Original Research Papers

Isolation, incidence and molecular characterization of drug-resistant *Escherichia coli* of goat milk

Running title: Drug-resistant *E. coli* of goat milk

Abstract

Background

Goat is regarded as poor man's cow and its milk is recognized for its high nutritive profile. Foodborne pathogen *Escherichia coli* causes public health problems. The practise of antimicrobials in foodstuff of animals produces a significant source of resistance in bacteria and raises the threat of cure disappointments. The present study was proposed to isolate *E. coli* from raw goat milk samples, detect the antimicrobial resistance profile of *E. coli* isolates and determine the genes responsible for this resistance.

Methods

A total of 250 raw milk samples were obtained from different farms of Taif province, Saudi Arabia. Collected samples were cultured on MacConkey agar. Various biochemical tests were achieved for the identification of isolates. Antimicrobial resistance pattern of *E. coli* was estimated by the disk diffusion method. The resistance genes *tet*(A) and *tet*(B), *ere*(A), *aadA1*, *bla*SHV, *aac(3)-IV*, sul1, catA1 and *cmlA*, were examined.

Results

Results of the present study have showed that out of the 250 samples examined, 100 (40%) were found to be infected with *E. coli*. Antimicrobial resistance profile evaluated showed a higher resistance against ceftriaxone (95.8 %) and ticarcillin (91.7%), followed by amikacin and cefotaxime (87.5%), and

augmentin and penicillin (85%). Lower percentage was observed for gentamicin (58%), ampicillin (66.7%), imipenem (70.8%) and bacitracin (75%), Furthermore, multi-drug resistance was observed in most of the total isolates. Among *E. coli* isolates 89% gave positive amplicons for the *bla*SHV gene followed by *tet*(A) and *tet*(B) genes (85%).

Conclusion

The results suggested a probability of possible public health risk of multi-drug resistance of *E. coli* strains collecting from raw goat milk samples. Consequently, appropriate handling of goat milk is significant in preventing *E. coli* infections.

Keywords: Antimicrobial-resistance, raw goat milk, *E. coli*, resistance genes, 16S rRNA.

1. Introduction

Consistent with EU regulation, "raw milk" is described as milk formed by the discharge of the mammary gland of farmed animals that has not been heated to more than 40°C or experienced any conduct that has an equivalent effect (853/2004). In everyday speech, "raw milk" is frequently agreed to mean milk that has not been pasteurized. Milk and dairy products are essential in the regime of humans, since they are a supply of many important nutrients such as proteins, fats, carbohydrates, vitamins and minerals [1]. Total eating of milk and dairy foodstuffs is great and rising in most parts of the world, exclusively in developing countries [2, 3, 4]. Goat has been referred as the "poor man's cow'' due to his great contribution to the health and nutrition of the landless and rural poor [5].

One of the foodstuffs supported as 'health food' is raw milk. Raw milk is described by European Union legislation as: "milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40°C or undergone any treatment that has an equivalent effect" [6]. The drinking of raw milk among the common population is rather low, while it seems to be high in case of

health-conscious people, who wish to consume natural, unprocessed food and believe that raw unpasteurized milk, which has not been subject to any heating process, is considered by specific healthy properties, a reduced susceptibility to allergies, improved nutritional quality and a better taste [7, 8]. This method results in milk drinking by persons, who may have lowered immunity, such as the very young, very old, immune-compromised or the people with specific dietary needs.

In Saudi Arabia, raw milk may be obtainable through many delivery stations, including direct sale to customers at the farm, sale through vending machines and the internet. The presence of food-borne pathogens in bulk tank milk has been demonstrated in many surveys and food-borne outbreaks associated with *Campylobacter*, *Salmonella* spp., *Listeria monocytogenes* and shigatoxin-producing *Escherichia coli* (STEC) have been traced to the consumption of raw milk [8].

Microbial pollution of milk can happen from three main sources: from within the udder, from the exterior of the udder, and from the surface of milk handling and storage equipment [9]. The development of bacterial resistance to antimicrobial agents poses a serious threat to human health. The antimicrobial-resistant zoonotic bacteria are of particular concern, as they might negatively affect the treatment of infections in humans [10]. Intramammary inflammation is the main cause of antimicrobial usage on dairy farms [11] and herd-level associations between the use of antimicrobial agents and antimicrobial resistance in some mastitis pathogens have been demonstrated [12, 13].

