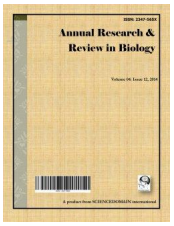

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Isolation, Incidence and Molecular Characterization of Drug-resistant *Escherichia coli* of Goat Milk

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Authors' contributions

This work was carried out in collaboration between both authors. Author AEAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Both authors managed the analyses of the study. Author JAA managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Goat milk is recognized for its high nutritive profile. The practise of antimicrobials in feeding of animals produces resistance in bacteria. Therefore, the present study was proposed to study the incidence of drug-resistant *E. coli* from raw goat milk samples and investigate the genes responsible for the 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. Morphological and 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*, *blaSHV*, *aac(3)-IV*, *sul1*, *catA1* and *cmlA*, were examined by PCR.
Results: Results of the present study showed that out of the 250 samples examined, 100 (40%)

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were found to be infected with *E. coli*. Antimicrobial resistance profile evaluated showed a higher resistance against ceftriaxone (90 %) and ticarcillin (86%), followed by amikacin and cefotaxime (87%), and augmentin and penicillin (85%). Lower percentage was observed for gentamicin (58%), ampicillin (66%), bacitracin (75%) and imipenem (32%). Furthermore, multi-drug resistance was observed in most of the isolates. Among *E. coli* isolates, 86% gave positive amplicons for the *blaSHV* 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 processing is significant to prevent *E. coli* infection.

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

1. INTRODUCTION

Milk and dairy products are essential for humans, since they are a supply of many important nutrients such as proteins, fats, carbohydrates, vitamins and minerals [1]. Drinking of milk and eating of dairy foodstuffs are increasing 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 poor rural [5].

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]. Consequently, raw milk drinking was preferred by persons, who may have lowered immunity, such as the very young, very old, immune-compromised or the people with specific dietary needs.

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

Microbial pollution of milk can be happened from inside and outside the udder, 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 might negatively affect the treatment of infections in humans [10]. Intramammary inflammation is the main cause of antimicrobial practice in dairy farms [11]. Herd-level associations between the use of antimicrobial agents and antimicrobial resistance in some mastitis pathogens have been demonstrated [12,13].

The possibility of public health threats associated to raw milk may be increased from the incidence of pathogens which are resistant to antimicrobials and having genes encoding the resistance. In addition, non-pathogenic bacteria that may move their resistance factors to pathogenic bacteria influence the selection and emergence 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 possible genes for triggering the resistance.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 250 raw milk samples were collected from goats from different farms by farms' owners at Taif province. The farms' owners usually sell directly 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.

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	*tetA*	F- CCTCAATTTCCTGACGGGCT	712	60.04	55	[18]
		R-GGCAGAGCAGGGAAAGGAAT		60.03		
	tetB	F- GAAAGACGGTGAGCTGGTGA	586	59.97	55	[18]
		R- TAGCACCAGGCGTTTAAGGG		60.04		
Erythromycin	*ereA*	F- CGATTCAGGCATCCCGGTTA	897	59.89	55	[18]
		R- CCATGGGGGCATCTGTCAAT		60.11		
Streptomycin	*aadA1*	F- TCGCCTTTCACGTAGTGGAC	816	60.04	55	[18]
		R-CAACGATGTTACGCAGCAGG		59.90		
β-lactams	*blaSHV-199*	F- CTATCGCCAGCAGGATCTGG	543	60.04	55	[18]
		R- ATTTGCTGATTTCGCTCGGC		59.90		
Gentamicin	*aac(3)-IVa*	F- ATGTCATCAGCGGTGGAGTG	454	60.11	55	[18]
		R- GGAGAAGTACCTGCCCATCG		59.89		
Sulfonamides	*sul1*	F- ACTGCAGGCTGGTGGTTATG	271	60.32	55	[18]
		R- ACCGAGACCAATAGCGGAAG		59.54		
Chloramphenicol	*catA1*	F- GTGACATTTACGCAGGTCGC	473	59.97	55	[18]
		R- TGCGAAGCCCATATTTCGGT		60.04		
	cmlA5	F- GTGACATTTACGCAGGTCGC	532	59.91	55	[18]
		R- TGCGAAGCCCATATTTCGGT		60.11		

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 in 15% glycerol contained tryptic soy broth at –80°C.

