of the product Figure 5(middle up).

Analysis of the *tetC* gene: Based on the PCR and electrophoresis results of the samples, the *tetC* gene was absent in all samples Figure 5(right up). The presence of different bands and smears according to the size of the PCR product indicated DNA fragmentation.

Analysis of the *tetD* gene: Based on the PCR and electrophoresis results of samples, the *tetD* gene was observed in two of 12 samples Figure 5, which is confirmed according to the PCR product length Figure 5(left down).

Analysis of the *tetG* gene: Based on the PCR and electrophoresis results of the samples, the *tetG* gene was present in none of the samples Figure 5(middle down).

Analysis of the *tetE* gene: Based on the results obtained from the PCR and electrophoresis of samples, the *tetE* gene was present in three out of 12 samples Figure 5(right down), which is confirmed by PCR product length.

In Analysis of the *tetH* gene: Based on the PCR and electrophoresis results of the samples, the *tetH* gene was visible in none of the samples Figure 5(row 3).

PCR examination of TRGs in different strains showed that *Tetg*, *Teth*, and *Tetc* genes were not present in all the strains

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Table 5. *Teta* was present in strains 1, 2, 6, and 12, but it was absent in the other strains. *Tetb* was found in most of the strains (1,2,

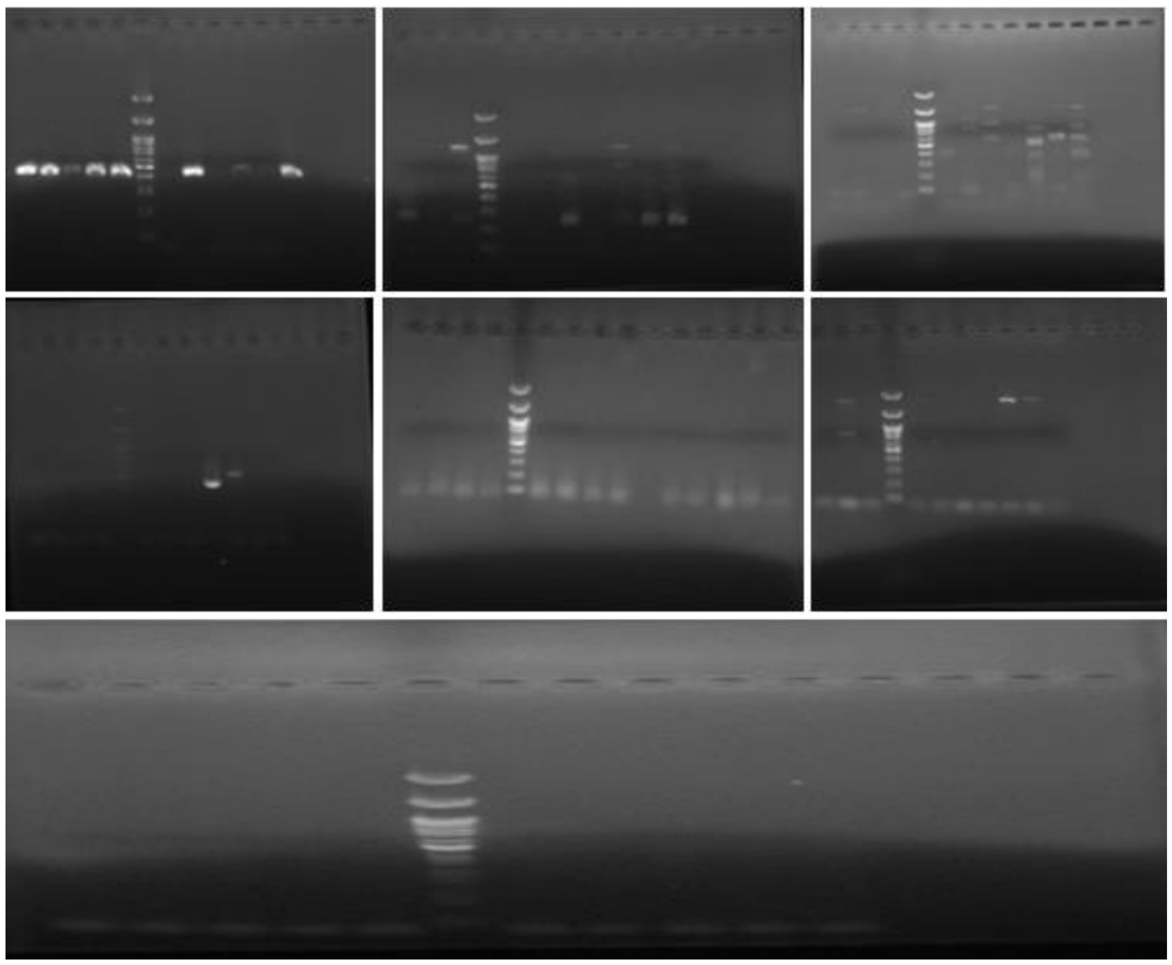

Figure 5. The gel showing the presence/absence of the *tetA* gene, the presence/absence of the *tetB* gene, presence/absence of the *tetC* gene, the presence/absence of the *tetD* gene, the presence/absence of the *tetG* gene, the presence/absence of the *tetE* gene and the presence/absence of the *tetH* gene

Table 5. Presence/absence of tetracycline genes in the studied strains

Column1	tetc	teta	tetb	Tete	tetd	Tetg	teth
atcc25922	0	0	0	0	0	0	0
st1	0	1	1	0	0	0	0
st2	0	1	1	1	0	0	0
st3	0	0	0	0	0	0	0
st4	0	0	0	0	0	0	0
st5	0	0	0	0	0	0	0
st7	0	0	0	1	0	0	0
st8	0	0	0	0	0	0	0
st10	0	0	1	0	0	0	0
st11	0	0	1	0	1	0	0
st12	0	1	1	1	0	0	0

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10, 11, and 12). The presence of the *Tete* gene was confirmed in strains 2, 7, and 12.

