

Zoonotic Importance of Some Pathogenic Bacteria Isolated from Small Ruminants' Milk and Hands of Dairy Workers

Heba S. El-Mahallawy^{1*}, Mahmoud Elhariri², Rehab Elhelw² and Dalia Hamza³

¹Animal Hygiene, Zoonoses and Animal Behaviour and Management Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, 41522, Egypt

²Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt

³Zoonoses Department, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt

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Abstract

Staphylococcus aureus and enteropathogenic *Escherichia coli* are among zoonotic bacterial food-borne pathogens causing illness ranged from diarrhoea to fatal conditions. This study was undertaken to evaluate the occurrence of *Staph. aureus* and *E. coli* in small ruminants' raw milk and hands of dairy workers at small-scale production units, Giza, Egypt. A total of 420 raw milk samples were obtained from apparently healthy sheep and goats, also, hand swabs (n=46) from workers at the units under investigation were examined. Overall, *Staph. aureus* (13.1%, 55/420) and *E. coli* (26.2%, 110/420) isolates were obtained from raw milk of sheep and goats. However, only *Staph. aureus* (15.2%, 7/46) was recovered from hand swabs of dairy workers. Using PCR, all the tested *Staph. aureus* isolates from milk and hand swabs yielded specific bands of *Thermonuclease (nuc)* gene at 279 bp, however, amplification of virulence (*eae*) gene encoding the intimin protein of *E. coli* produced an amplicon of 917 bp in 45% of the tested *E. coli* isolates. In conclusion, our findings provide an overview about *Staph. aureus* and *E. coli* contamination in raw milk of small ruminants locally bred by smallholders and suggest probably transmission of *Staph. aureus* from hands of dairy workers that contaminate the milk or udder of apparently healthy animals. Reporting of such organisms from milk indicates the need for strict hygienic measures during milking at these production units.

Keywords: Raw milk, Dairy workers, Sheep, Goat, *E. coli*, *Staph. aureus*.

Introduction

Milk is a highly nutritious food for human beings from childhood to senility. It also serves as a good medium for the growth of many microorganisms especially bacterial pathogens particularly in tropical areas. *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Escherichia coli* and *Micrococcus* spp. are among the commonly detected bacteria in fresh milk [1].

Sheep and goats are considered the second most important milk producers after dairy cattle and buffaloes, in both temperate and tropical agriculture [2]. In Mediterranean countries, milk from small ruminants has always been a part of people's traditional food [3]. The need for milk from sheep is increasingly rising, moreover, dairy goat industry currently gaining more attention worldwide [4].

There have been few attempts to determine the microbial loads in small ruminants' raw milk in Egypt, since most of dairy industry mainly rely on milk from cattle and. Although the burden of various etiological agents in milk-borne diseases has dramatically changed over time, more than 90% of all reported cases of dairy-related illnesses continue to be of bacterial origin [5].

Presence of microorganisms in milk and milk products greatly affect its quality and safety, furthermore, it has a considerable public health implications. Staphylococci are frequently recovered from raw milk of animals. *Staphylococcus aureus* has been reported as an important etiological agent of subclinical and clinical mastitis [6]. Moreover, this organism usually present among the normal flora in the anterior nares and skin of humans and animals [7], and interchange of

*Corresponding author email: dr_ba1012_2@hotmail.com), Animal Hygiene, Zoonoses and Animal Behaviour and Management Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, 41522, Egypt.

Staph. aureus between humans and animals due to close contact has been previously reported [8-10], posing a zoonotic risk and notable threats regarding food hygiene. The presence of high numbers of these bacteria in milk increases the risk of staphylococcal toxin production which is highly resistant to heat treatment by pasteurization [11].

E. coli is recognized as a serious food-borne pathogen and has been associated with numerous disease outbreaks worldwide [12]. Raw milk and dairy products such as pasteurized milk and yoghurt contaminated with *E. coli* have been reported as the main cause of several food borne disease outbreaks since the 1980s and thus constitutes a serious health threat [13,14].

In Egypt, large number of sheep and goats are bred in small groups (usually 20-30 animals/herd) by smallholders in peri-urban areas where they share in pasture and shelter. On contrary to machine milking in large ruminants' dairy farms, small ruminants in local small-scale units are managed and milked by hands of their smallholders, thus increasing the probable risk of milk contamination or disease transmission. This study aimed to determine the frequency of two of the most important zoonotic bacterial pathogens, *Staph aureus* and *E. coli*, in small ruminants' raw milk and hands of their dairymen at small-scale production units in Giza, Egypt to evaluate the level of sanitary measures fulfilled by dairy workers during milking process.