2.3 Susceptibility Assay

Antimicrobial susceptibility assay was achieved by the Kirby-Bauer method as described previously by CLSI [16]. Antimicrobials such as 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 were used in this study. 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. The assay results were verified as recommended by the CLSI [16].

2.4 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 PCR Detection of Antibiotics Resistance Genes

The resistance genes of tetracycline [*tet(A)*, *tet(B)*], erythromycin [*ere(A)*], streptomycin (*aadA1*), β-lactams (*blaSHV*), 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 primers were designed by the method of Primer-BLAST web site according to Ye et al. [17]. 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.

2.6.1 PCR of 16S rRNA gene

To confirm the morphological and biochemical identification of some *E. coli* isolates with high resistance 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 [18]. 35 cycles were achieved at 94 °C for 45 sec, 55 °C for 60 sec, and 72 °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.

2.6.2 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 obtained 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.

3. RESULTS

3.1 Isolation and Identification *of E. coli*

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

3.2 Antimicrobial Susceptibility

One hundred *E. coli* isolates from goat milk samples were screened for antimicrobial susceptibility (Table 3). 90% of *E. coli* isolates were resistant to ceftriaxone and 86% resistant to ticarcillin. Moreover, 87% of isolates were resistant to amikacin and cefotaxime, and 85% for augmentin and penicillin. In addition, 83%

were resistant to trimethoprim-sulfamethoxazole, neomycin, and cefepime. However, lower resistances were observed for imipenem (32%), gentamicin (58%), ampicillin (66%), 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, *blaSHV* for β-lactams, *aac(3)-IV* for gentamicin *catA1, sul1* for sulfonamides, and *catA1, cmlA* for chloramphenicol were investigated. Among *E. coli* isolates, 86% gave positive amplicons for the *blaSHV* 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 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
Augmentin, AUG	85
Gentamicin, GM	58
Cefoxitin, FOX	79
Cephalothin, CF	77
Trimethoprim-sulfamethoxazole, TS	83
Bacitracin, BA	75
Chloramphenicol, C	77
Penicillin G	85
Polymyxin, PB	79
Ceftriaxone, CRO	90
Neomycin, NE	83
Amikacin, AK	87
Cefotaxime, CTX	87
Cefepime, CMP	83
Ticarcillin, TC	86
Piperacillin, PRL	81
Imipenem, IMI	32

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	*blaSHV*	86%
Gentamicin	*aac(3)-IV*	25%
Sulfonamides	*sul1*	13%
Chloramphenicol	*catA1, cmlA*	75%

3.4 Phylogenetic Tree of *E. coli* Isolates

For additional categorization of some *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 Fig. 1. Results showed highest matching of isolates GM1, GM2, 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, GM2, GM3, GM4, GM5, GM6, GM7, GM8, GM9 and GM10 shares 99% identity with that of *Escherichia coli* strain M-N1.

4. DISCUSSION

Milk is considered 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 recovery 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].

Furthermore, previous results stated that 44%% of raw milk samples were found to harbour *E. coli* [23]. The present study showed that 90% and 86% of isolates were resistant to ceftriaxone