Examination of TRGs by PCR in different strains showed that *Tetg*, *Teth*, and *Tetc* genes were not found in any of the strains Table 5. *Teta* was present in strains 1, 2, and 12, but it was not seen in the other strains. *Tetb* was visible in most of the strains (1,2, 10, 11, and 12). The presence of the *Tete* gene was confirmed in strains 2, 7, and 12.

Figure 6 shows the cluster analysis and similarities of strains regarding the presence of gene bands. By dividing the dendrogram into a distance of 10 units, the samples were classified into four sub-clusters based on the presence of TRGs. The first cluster consisted of St8, St12, and St2. St12 and St2 strains contained *teta*, *tetb*, and *tete* resistance genes, but all resistant genes were not observed in st8 strain.The second sub-cluster comprised strains St5, St7, and St4, all of which contained SUL2 resistance genes; however, *nfsa* was also present in the St7 strain.

The third sub-cluster included St11, St10, ATCC, and St3, which shared the *tetb* resistance gene. However, no resistance gene was found in strain 3, and Atcc carried the sul2 gene. Sub-cluster 4 comprised strain 23 that possessed *tetc*, *tetb*, and *tete* genes.

Examination of the *sulI* gene: The PCR and electrophoresis results of samples indicated the presence of the *sulI* gene in samples 1, 2, 4, 5, 7, and 10 (60% of samples), Figure 6, which is confirmed based on the PCR product length. Analysis of the *sulII* gene: The PCR and electrophoresis results of samples revealed that this gene was seen in six samples Figure 6(left up), which is confirmed according to the PCR product length.

The presence of smear bands in some samples indicates the possible presence of different copy numbers in different positions on the plasmids or DNA fragmentation of the samples.

Analysis of the *sulIII* gene: The PCR and electrophoresis results of the samples showed that the *sulIII* gene was present in none of the samples Figure 6 (middle up). Analysis of the *nfsA* gene: The results obtained from the PCR and electrophoresis of samples indicated that the *nfsA* gene was found in four samples according to the PCR product length Figure 6(right up).