Materials and Methods

Sample collection

Milk samples

A total of 420 raw milk samples (220 sheep and 200 goats) were collected from small locally bred herds in Giza, Egypt. Teats of the udder were disinfected with 70% ethyl alcohol and left to dry. First milk strips were discarded and 10 mL of milk from each animal was collected by its owner in sterile labelled capped cups then placed directly in an ice box.

Hand swabs from dairy workers

Pre-moistened saline swabs were rolled on the palm of hands and fingers of forty-six dairy workers prior to milking in the visited

units. Swabs were aseptically placed back in their labelled tubes. Following collection, all milk samples and swabs were shipped on ice within 2 h of collection to the Microbiology Department's Laboratory, Faculty of Veterinary Medicine, Cairo University. Informed verbal/written consent for participation was obtained from all the participants.

Isolation and identification of Staph. aureus

One millilitre from each well mixed sample was aseptically enriched into Brain heart infusion broth tubes (Oxoid, Hampshire, UK) and incubated at 37°C for 24 h. Two loopfuls from each enrichment broth was streaked onto mannitol salt agar plates (Oxoid, Hampshire, UK) and incubated at 37°C for 24 h. Positive *Staph. aureus* colonies were identified by mannitol fermentation with yellow colour production. Each suspected isolate was tested for its haemolytic activity by streaking on 5% defibrinated sheep blood agar (Oxoid Ltd, Hampshire, UK) incubated at 37°C for 24 h. Typical colonies were further identified by Gram-stained smears and examined for their catalase activity as described previously [15]. Production of coagulase by *Staph. aureus* strains was also determined using rabbit plasma. Briefly, 0.2 mL of overnight grown tested broth was added to tubes containing 1 mL of 1:10 diluted rabbit plasma. Stable gelling of plasma was observed during incubation at 37°C with half hour intervals for 4 hours.

Isolation and identification of Enterobacteriaceae

One millilitre from each well mixed sample was inoculated into buffered peptone water at 37°C for 24 h. Two loopfuls from each enrichment broth were streaked on each MacConkey and Eosin methylene blue (EMB) (Oxoid, Ltd, Hampshire, UK) agar plates and incubated at 37°C for 24 h. Morphologically typical *E. coli* colonies with green metallic sheen on EMB were transferred into nutrient agar plates and broth for further morphological identification and Gram staining as described previously [16,17] and biochemical identification using RapID ONE test (Oxoid, remel, USA) was carried out.

Table 1: Primer sequences of *nuc* gene specific for *Staph. aureus* and *eae* gene specific for EPEC *E. coli*

Target gene	Oligonucleotide sequence	Size of amplified product
<i>nuc</i> ¹	5'-GCGATTGATGGTGATACGGTT-3' 5'-AGCCAAGCCTTGACGAACTAAAGC-3'	279 bp
<i>eae</i> ²	5-CTGAACGGCGATTACGCGAA-3 5-CCAGACGATACGATCCAG -3	917 bp

¹The *nuc* primer pair were used for identification of *Staph. aureus*,

²primer pair specific for *eae* gene encoding intimin for identification of EPEC *E. coli*.

Polymerase chain reaction

Genomic DNA was extracted from crude cell lysate of bacterial isolates using rapid boiling procedures [18]. Briefly, two to five bacterial colonies were randomly picked up from fresh grown positive cultures and were then suspended in 200 µL DNA-RNA free water. After boiling for 10 min in water bath, the supernatant was separated following centrifugation at 10.000 rpm for 5 min. The extracted DNA template was kept at -20°C until being used in PCR assay.

Amplification of specific *Staph. aureus nuc* gene [19] and specific *eae E. coli* gene encoding intimin sequence [20] was performed as previously described. All oligonucleotide primers were purchased from Sigma Genosys (Sigma) and their sequences are listed in Table 1.