The possible public health threats associated to milk may result from the incidence of pathogens which are resistant to antimicrobials or have genes encoding resistance to such antibiotics. In addition, non-pathogenic bacteria that may move their resistance factors to pathogenic bacteria, which influence the appearance and selection of multi-drug resistant food-borne pathogens. Raw milk may be a source of bacteria that are resistant to antimicrobials, depending on the reservoir of antimicrobial-resistant bacteria in the farm and animal environment [14]. Therefore, this project was proposed to investigate the

incidence of drug-resistant *E. coli* of raw goat milk at Taif province and study the genes responsible for their resistance.

2. Materials and Methods

2.1. Sample Collection

A total 250 raw milk samples were collected from healthy goats from different farms by farms' owners at Taif province. The farms' owners usually sell the milk to the publics. After collection, the samples were transferred directly to the laboratory in an ice box and stored at 4°C until use.

Ethical Considerations

An authorization to carry out the study and collect the samples was obtained from Taif University.

2.2. Isolation and identification of E. coli

Different dilutions of milk samples were inoculated on MacConkey agar plates (Oxoid UK) and incubated at 37°C for 18 to 24 hours. Smooth pink colonies on MacConkey were primitively characterized as *E. coli*. The isolates were characterized as described according to Bergey's Manual of Systematic Bacteriology (Table 2) [15]. The *E. coli* isolates were kept (Merck, Germany) in 15% glycerol of tryptic soy broth at –20 °C.

2.3. Susceptibility assay

Antimicrobial susceptibility assay were achieved by the Kirby-Bauer disk diffusion method as described previously by CLSI [16] on Mueller-Hinton agar plates. The following antimicrobials were used: ampicillin, AM; augmentin, AUG; gentamicin, GM; cefoxitin, FOX; cephalothin, , CF; trimethoprim-sulfamethoxazole, TS; bacitracin, BA; chloramphenicol, C; penicillin G, PG; polymyxin, PB; ceftriaxone, CRO; neomycin, NE; amikacin, AK; cefotaxime, CTX; cefepime, CMP; ticarcillin, TC; piperacillin, PRL and imipenem, IMI. The plates were incubated for 24 h at 37°C, and the diameters of inhibition zones were measured and verified as recommended by the CLSI [16]. These antibiotics were

chosen on the basis of their importance in treating human or animal *E. coli* infections and their use as feed additives to promote growth in animals and on the basis of their ability to provide diversity for representation of different antibiotics classes.

2.4.9 Extraction of DNA

DNA was isolated from *E. coli* isolates by using a Genomic DNA purification kit according to the manufacturer's instructions.

2.5.10 PCR of 16S rRNA gene

In order to confirm the identification of *E. coli* isolates having resistance of the highest numbers of antibiotics, the 16S rRNA analysis was achieved. The primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGYTACCTTGTTACGACTT-3') were employed. 1 µl of template DNA (1 µg) was included in 20 µl- PCR reaction. 35 cycles were achieved at 94 ^{o}C for 45 sec, 55 ^{o}C for 60 sec, and 72 ^{o}C for 60 sec. PCR products were ~ 1,400 bp. Unincorporated PCR primers and dNTPs were removed from PCR products using PCR Clean up kit.

2.5.2 Sequencing of 16S rRNA gene

The PCR-products of 16S rRNA gene (~ 1,400 bp) were sequenced by the following tow primers: 785F (5'-GGA TTA GAT ACC CTG GTA-3') and 907R (5'-CCG TCA ATT CMT TTR AGT TT-3'). Sequencing was accomplished by Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). The products sequencing were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Selected sequences of other microorganisms with highest match to the 16S rRNA sequences of our bacterial isolates were obtainede from the nucleotide sequence databases and aligned using CLUSTAL W (1.81) Multiple Sequence Alignment generating phylogenetic tree. The 16S rRNA gene sequences of

the bacterial isolates which described in the present study were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases.