Analysis of the *nfsB* gene: The PCR and electrophoresis results of the samples revealed that the *nfsB* gene was not present in all the samples Figure 6(left down).

Based on the Jaccard coefficient and Euclidean distance, the results of grouping multidrug-resistant strains having resistance genes against tetracycline, sulfonamide, and nitrofurantoin are represented in Figure 6(right down). Dividing the dendrogram into a distance of 10 units indicated that the strains St8, St12, St3, and St10 belonged to a sub-cluster and did not possess *tetd, tedc, tetg, teth, Sul1, Sul3, nfsa*, and *nfsb* genes, but the *tetb* and sul2 were present in St10 and St12 contained *tet a*, *b*, and *e* genes.

The second sub-cluster consisted of atcc and st11. These strains were similar regarding the absence of all the studied genes, except tetb and sul2. The third sub-cluster only contained one st1 strain that possessed at least one resistant gene to each of the antibiotics. This strain contained *teta/tetb*, *sul2*, and *nfsa* genes from tetracycline, sulfonamide, and nitrofurantoin, respectively, but it lacked the other genes. The st2 strain was grouped alone in the sub-cluster and did not possess all the studied resistance genes.

The st4 and st5 strains were grouped in a different sub-cluster and did not have all the genes studied, except the *sul2* gene. The last sub-cluster included the st7 strain, which was grouped farther than the other strains, and contained the *sul2*, *tete*, and *nfsA* genes,

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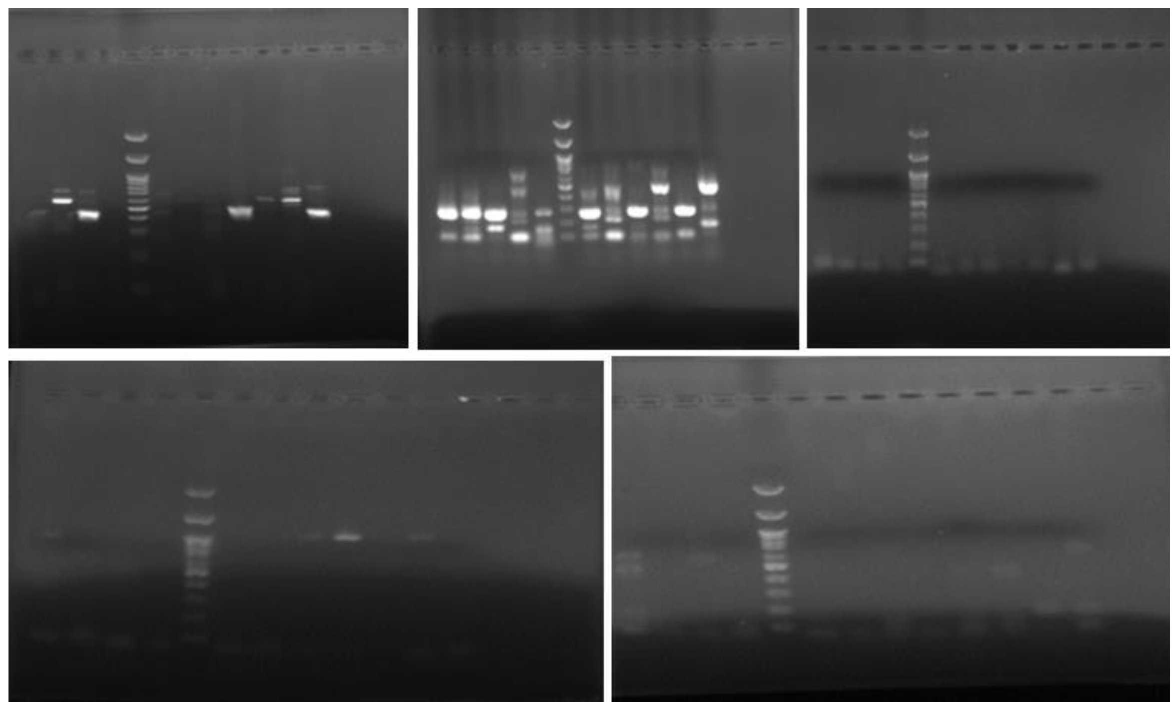