The reaction mixture consisted of 12.5 µL 1× of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science), 3 µL bacterial DNA template, 0.5 µL of each primer with concentration of 50 pmol and nuclease free water up to 25 µL. The reaction was run in PCR thermal cycler (Swift MiniPro, ESCO. Philadelphia, USA). Positive control was kindly provided from Zoonoses Department, Faculty of Veterinary Medicine, Cairo University, Egypt was involved in each PCR run. DNase RNase free water was used as negative control. The cycling conditions for *Staph. aureus nuc* gene involved initial

denaturation at 98°C for 5 min followed by 37 PCR cycles under the following conditions: denaturation at 94°C for 1 min, primer annealing at 53°C for 0.5 min, and DNA extension at 72°C for 1.5 min and the reaction was terminated by a final extension at 72°C for 3.5 min. For *E. coli* specific *eae* gene, the cycling conditions involved 95°C for 5 min followed by 40 cycles of 95°C for 45 sec, 50°C for 1 min, 72°C for 1 min and final extension at 72°C for 7 min. Amplified products (10 µL) were then visualized under UV light and photographed after being electrophoresed on 1.5% agarose gel stained with ethidium bromide and 0.5X TBE buffer for 45 min.

Results

In this study, the frequency of bacterial isolation from raw milk and hand swabs of dairy workers is shown in Table 2. Colony characteristics, gram stain and biochemical identification, conventionally confirmed that the isolates were classified as *Staph. aureus* or as *E. coli*. The frequency of *Staph. aureus* isolated from raw milk and dairy workers' hand swabs was 13.1% (55/420) and 15.2% (7/46), respectively. However, no *E. coli* isolates were obtained from dairy workers' hand swabs (Table 2). Notably, sheep milk (14.1% and 29%) showed higher rate of contamination with *Staph. aureus* and *E. coli* than goats' milk (12% and 23%), respectively.

Table 2: Total *Staph. aureus* and *E. coli* isolates obtained from raw milk of sheep and goats and hand swabs of dairy workers.

Source of sample	Total No. examined	<i>Staph. aureus</i>		<i>E. coli</i>	
		+ve	%	+ve	%
Sheep milk	220	31	14.1	64	29
Goat milk	200	24	12	46	23
Dairymen hand swabs	46	7	15.2	--	--

Molecular identification of randomly selected *Staph. aureus* isolates obtained from 10 sheep milk, 10 goats' milk and 7 human hand swabs, revealed the amplification of specific band of Thermonuclease *nuc* gene at 279 bp (Figure 1). However, specific *eae* gene encoding intimin of *E. coli* was detected in 40% (4 out of 10) and 50% (5 out of 10) of randomly selected *E. coli* isolates from raw milk of sheep and goats, respectively (Figure 2).

Discussion

This study provides an overview about the frequency of two zoonotic bacterial pathogens that may be present in sheep and goat's raw milk and the probable risk of their transmission from their dairy workers at small-

scale bred herds in Giza, Egypt. In this study, overall, *Staph. aureus* (13.1%, 55/420) and *E. coli* (26.2%, 110/420) were isolated from raw milk of small ruminants. This was lower than previous reports of *Staph. aureus* of milk from several ruminant species 56% [21], 40% [22], and 40% [23]; however, our findings were higher than 7.3% [24] and 6.6% [25] reported in other studies. Although these variable prevalence rates mentioned in the literature vary in different geographical regions and with variable sample sizes; contamination and spoilage of sheep and goats' milk with these pathogens can generally be encountered due to poor hygienic conditions and management maintained at these small local backyards during milking or due to improper handling, inadequate storage and transport [26].

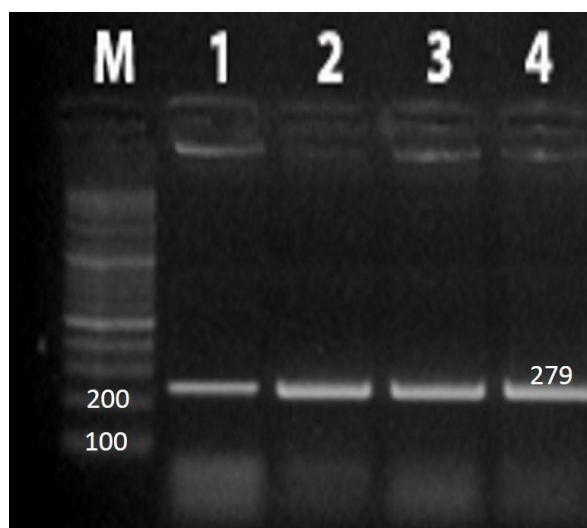


Figure 1: Typical amplification of the specific 279 bp fragment of *nuc* gene from some *Staph. aureus* isolates. Lane Marker: molecular DNA ladder, Lanes 1,2: *Staph. aureus* isolates from sheep milk, Lane 3: *Staph. aureus* isolate from goat milk, Lane 4: *Staph. aureus* isolate from dairy workers' hand swabs.