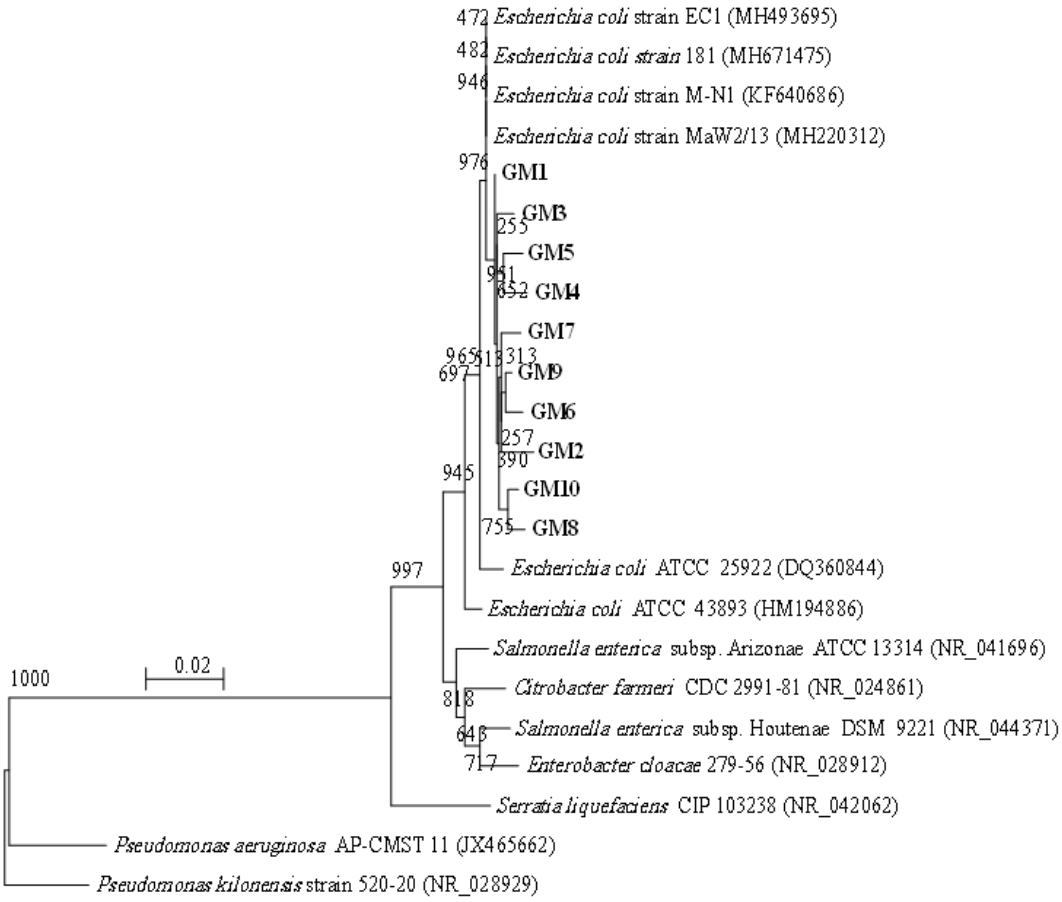

Fig. 1. A phylogenetic tree of drug-resistant *E. coli* isolates from raw goat milk based on the nucleotide sequences of 16S rRNA genes was constructed by neighbor-joining method. The scale bar shows the genetic distance. The number presented next to each node shows the percentage bootstrap value of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

and ticarcillin, respectively. Furthermore, 87% and 85% were resistant to amikacin & cefotaxime and augmentin & penicillin, respectively. Moreover,83% were resistant to trimethoprim-sulfamethoxazole, neomycin, and cefepime. Nevertheless, lower resistances were detected for imipenem (32%), gentamicin (58%), ampicillin (66%), bacitracin (75%), chloramphenicol and cephalothin (77%), cefoxitin and polymyxin (79%) and piperacillin (81%). The increase 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 pathogens from milk and other human food sources.

Generally, 97% were multi-drug resistant (MDR) strains to at least three different classes of antimicrobials in the panel of drugs studied. Bacterial isolates showed a multi-drug 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% of isolates were multi-drug resistant. Moreover, various authors [30, 31] reported multidrug resistance patterns.

The multi-drug 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 (86%) 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%). The results indicated that there was a high percentage of *E. coli* harbouring *blaSHV*. 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%), 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% of *E. coli* isolates. 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* isolates 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% of *E. coli* [35].

5. CONCLUSION AND RECOMMENDATION

It can be concluded that the microbial quality and safety of the raw milk samples collected from goats for the local community was commonly slightly dangerous. That is, goat milk is not only of potential public health threat of *E. coli* strains, but also a source of a multi-drug antimicrobial resistance to the publics. 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 use.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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