Figure 6. The gel for the presence/absence of the *sulI* gene(left up), the presence/absence of the *sulII* gene(middle up), the presence/absence of the *sulIII* gene(right up), the presence/absence of the *nfsA* gene(left down)and the presence/absence of the *nfsB* gene(right down)

but the other genes were absent in this strain.

There was a maximum of three antibiotic-resistant genes for tetracycline in some of the studied strains, including St12 and St2, and the studied tetracycline genes were absent in some strains despite their MDR Figure 6. The source of resistance in these strains seems to be other TRGs that were not among the genes studied here.

The Number of tetracycline resistance genes in multidrug-resistant strains of *E. coli demonstrated that* the sensitivity percentage of the strains to four types of antibiotics indicated that St3 was the most sensitive strain with the largest halo diameter in all four types of antibiotics (oxycycline, doxycycline, tetracycline, and chlorocycline), similar to the ATcc strain. The St8 strain was also highly sensitive to all four antibiotics. The most resistant strains were St12, St11, St7, St10, and St4 to all four antibiotics with no halos in the growth of the above bacteria in the antibiogram test.

Examination of the resistance dose of the strains to various antibiotics idicated the same trend in both sensitive and resistant strains for all four antibiotics. Evaluation of the various MDR in the studied strains based on the tested antibiotics is presented in Table 6.

Table 6. Percentage of relative resistance to various antibiotics

Strain	Oxy	tet	clro	Doxy
atcc25922	100	100	100	100
st1	21.74	21.74	23.81	31.25
st2	21.74	21.74	23.81	31.25
st3	100	100	100	100
st4	0	0	0	0
st5	8.7	8.7	9.52	12.5
st7	0	0	0	0
st8	91.3	91.3	100	100
st10	0	0	0	0
st11	0	0	0	0
st12	0	0	0	0

In this study, an MDR grade of 4 was obtained for strains St4, St10, St7, St11, and St12, but it was equal to 1 for strains St3 and St8. Relative resistance of 25% for all

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four antibiotics was estimated for St2 and St3.

Discussion

Examination of 12 samples of drug-resistant *E. coli* in terms of resistance genes by the PCR method showed that there were a limited number of resistance genes in each strain. The highest number of resistant genes belonged to St2 and St12 strains with three genes from the resistant group. In the same group, TRGs were not seen in aTcc St8, St4, and ST3 strains.

Among the studied strains, there was a maximum of one gene from each of the sulfonamide and nitrofurantoin resistance groups. The atcc, St3, and ST8 were the most susceptible strains, which were grouped significantly compared to the other strains (P-value ≤ 0.05).

The maximum resistance belonged to St4, ST7, St11, St10, and ST12 strains, meaning that inhibitory changes were not observed in *E. coli* with the application of various antibiotics. The study of the number of resistance genes and susceptibility indicated a direct relationship between these two variables. The studies related to the present study are described below.

Khannazer et al., examined antibiotic residues in 250 samples of poultry carcasses, which were found to be usable, collected from slaughterhouses around Shiraz in Iran. They tested the antibiotic contamination level using the four-plate test, which is considered one of the standard quality methods for determining antibiotic residues in carcasses. Two bacteria, *Bacillus subtilis,* and *Staphylococcus aureus* were cultured in Mueller-Hinton agar culture media with pHs of 6, 7, 7, and 8. To detect minor concentrations of sulfonamides, 0.01, 0.8, and 0.8 μg/ml of trimethoprim (TMP) were added to the culture media of *B. subtilis, B. subtilis*, and *S. aureus*, respectively. Out of 250 chicken carcasses, impermissible infections with antibiotics were detected in chest muscles (54.2%), kidneys (2.6%), and the liver (9.9%). In 250 samples of chest, liver, and kidney muscles cultured in the TMP-containing Mueller-Hinton medium, sulfonamide residues were observed in 7%, 3%, and 1%, respectively (4).