Raw milk might get contaminated by *Staph. aureus* from infected mammary glands of the animals [27]. Also, *Staph. aureus* is a well-known flora of the anterior nares and skin of human beings [28]. Detection of a high rate (15.2%, 7/46) of *Staph. aureus* isolates from dairy workers' hand swabs in our study, might suggest the probable risk of *Staph. aureus* direct transmission through their contaminated hands to the animals' mammary glands or the milk itself [29]. Referring to our observations in the visited farms and during sample collection, dairy workers were applying hand milking and unhygienic practices in the milking process. In other studies, from Egypt, lower isolation rate of *Staph. aureus* from dairy workers' hands was reported in Sharkia Governorate (10%) [30]; however, *Staph.*

aureus was isolated at higher rates from dairy workers in Ismailia (60%) [31] and Aswan (44.1%) [32].

On the other hand, although the sampled animals were clinically normal at the time of sample collection, we believe that subclinical mastitis may also share with a considerable percentage in the *Staph. aureus* positivity of milk. In this regard, reports stated that *Staph. aureus* represented 37% of the isolates causing subclinical mastitis in goats [33]. Previously reported outbreak of foodborne illness associated with consumption of raw goats' milk has been attributed to its increased favourable nutrient contents, which in turn encourages the growth and proliferation of microorganisms [9].

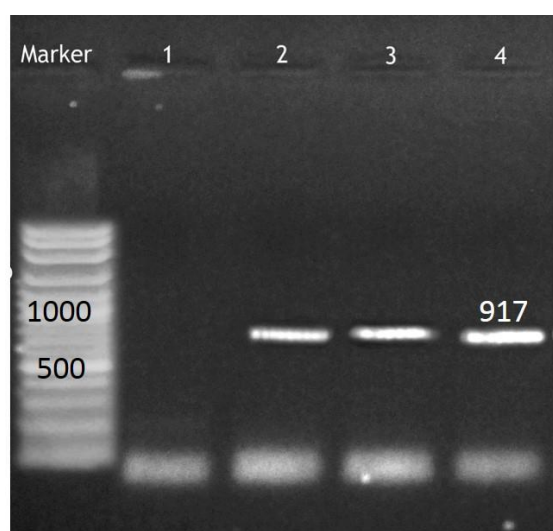


Figure 2: Typical amplification of the specific 917 bp fragment of *eae* gene from some *E. coli* isolates. Lane Marker: molecular DNA ladder, Lane 1: negative sample, Lanes 2,3: *E. coli* isolates from sheep milk, Lane 4: *E. coli* isolate from goat milk.

The microbial load of the examined milk samples revealed higher frequency of *E. coli* (26.2%, 110/420) than (13.1%, 55/420) *Staph. aureus* (Table 2). Ruminants are usually identified as one of the important reservoirs for EPEC/STEC (also called verotoxin-producing *E. coli*). In Egypt, cattle, goats and sheep are considered a potential source of *E. coli* infection to humans, with the important serotypes are O26, O86, O111, O126, O127, O157, O158 and O164 [34]. They shed the

bacteria into the environment throughout their faeces without even suffering from clinical disease, thus allowing meat and milk contamination causing human foodborne illness following consumption of such food [35]. In this regard, Food Regulations Act stated that coliform count shouldn't exceed 1.7 log CFU/mL and *E. coli* shouldn't be present in even one mL of a milk sample [29]. Therefore, high detection rate of *E. coli* from milk in this study is an evidence for faecal

contamination. Furthermore, other contributing factors such as poor hygiene and sanitary practice at the farm could also play a significant role. Since dairy farms have complex surroundings, coliforms present in the faeces, manure and soil, might be easily dispersed throughout the farm [29,36]. In other studies, *E. coli* was isolated from milk at higher rate (76.4%) from Egypt [37] and 33.9% from Western Ethiopia [38]. Notably, sheep milk showed high bacterial contamination with both pathogens than goats' milk. This may be due to the nature of each animal species coat, where heavy wool of sheep may help in carrying more contaminants from the environment that contaminate the milk during milking.