2.6. PCR detection of antibiotics resistance genes

The resistance genes of tetracycline [*tet*(A), *tet*(B)], erythromycin [*ere*(A)], streptomycin (*aadA1*), β-lactams (*bla*SHV), gentamicin [*aac(3)-IV*], sulfonamides (*sul1*) and chloramphenicol (*catA1*, *cmlA*) and was determined by PCR. The set of primers employed is shown in Table 1. The method of Primer-BLAST web site according to Ye et al. [17] was used to design the primers. PCR reactions were performed as described previously by Abo-Amer et al. [18]. PCR products were analyzed by electrophoresis in 1.5% agarose gel. A molecular weight ladder of 100 bp increments (100 bp DNA ladder) was employed.

3. Results

3.1. Isolation and identification of E. coli

According to morphological and biochemical description of bacterial isolates (Table 2), out of the 250 samples tested of raw goat milk, 100 samples (40%) were found to be infected with *E. coli*.

3.2. Antimicrobial susceptibility

One hundred of *E. coli* isolates from goat milk samples were examined for antimicrobial susceptibility (Table 3). For 100 *E. coli* isolates, 95.8 % were resistant to ceftriaxone and 91.7% resistant to ticarcillin. Moreover, 87.5% were resistant to amikacin and cefotaxime while 85% for augmentin and penicillin. In addition, 83% were resistant to trimethoprim-sulfamethoxazole, neomycin, and cefepime. However, lower resistances were observed for gentamicin (58%), ampicillin (66.7%), imipenem (32.5%), bacitracin (75%), chloramphenicol and cephalothin (77%), cefoxitin and polymyxin (79%) and piperacillin (81%). Generally, 97% were multidrug resistant (MDR) strains resistant to at least three different classes of antimicrobials in the panel of drugs studied.

3.3. Antibiotic resistance genes

The prevalence of resistance genes in phenotypically-resistant *E. coli* isolates recovered from goat milk samples is presented in Table 4. The resistance genes *tet*(A) and *tet*(B) for tetracycline, *ere*(A) for erythromycin, *aadA1* for streptomycin, *bla*SHV for β-lactams, *aac(3)-IV* for gentamicin *catA1*, *sul1* for sulfonamides, and *catA1, cmlA* for chloramphenicol were investigated. Among *E. coli* isolates 89% gave positive amplicons for the *bla*SHV gene followed by *tet*(A) and *tet*(B) genes (85%). Moreover, 75% of *E. coli* isolates carried *catA1* and *cmlA* genes. However, *E. coli* carried *aac(3)-IV* gene (25%), *ere*(A) gene (20%), *aadA1* gene (15%), and *sul1* gene (13%).

Table 1. Resistance genes and their primers employed in this study.

Antimicrobials	Resistance gene	Sequence, 5-3	Product size (bp)	Melting temperature (°C)	Annealing temperature (°C)	References
Tetracycline	*tet*A	F- CCTCAATTTCCTGACGGGCT	712	60.04	55	Abo-Amer
		R-GGCAGAGCAGGGAAAGGAAT		60.03		et al., 2018
	*tet*B	F- GAAAGACGGTGAGCTGGTGA	586	59.97	55	Abo-Amer
		R- TAGCACCAGGCGTTTAAGGG		60.04		et al., 2018
Erythromycin	*ere*A	F- CGATTCAGGCATCCCGGTTA	897	59.89	55	Abo-Amer
		R- CCATGGGGGCATCTGTCAAT		60.11		et al., 2018
Streptomycin	*aad*A1	F- TCGCCTTTCACGTAGTGGAC	816	60.04	55	Abo-Amer
		R-CAACGATGTTACGCAGCAGG		59.90		et al., 2018
β-lactams	*bla*SHV-199	F- CTATCGCCAGCAGGATCTGG	543	60.04	55	Abo-Amer
		R- ATTTGCTGATTTCGCTCGGC		59.90		et al., 2018
Gentamicin	*aac(3)-IVa*	F- ATGTCATCAGCGGTGGAGTG	454	60.11	55	Abo-Amer
		R- GGAGAAGTACCTGCCCATCG		59.89		et al., 2018
Sulfonamides	*sul1*	F- ACTGCAGGCTGGTGGTTATG	271	60.32	55	Abo-Amer
		R- ACCGAGACCAATAGCGGAAG		59.54		et al., 2018
Chloramphenicol	*cat*A1	F- GTGACATTTACGCAGGTCGC	473	59.97	55	Abo-Amer
		R- TGCGAAGCCCATATTTCGGT		60.04		et al., 2018
	*cml*A5	F- GTGACATTTACGCAGGTCGC	532	59.91	55	Abo-Amer
		R- TGCGAAGCCCATATTTCGGT		60.11		et al., 2018

Table 2: Characteristic tests of *E. coli* isolates.