Italian researchers Cavaliere et al. used liquid chromatography-mass spectrometry to examine the presence of 14 sulfonamide antibiotic residues in milk and eggs. The sulfonamides were extracted and purified using a solid-phase extraction (SPE) cartridge. The protocol of this study extracted a higher amount of sulfonamide antibiotics from the eggs and reduced the analysis time. Sulfonamide antibiotics in eggs were obtained at a level of 50 ppb ranging from 68 to 106% with a relative standard deviation (RSD) of < 12%. In this method, 5-13 ppb of sulfonamide was estimated quantitatively (S/N = 10) in eggs. After one week of hatching, sulfaquinoxaline was still detectable in chickens' eggs at a concentration of 150 ppb, according to an examination (5).

Mohammadian et al., determined antibiotic residues in liver, breast, thigh, and wings samples of 100 pieces of usable poultry carcasses in slaughtered poultry of Sanandaj, Iran. They employed Mueller-Hinton agar media with three different pHs (6, 7.2, and 8) together with B. subtilis and Micrococcus latus to perform the four-plate method, a qualitative technique for identifying antibiotic residues in food. Based on the results, 72 (72%) out of 100 tested carcasses contained antibiotic residues in one or more parts of carcasses, and 28 (28%) presented no residues or showed residues less than the maximum allowable amount. Infection with antibiotic residues were observed in 44 cases (60.91%) in one organ, 11 cases (15.15%) in two organs, 8 cases (11.09%) in three organs, and 9 cases (12.50%) in all four tested organs. Moreover, the numbers of carcasses infected with antibiotic residues were 19 (76%), 18 (72%), 15 (60%), and 20 (80%) in spring, summer, autumn, and winter, respectively (6).

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In France, Gaudin et al., compared three screening methods, two microbiological methods (the Premi test and explorer kit), and an ELISA kit to detect sulfonamide residues in eggs using Premi and explorer tests with an ELISA kit. Of the 55 egg samples tested for the Premi test, only one sample (1.82%) was false positive with a satisfactory level. In the end, 33 examined egg samples had a false-positive reaction rate of zero. The ELISA kit was used in this work to test 12 egg samples for a variety of antibiotics, including tetracycline and sulfonamide. Finally, for all kits, the false-positive rate was under 2%. The sensitivity of the Premi test was superior to the Explorer's. The Premi test indicated that detectable values for sulfonamide antibiotics (≥ 100 µg/kg) were satisfactory. Tetracycline and doxycycline antibiotics had detectability levels that were nearly as high as the MRL or twice as high as the MRL. In comparison to microbiological testing, the ELISA kit's sulfonamide determination and detection rates were substantially lower. For specific sulfonamide screening of eggs, ELISA kit is advised. The Explorer and Premi tests, on the other hand, can also be employed as comprehensive screening tests that enable the identification of the majority of antibiotic families (7).

Antibiotic usage and antibiotic residues in commercial eggs in Tanzania were examined by Nonga et al. Twenty chicken farmers were questioned about their understanding of the antibiotic withdrawal time, the types of antibiotics they use, and the justifications for doing so. For a qualitative examination of antibiotic residues in 70 egg samples, they used the Delvotest and the agar-well diffusion method. The researchers discovered that farmers utilised antibiotics to prevent and treat common poultry ailments. Conventional antibiotics consisted of oxytetracycline (75%), egg booster (50%), amprolium (35%), sulfamotoxy pyridazine (35%), sulfonamide (25%), chlorotrotracycline (10%), chloramphenicol (10%), sulfadiazine-trimoprim (20%), doxycycline (20%), sulfadiazine (25%), and flumquin (10%), which accounted for 85% of commonly used medicines. The Delvotest kit detected antibiotic residues in all 70 eggs, whereas the agar well diffusion test detected residues in 21.4% of them (8).

