RESEARCH ARTICLE

The Prevalence and Etiology of Subclinical Mastitis in Sheep and Goats

El-Shymaa A. Abdallah1*, Mohamed I. Eissa2 and Afaf M. Menaze2

1Veterinary Hospital, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt
2Animal Medicine Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt

Abstract

From September 2016 to August 2017, four hundred and twenty four milk samples were collected from mammary halves of 212 apparently healthy lactating animals (108 ewes and 104 does) from small private flocks in different localities at Sharkia Governorate. All samples were investigated by California mastitis test (CMT) and bacteriological examination to determine prevalence and etiology of subclinical mastitis (SCM) in sheep and goats and to estimate the mean somatic cell count (SCC) of infected milk samples with different pathogens. The prevalence rates of SCM using CMT were 44 (40.7%) in ewes [59 milk samples, (27.3%)] and 50 (48.1%) in does [62 milk samples, (29.8%)]. Furthermore, out of ewes’ and does’ milk samples examined by bacteriological culture 54 (25.0%) and 56 (26.9%) samples were bacteriologically positive, respectively. The isolated bacterial causing SCM in ewes’ milk samples were E. coli (44.4%), S. aureus (38.9%), Streptococcus spp. (27.8%), coagulase negative Staphylococci (26.0%), Citrobacter spp. (3.7%) and Enterobacter spp. (1.9%). The identified isolates from does’ milk samples were S. aureus (46.4%), E. coli (26.8%), Streptococcus spp. (25.0%), coagulase negative Staphylococci (19.6%), Citrobacter spp. (5.4%) and Enterobacter spp. (3.6%). The geometric mean of SCC of milk samples harbored major pathogens was significantly (P < 0.05) higher than those harbored minor pathogens. Polymerase Chain Reaction (PCR) was applied on a total of 26 isolates from the most recovered species associated with mastitic milk samples of ewes and does. Suspected S. aureus, E. coli, Strept. agalactiae, Strept. dysgalactiae, and Strept. uberis isolates showed characteristic bands at 270 bp, 366 bp, 487 bp, 279 bp, and 723 bp which were specific for the used genes, respectively. In conclusion: a great attention should be directed to the early diagnosis of SCM in ewes and does.

Keywords: Ewes, Does, Mastitis, Somatic cell count, Bacteriology, PCR.

Introduction

Subclinical mastitis (SCM) is one of the most serious economic diseases of the mammary glands of goats [1] and ewes worldwide. The main reasons for its economic impact include higher prevalence rates [2] and adverse effects such as reduced milk yield in consequence with growth retardation and higher mortality rate among suckling lambs [3] and kids [4]. The disease is characterized by evolving of intramammary infection (IMI) without clinical signs [5] and is often associated with an increase in somatic cell count (SCC) [3]. The disease is usually passed undetectable and animals with SCM remain untreated.

Therefore, early detection of SCC is necessary to avoid persistent udder infection and the spread of the disease [6]. PCR assay is a reliable, accurate and confirmatory technique for the identification of pathogens especially Staphylococcus aureus recovered from mastitic milk samples of sheep and goats [7]. The objectives of this study were to investigate the prevalence and etiological agents of subclinical mastitis among sheep and goats in Sharkia Governorate using California mastitis test (CMT), bacteriological and molecular identification of the bacterial isolates as well as studying the relation between udder...
infection and the level of milk somatic cell count have been carried out.

Materials and Methods

Animals

From September 2016 to August 2017, a total of 212 apparently healthy lactating small ruminants comprising 108 ewes and 104 does from small private flocks in different localities at Sharkia Governorate were selected for this study based on acceptance of the owners to participate in the study.

Milk samples

A total of 424 milk samples comprising 216 ewes’ and 208 does’ milk samples were collected under aseptic conditions after cleaning and disinfection of teat tips with 70% ethyl alcohol [8].

California mastitis test

Each milk sample was screened with CMT as described by Schalm et al. [9]. An equal volume of CMT reagent was added to 2 mL of milk samples in a black plastic cup paddle and then rotated for 10 sec. The detergent in CMT lysed somatic cells and released DNA with the formation of viscous gel. The results were interpreted according to the grade of gel formation as follows; negative (no trace), trace, +1 (weak positive), +2 (distinct positive) and +3 (strong positive).