Characteristic tests	*E. coli* isolates	Percentage
Gram Staining	G-v, short bacilli	100
Oxidase Test	-	95
Catalase Test	+	97
Methyl Red Test	+	99
Indole Test	+	97
Citrate Test	-	98
Voges-Proskauer Test	-	98
H_2S production	+	97
Motility	+	98
Nitrate Reduction Test	+	96
Urea Hydrolysis test	+	99
Lipase	+	99
DNase Production	-	98
Acid and gas from:		
Maltose	+	97
Lactose	+	100
Glucose	+	98
Sucrose	+	97
Arabinose	+	98

Table 3: Incidence of antimicrobial resistance of *E. coli* isolates.

Antimicrobials/code	Percentage
Ampicillin, AM	66.7
Augmentin, AUG	85
Gentamicin, GM	58
Cefoxitin, FOX	79
Cephalothin, CF	77
Trimethoprim-sulfamethoxazole, TS	83
Bacitracin, BA	75
Chloramphenicol, C	77
Penicillin G, PG	85
Polymyxin, PB	79
Ceftriaxone, CRO	95.8
Neomycin, NE	83
Amikacin, AK	87.5
Cefotaxime, CTX	87.5
Cefepime, CMP	83
Ticarcillin, TC	91.7
Piperacillin, PRL	81
Imipenem, IMI	32.5

Table 4: Incidence of resistance genes of *E. coli* isolates.

Antibiotic class/agent	Resistance gene	Percentage
Tetracycline	*tet*(A), *tet*(B)	85%
Erythromycin	*ere*(A)	20%
Streptomycin	*aadA1*	15 %
β-lactams	*bla*SHV	89%
Gentamicin	*aac(3)-IV*	25%
Sulfonamides	*sul1*	13%
Chloramphenicol	*catA1, cmlA*	75%

3.4. Phylogenetic tree of E. coli Isolates

For additional categorization of the *E. coli* isolates having resistance of the highest numbers of antibiotics, 16S rRNA encoding genes of the isolates GM1, GM2, Gm3, GM4, GM5, GM6, GM7, GM8, GM9 and GM10 were PCR-amplified and sequenced. The 16S rRNA gene sequences of the bacterial isolates were deposited in the DDBJ/EMBL/GenBank nucleotide sequence data bases with the accession numbers: LC431219 (*E. coli* GM1), LC431220 (*E. coli* GM2), LC431221 (*E. coli* GM3), LC431222 (*E. coli* GM4), LC431223 (*E. coli* GM5), LC431224 (*E. coli* GM6), LC431225 (*E. coli* GM7), LC431226 (*E. coli* GM8), LC431227 (*E. coli* GM9) and LC431228 (*E. coli* GM10).

The nucleotide sequences of *E. coli* isolates were compared to current sequences in the databases. A dendrogram demonstrating the results of 16S rRNA analysis is exhibited in Figure 1. Results showed

highest matching of isolates GM1, GM22, GM3, GM4, GM5, GM6, GM7, GM8, GM9 and GM10 to members of the *Escherichia* group. As verified, the 16S rRNA sequences of the *Escherichia* isolates are highest strictly related to *Escherichia coli.* These results are similar with the decisions of the morphological and biochemical classification. The 16S rRNA gene of isolates GM1, GM22, GM3, GM4, GM5, GM6, GM7, GM8, GM9 and GM10 shares 99% identity with that of *Escherichia coli* strain M-N1.