In Belgium, antibiotic residues and food contamination were studied by Vandenberge et al. In this investigation, sulfadiazine, or doxycycline, was cross-contaminated at levels of 2.5, 5, and 10% of therapeutic concentrations in experimental feed given to laying hens. The concentration of cross-contamination in feed was estimated to be 6.25, 12.5, and 25 mg/kg in each drug, respectively, because the therapeutic dose for both antibiotics was 250 mg/kg. During the purification and emptying periods, samples of eggs, egg whites, and egg yolks were collected and examined using liquid chromatography and sequential mass spectrometryRemaining sulfadiazine concentrations of 208, 299, and 60 g/kg were found in the 10% cross-contamination group on day 1, while doxycycline residual concentrations of 455, 332, and 206 g/kg were found in whole eggs, egg whites, and yolks on day 13. The concentrations of sulfadiazine and doxycycline in egg white were higher than those in egg yolk during the treatment period, although the ratio of sulfadiazine to egg white was larger than that of doxycycline. Residues could pose a risk to the safety of the food supply, as none of the medications are approved for use in Belgian laying hens (9).

El Nasr et al., identified antibiotic residues in eggs consumed in Khartoum, Sudan. A total of 157 egg samples were collected from 32 chicken farms and 74 outlets. Information related to farm health status, extensive symptoms of diseases, awareness of farmers and type of antibiotics was collected using a structured questionnaire. They found that diarrhea was present in 34.6% of the farms and tetracycline was the

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most used in 36.4% of the farms. Farmers monitored their fields in 57.6% of farms and insufficient knowledge about harvest period was observed in 94% of farmers. Disc and Premi test kits were used to identify antibiotic residues. Out of 157 samples, the total number of positive cases was 49.6% and 12.7% using disk method and Premi kit, respectively. Using the Kappa statistic, the agreement between the two tests and the percentage of agreement were 0.30 (weak agreement) and 61.7%, respectively. The data showed that the health status of poultry farms has led to an increase in the percentage of positive samples with the simultaneous use of antibiotics to treat diseases and lack of knowledge about the use of antibiotics. Although antibiotics were used excessively in poultry farms, a lower positive percentage of positive samples was detected by the Premi test, while the disc method was significantly associated with excessive use of antibiotics (10).

By examining 120 poultry carcasses from five different slaughterhouses in Kermanshah, Iran, Shahbazi et al. claimed to have measured MIC in cultured samples and run ELISA and HPLC tests. To examine the tetracycline (TC) group residues in 120 grilled chicken carcasses (including breast, liver, and thigh tissue), the main quantitative screening procedures included high-performance liquid chromatography (HPLC), four-plate methods (FPT), and ELISA. The FPT findings showed that 30 corpses (about 25%) had antibiotic residues. ELISA results showed that 21, 16, and 12 liver, thigh, and chest muscle samples, respectively, were positive for TC residue out of 30 positive samples in FPT. In samples that tested positive for ELISA, the HPLC method was utilised to evaluate the prevalence of TC pseudo-residues and the range of their concentrations. FPT and HPLC had a higher correlation (0.940) than ELISA and HPLC (-0.726), according to a comparison of the data. The results of this investigation also showed that the medium inhibition test with B. subtilis at pH 6 exhibited the maximum sensitivity for detecting TC residues (11).

Lalawmpuia conducted a study on antibiotic residues in poultry feed, eggs and water. In this study, two samples of poultry feed and water and three samples of eggs were collected from 27 different poultry units in order to investigate antibiotic residues in feed, water and eggs and to investigate the effect of different heat treatment methods on these residues. Of these numbers, 81.7 (6.8%) and 54.7 (9.12%) of egg and feed samples were positive for oxytetracycline, while 81.4 (9.4%) and 54.2% (5.2%) of egg samples Chicken and food were positive for chlortetracycline, respectively. . Among the 106 eggs available in the market, 106.5 (4.7%) and 106.3 (2.8%) were positive for oxytetracycline and chlortetracycline, respectively. However, none of the egg albumin samples were positive among the sulfonamide groups, including sulfadiazine and sulfamethoxazole. Also, 106.2 (1.8%) and 106.1 (0.9%) samples had oxytetracycline and chlorotetracycline, respectively (12).