Somatic cell count

Milk samples were examined automatically using somatic cell count MT05 apparatus [10] at Food Health Department, Animal Health Research Institute, Zagazig, Sharkia. The samples were warmed at 40°C for 5 min and then mixed before reading the results automatically.

Bacteriological examination

Milk samples were incubated aerobically at 37°C for 24 h and then were centrifuged at 3000 rpm for 20 min. The supernatant fluid was discarded and a sterile loopful from the sediment was streaked onto blood agar base (Oxoid, CM3) enriched with 5-10% defibrinated sheep blood and then sub-cultured on selective media for different bacteria; Mannitol Salt Agar (Oxoid, CM 85) for Staphylococcus, Eosine Methyline Blue Agar (EMB) (Oxoid, CM 69) for Escherichia coli and Edwards medium (Oxoid, CM 27) for Streptococci spp. In addition, the samples were also cultured on MacConkey Agar (Oxoid, CM115) for differentiation of Enterobacteriaceae. All the plates were incubated aerobically at 37°C for 24–48 h and then examined for gross colony morphology, pigmentation and hemolytic characteristics. Suspected colonies were purified on nutrient agar plates and subjected to Gram staining and biochemical identification using; catalase test, coagulase test, Christie, Atkinson, Munch and Peterson test (CAMP), esculin hydrolysis test, haemolysis, indole, methyl red, Voges-Proskauer, triple sugar iron, citrate utilization tests, and sugar fermentation [11-14].

Statistical analysis

The data analysis was conducted using SPSS version 21 for windows [15]. Means and Standard Error values of variables were calculated. The Mann-Whinty U-test was used for comparison between major and minor pathogens groups in terms of the SCC values. The p-value < 0.05 was considered statistically significant.

Molecular identification of the suspected isolates

A total of 26 suspected isolates (5 S. aureus, 6 E. coli and 15 Streptococci isolates) recovered from milk samples of subclinically infected ewes and does were subjected to further molecular identification. Molecular identification was carried out at Food Analysis Center, Faculty of Veterinary medicine, Benha University, Egypt.

The DNA from the suspected colonies was extracted using Qiagen extraction kits (Qiagen, Germany) according to the manufacturer’s guidelines. The sequences of S. aureus [16], E. coli [17] and Streptococci [18] specific primers are listed in Table (1).
Amplification of nuc gene of S. aureus

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 μL of PCR mixture containing 3 μL of DNA extract, 200 M of deoxynucleotide triphosphate (dNTP mixture), 1.4 U of Taq DNA polymerase (Biotools, Madrid, Spain), buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl and 3 mM MgCl2, Biotools) and 20 M of each primer (nuc). The amplification conditions included denaturation for 5 min at 94°C, followed by 25 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and then a final extension at 72°C for 10 min. PCR amplified products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide then visualized and captured on ultraviolet transilluminator. The samples were electrophoresed at 100 volts for one hour. A 100 bp DNA Ladder (Fermentas, Germany) was used to determine the fragment sizes. Presence of 270 bp fragments indicated the presence of S. aureus.

Amplification of alr gene of E. coli

The PCR reaction mix (50 μL) for each sample was consistent of 10 μL extracted DNA, 2.5 μL primers mix., 1μL deoxynucleoside triphosphate (dNTP-mix), 5μL 10x buffer, 1 μL Taq-DNA polymerase enzyme (5000 U/ml) and 30.5μL ultra pure deionized water. The first initial cycle: 95 °C for 6 min (denaturation), 35°C for 2 min (annealing) and 72 °C for 1.5 min (extension).

The consequent 35 cycles: 95°C for 20 s (denaturation), 35 °C for 60 s (annealing) and 72 °C for 60 s (extension). A final extension at 72 °C for 5 min and then holding at 4 °C. The amplified product was analyzed on agarose gel (2% agarose and 5 μL of ethidium bromide in 1 x Tris –Acetate EDTA (TAE) buffer). The samples were electrophoresed at 100 volts for one hour, shown under ultra violet transilluminator and photographed. Visible bands of appropriate size of 366 bp were considered positive.