In this study, PCR was used for molecular characterization of the bacteriologically suspected *Staph. aureus* and *E. coli* isolates. Amplification revealed specific 279 bp fragment of *Staph. aureus* thermonuclease *nuc* gene from all the tested 27 isolates (10 from sheep, 10 from goats and 7 from hand swabs). Similar results to our findings were previously reported [23,39,40].

Specific 917 bp fragment of *E. coli eae* intimin gene have been detected in 9 of the 20 testes *E. coli* isolates obtained from milk samples (10 from sheep and 10 from goats). The detection of intimin gene in the tested *E. coli* isolates is of special concern regarding food safety and human health since its detection is linked to the presence of enteropathogenic *Escherichia coli* (EPEC) [41], the strains which have been identified as one of the major foodborne pathogens in humans. However, lower percentages of *E. coli* isolates carrying intimin gene by PCR were previously reported (65.9%) [42] and (21%) [43].

Conclusion

This study provides an evidence of *Staph. aureus* and *E. coli* as hazardous in raw milk from apparently healthy sheep and goat and the probable risk of *Staph. aureus* trans-infection from their milk handlers in Giza, Egypt. Improvement of hygienic and management practices at small backyards will reduce milk contamination with these pathogens and consequently their transmission to humans. More effort is needed to increase

the awareness of smallholders about the importance of obtaining a safe good quality milk at their production and sale units. Ultimately, milk testing programs should be a component in small-scale production units to production of high quality milk.

Conflict of interest

Authors declare that they don't have any conflict of interest.

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الملخص العربي

الأهمية المشتركة لبعض أنواع البكتيريا المعزولة من ألبان المجترات الصغيرة وأيدي عمال الحلابة

هبة سيد المحلاوي^{١*}، محمود الحريري^٢، رحاب الحلواني^٣ وداليا حمزة^٣

^١ قسم الصحة والأمراض المشتركة وسلوكيات الحيوان ورعايته، كلية الطب البيطري، جامعة قناة السويس

^٢ قسم الميكروبيولوجي، كلية الطب البيطري، جامعة القاهرة

^٣ قسم الأمراض المشتركة، كلية الطب البيطري، جامعة القاهرة

تعد المكورات العنقودية والإيشيريشيا كولاي ذات الأمراض المعوي من ميكروبات التسمم الغذائي البكتيرية المشتركة والتي تسبب أعراض تتراوح ما بين الاسهال العادي إلى الصورة الخطيرة والتي قد تؤدي إلى الوفاة. أجريت هذه الدراسة لتقييم مدى انتشار هذه الميكروبات في ألبان الماعز والأغنام وأيدي الحلابين في المزارع الصغيرة بالجيزة، مصر. تم تجميع عدد ٤٢٠ عينات لبن من الأغنام والماعز السليمة ظاهرياً وعدد ٤٦ مسحة من أيدي الحلابين بالمزارع محل الدراسة. أسفرت النتائج عن وجود الميكروب المكور العنقودي (١٣,١%) وميكروب الإيشيريشيا كولاي (٢٦,٢%) في ألبان الماعز والأغنام، كما تم عزل الميكروب العنقودي (١٥,٢%) من أيدي الحلابين مما قد يشكل احتمالية انتقال هذه الميكروبات من أيدي الحلابين للحيوانات أو للبلبن أثناء الحلابة اليدوية أو أثناء تداول وتخزين وبيع هذه الألبان ومنتجاتها. باستخدام التصنيف الجيني تأكد تصنيف ميكروب المكور العنقودي في كل العزلات الإيجابية له، كما تم تعيين الجين المسؤول عن تعلق والتصاق الإيشيريشيا كولاي بالأمعاء في ٤٥% العزلات المفحوصة. بصفة عامة تسجل هذه الميكروبات في عينات الألبان من المجترات الصغيرة يدل على تدني مستوى النظافة في هذه الحظائر وتلوثها ببعض من روث الحيوانات، كما يدل على عدم وعي الحلابين باسترشادات الحلابة الصحية الصحيحة.