In South Africa, Ramatla et al., examined antibiotic residues in three types of meat, including chicken, in 150 samples (50 samples of each type of meat). Sulfonamides and tetracyclines were also among the antibiotics used in this study. The levels of antibiotics were evaluated by three methods of ELISA, TLC, and HPLC. ELISA results indicated that 18% and 25.3% of the samples were positive for the presence of sulfonamide and tetracycline antibiotics, respectively, while values of 88.8% and 14.6% were obtained in TLC and HPLC results for these antibiotics, mostly belonging to sulfonamide. In the ELISA method, the concentration of antibiotic residues ranged from 19.8 to 92.8 μg/kg and 14.2 to 1280.8 μg/kg for sulfonamide and tetracycline, respectively. In the HPLC method, this concentration ranged from 20.7 to 82.1 μg/kg and 41.8 to

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320.8 μg/kg for the same antibiotics, respectively (13). In Poland, Piatkowska et al., simultaneously studied antibiotic residues used in veterinary medicine, food additives, and illegal dyes on eggs using HPLC and mass spectrometry. In this experiment, 150 egg samples were examined to simultaneously determine 120 analytes, including tetracycline and sulfonamide. The results of linearity ($r \leq 0.99$), recovery (75-108%), reproducibility (CV 1.60-15.9%), repeated production (CV 2.60-15%), decision limit (CCα 2.25-11156 μg/kg) and detection ability (CCβ 2.04-1316 μg/kg). A monitoring control program for the proposed method was used to examine 150 real egg samples (14).

Krisova & Kozarova in Slovakia compared two techniques for quickly finding antibiotic residues in eggs. Out of 66 egg samples obtained from 11 different countries in Europe, 22 egg samples were chosen at random for this comparison and tested using the Premi and EXP kits. The findings of this investigation showed that whereas the Premi kit was unable to detect antibiotic residues in any of the samples, the EXP kit was able to detect them in eight and six samples, respectively, suggesting that this assay was more sensitive (15).

According to Jammoul & El Darra's study, Antibiotic Residues in Chicken Meat in Lebanon, chicken samples obtained from Lebanese farms show antibiotic residues. The study tested 80 chicken samples from farms located around Lebanon for antibiotic residues. Using an improved multi-class method, HPLC mass spectrometry was used to identify and quantify 30 antibiotics from four different chemical classes (sulfonamides, tetracyclines, quinolones, and beta-lactams). Following tetracyclines (17.5%), amoxicillin (beta-lactam), and ciprofloxacin (quinolones), the screening of four antibiotic families revealed that quinolones were associated with the highest percentage (32.5%). The maximum residue limit (MRL) set by the EU standard was surpassed by the residue levels of sarafloxacin, amoxicillin, and penicillin G. Therefore, recommendations are given for the prudent application of antimicrobial agents to chickens in order to decrease the incidence of Salmonella resistance in chickens (16).

Eggs were examined for the presence of bacteria using standard techniques in the study by Adesyun et al., which was based on the prevalence of Salmonella and E. coli species, the resistance of anthropopathogens, and antibiotic residues in eggs collected from 39 farms. Using the disc diffusion approach, bacterial resistance to eight antimicrobial drugs was identified. ELISA, HPLC, and the microbial inhibition test were used to find antimicrobial residues in eggs. Salmonella and E. coli were present in eggs at rates of 7.7 and 48.7%, respectively. Only 2.0% (4 of 196) of the 19 E. coli species found in the eggshells and egg contents, respectively, tested positive for the presence of E. coli. 49 E. coli isolates were examined, and 71.4% of them revealed resistance to one or more antibiotics. Sulfonamide (ppb79) and oxytetracycline (ppb106) were found to be present in eggs, bringing the prevalence and residual level of antibiotics to 2.6 and 0.5%, respectively (17).