4. Discussion

Milk is measured to be a good medium of growing for several microorganisms [19]. *E. coli* is a normal inhabitant of the intestines of animals and humans. Nevertheless, its retrieval from food may be of public health concern because of the potential incidence of enter-pathogenic and/or toxigenic strains like *E. coli* O157:H7 which can lead to dangerous gastrointestinal disorders [20] and other life threatening diseases on the consumer [21]. The present study showed 100 samples (40%) of raw goat milk were found to be infected with *E. coli* out of the 250 samples examined. Recent results reported that out of 200 samples tested, 40 (20%) and 7 (3.5%) of the samples were positive to *E. coli* and *E. coli* O157: H7 respectively [22].

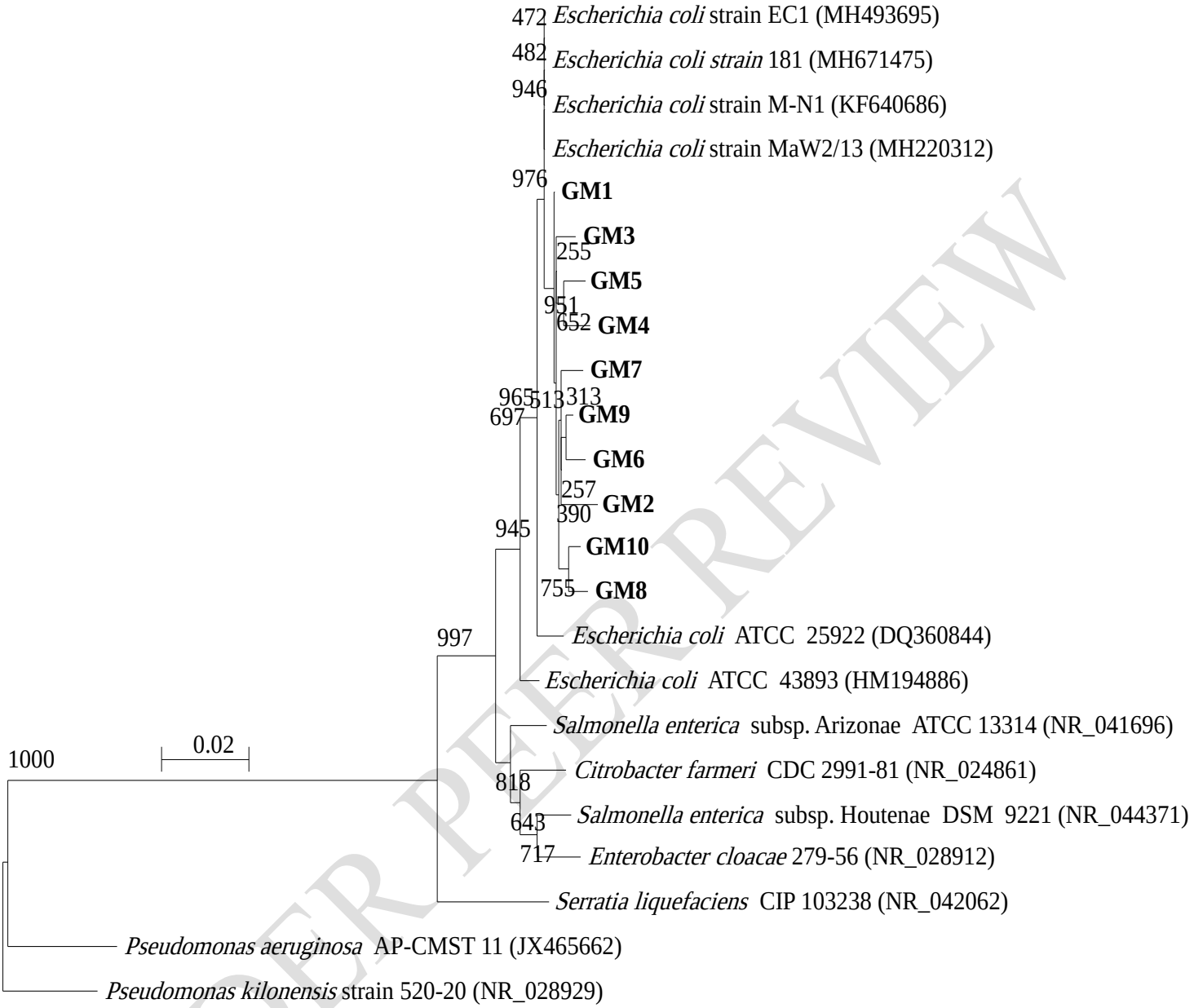

Figure 1. A phylogenetic tree of antibiotic-resistant isolates from raw goat milk based on the nucleotide sequences of 16S rRNA genes was constructed by neighbor-joining method. The scale bar shows the genetic distance. The number presented next to each node shows the percentage bootstrap value of 1000 replicates. The *Pseudomonas kilonensis* was treated as the out-group. The GenBank accession numbers of the bacteria are presented in parentheses.