In a 2017 study by Chaiba et al., 120 samples of poultry meat (muscle, liver, gizzard, and egg) were collected from various farms and subjected to regular microbiological analysis (four pages), which was encouraged and approved by the Food Safety Agency. The French (AFSSA) were examined. Liquid chromatography-mass spectrometry (HPLC-UV-MS) was used to do a quantitative analysis of the chemicals found in the muscle samples that tested positive for antibiotic residues. According to the findings, antibiotic traces were found in 36.15% of poultry meat samples from intensive livestock. Regarding conventional livestock samples, every sample came back negative. The maximum residual limit of tetracycline set by the FAO and WHO, which is 100 g/kg,

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was not exceeded in these samples. The obtained level ranged from 37 to 74 g/kg (18).

In a study by Van den Meersche et al., nine antibiotic resistance genes (tet (B), tet (L), tet (M), tet (Q), tet (W), erm (B), erm (F), and sul2), as well as commensal bacteria (Salmonella typhimurium), were examined to determine their presence and fate during the biological removal of nitrogen from pig manure over time. On six occasions, separated by two weeks, samples of animal dung and storage lagoons were examined on two farmsThe only antibiotics that were used in the three months before the initial sampling, before, and after the biological removal of nitrogen from pig faeces were those that could be recognised. Nearly all of the samples from both farms contained doxycycline and sulfadiazine, two of the examined antibiotics. The second field sample for sul2 and erm (F) revealed a considerable increase in relative abundance; however, there were no differences between samples for tet (L). The results point to a potential reduction in the levels of various drug residues, some antibiotic resistance genes, and common bacteria in pig manure as a result of biological nitrogen removal (19).

To ensure the health of eggs for human consumption, eggs should be obtained from hens that have already used approved antibiotics, and the number of antibiotic residues that accumulate in the egg yolk and albumin and the time they remain in the egg components after the antibiotics are removed is recorded (20).

Recommendation

The present study, which was conducted on the contamination level of Iranian eggs, indicates that some of the selected strains were resistant to several drugs, having resistance genes to tetracycline, sulfonamide, and nitrofurantoin, although some strains were resistant. The studied resistance genes did not exist, which justifies the need to study other genes.

Conclusion

Since eggs are distributed without proper quarantine in Iran, this factor causes the spread of infection to various pathogens, including *E. coli*. The purpose of this investigation was to find out how contaminated Iranian-brand eggs were. The sources of infection showed that certain chosen strains had some tetracycline, sulfonamide, and nitrofurantoin resistance genes, indicating that they were multidrug-resistant. However, some resistant strains did not contain the studied resistance genes, which supports the need to investigate additional genes. The present investigation found that efflux pumps were the sources of tetracycline resistance in 60% of the strains, with Sul2 and nfsa genes being responsible for 60% and 40% of the strains' resistance to sulfonamide and nitrofurantoin, respectively. Given that more than 60% of the animal protein required by society is provided by chicken and eggs, producers and managers of the food industry should be specifically concerned about the increased bacterial resistance to frequently used antibiotics based on the present results. Furthermore, resistance monitoring programs should be implemented aiming at protecting the health of humans and animals.

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Authors' contribution

Reza Abolghasemi and Arash Chaichi Nosrati developed the study concept and design Leila Modiri acquired the data. Mirsasan Mirpour and Reza Abolghasemi analyzed and interpreted the data, and wrote the first draft of the manuscript. All authors contributed to the intellectual content, manuscript editing and read and approved the final manuscript.

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Informed consent

Questionnaires were filled with the participants' satisfaction and written consent was obtained from the participants in this study.

Conflict of interest

The authors declare that they have no conflict of interests.

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