Furthermore, previous results stated that 44%% of raw milk samples were found to harbor *E. coli* [23]. The present study showed that 95.8 % and 91.7% of isolates were resistant to ceftriaxone and ticarcillin, respectively. Furthermore, 87.5% and 85% were resistant to amikacin & cefotaxime and augmentin &d penicillin. Moreover, 83% were resistant to trimethoprim-sulfamethoxazole, neomycin, and cefepime. Nevertheless, lower resistances were detected for gentamicin (58%), ampicillin (66.7%), imipenem (70.8%), bacitracin (75%), chloramphenicol and cephalothin (77%), cefoxitin and polymyxin (79%) and piperacillin (81%). The enlargement of antimicrobial resistance among the pathogenic bacteria causes a problem of high concern. *E. coli* isolates have shown higher resistance rates to amoxicillin, gentamicin and tetracycline which are in agreement with findings of Zuleka et al. [24], Briscoe *et al*. [25] and Thaker *et al.* [26] who have reported different antimicrobial resistance patterns against isolated challenged pathogens from milk and other human food sources.

Generally, 97% were multidrug resistant (MDR) strains resistant to at least three different classes of antimicrobials in the panel of drugs studied. Isolates showed a multidrug resistance to amoxicillin, gentamicin, tetracycline, erythromycin and chloramphenicol. Similar findings were also reported by Orrett and Shurl [27] and Kurutepe *et al*. [28] and Zuleka et al. [24]. In addition, this is in agreement with the report of Mude et al. [29], who showed 92.3% of isolates were multidrug resistant. Moreover, various authors [30, 31] reported multidrug resistance patterns.

The multidrug resistance detected in this study might be mediated by genetic mobile elements such as resistance genes. Commonly, in the present study, 89% of *E. coli*

isolates gave positive amplicons for the *bla*SHV gene followed by *tet*(A) and *tet*(B) genes (85%) and *catA1* and *cmlA* genes (75%). However, *E. coli* carried *aac(3)-IV* gene (25%), *ere*(A) gene (20%), *aadA1* gene (15%), and *sul1* gene (13%). There was a high percentage of *E. coli* harbouring *bla*SHV (89%). previous study reported that the most prevalent β-lactamase genes of *E. coli* isolated from environmental, human and food samples in Spain were *blaCTXM-14* (26%) and *blaCTXM-1* (21.4%), followed by *blaSHV-12*, *blaCTX-M-15* and *blaTEM-42* [32]. The present study reported that the *aadA1* and *aac(3)-IV* genes were prevalent in 25% *E. coli*. Aminoglycoside nucleotidyl-transferases can give resistance to gentamicin, tobramycin or streptomycin including *aad* among Gram-negative bacteria [33]. The *sul1* gene was observed for 13% of *E. coli* in the present study. The incidence dissemination of the *sul* genes in the three environments investigated, swine farms, shrimp ponds, and a city canal generally followed sul1 > sul2 > sul3 [34]. The *tet*(A) and *tet*(B) genes were noticed in 85% *E. coli* isolates in our study. Recent results stated that the *Tet (A)* resistance gene was prevalent in 86% *E. coli* [35].

Conclusion and Recommendation

It can be concluded that the microbial quality and safety of the raw milk produced from goats for the local community was commonly dangerous. That is, goat milk is not only of potential public health threat of *E. coli* strains, but also a source of a multidrug antimicrobial resistance to the public of the Taif area. The incidence of *E. coli* in raw goat milk may result from infected animals or polluted conditions during processing, handling and distribution. Suitable hygienic practise should be followed during milking and handling of goat's raw milk before drinking.

Competing interests

None declared.

Ethical approval

Not required

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