Amplification ofGsag, Gsdy and Guber genes of Streptococcus species

The multiplex PCR was performed using 25 μL reaction mix, 1x HotStarTaq Master Mix, 2.5 μL of diluted lysate, and 300 nM of each primer. The reaction was performed on a Thermal Cycler. Amplification conditions were denaturation at 95 °C for 15 min followed by 35 cycles including 94 °C for 60 s, 58 °C for 60 s and 72 °C for 60 s. The PCR reaction was terminated by a final extension at 72 °C for 10 min followed by cooling down to 4 °C. The PCR products were analyzed by electrophoresis using a 1.3% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH = 8.3). The samples were electrophoresed at 100 volts for one hour. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. Visible bands of appropriate size of 487 bp, 279 bp and 723 were considered positive for Strept. agalactia, Strept. dysagalactia and Strept. uberis, respectively.

Results and Discussion

Results of Table (2) reveal that the prevalence rates of SCM on the basis of CMT were 44 (40.7%) in ewes [59 udder halves, (27.3%)] and 50 (48.1%) in does [62 udder halves, (29.8%)]. In accordance, percentages
of 43.2% in ewes and 41% in does in Sharkia Governorate, Egypt [19]. 40.5% in 42 first partum Santa Ines ewes in Brazil [20] and 45.8% among goats in China [21] were previously recorded. Higher prevalence rates of 66.9% in Awassi sheep in Jordan [22] and 61% among goats in Kenya [23] were previously reported. In contrary, lower prevalence rates of 6.9% among sheep and 14% among goats were recorded in El-Behera Governorate, Egypt [7]. The differences in the managemental practices, geographical distribution, health status of the flock, weather, nutritional status and finally the size of the study samples may be the reason for the variation in the prevalence rates between the present study and the previous ones.

Table 2: Prevalence rate of subclinical mastitis based on California Mastitis test

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of examined animals</th>
<th>Positive cases</th>
<th>No. of examined halves</th>
<th>Positive CMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewes</td>
<td>108</td>
<td>44</td>
<td>216</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.7%</td>
<td></td>
<td>27.3%</td>
</tr>
<tr>
<td>Does</td>
<td>104</td>
<td>50</td>
<td>208</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.1%</td>
<td></td>
<td>29.8%</td>
</tr>
</tbody>
</table>

Table 3: Prevalence rate of subclinical mastitis in ewes’ and does’ milk samples based on the results of bacteriological examination

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of examined milk samples</th>
<th>Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewes</td>
<td>216</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Does</td>
<td>208</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.9</td>
</tr>
<tr>
<td>Total</td>
<td>424</td>
<td>110</td>
</tr>
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<td></td>
<td></td>
<td>25.9</td>
</tr>
</tbody>
</table>

Results in Table (3) show that the prevalence rates of bacteriologically positive milk samples of subclinically mastitic ewes and does were 25% and 26.9%, respectively. Higher prevalence rates of 43.3% in ewes and 58.5% in does were reported in Sharkia Governorate, Egypt [19]. However, lower prevalence rates of 8.08% in ewes and 15.2% in does were recorded in Assiut Governorate, Egypt [24].

Results in Table (4) illustrate that the most prevalent bacterial species isolated from milk samples of ewes was *E. coli* (44.4%) followed by *S. aureus* (38.9%). Lower isolation rates of *E. coli* from milk samples of subclinically infected sheep were 11.3% in Turkey [25] and 19.6% in Jordan [26]. *S. aureus* was isolated from subclinically mastitic milk samples of sheep with the percentages of 23.1 in Egypt [7] and 39 in Jordan [26].

Concerning goats’ milk samples, the results reveal that *S. aureus* represents the most predominate isolated bacterial species (46.4%) followed by *E. coli* (26.8%). These results are in agreement with those previously recorded in Sharkia Governorate, Egypt [27] where the main isolates recovered from subclinically mastitic goats' milk samples were *S. aureus* (40%) followed by *E. coli* (26.67%). Lower isolation rates of 23.4% for *S. aureus* and 21.3% for *E. coli* were reported in Ethiopia [28]. *S. aureus* and *E. coli* were respectively recovered from 69.6% and 30.23% of goats’ milk in Turkey [29] and Assiut Governorate, Egypt [30]. The highest isolation rate of *E. coli* as environmental pathogen recovered from milk samples of subclinically infected ewes may be attributed to managemental mistakes as overcrowding, bad ventilation, inadequate manure removal and general lack of farm cleanliness and sanitation as recorded in Jordan [22].
Table 4: Frequency of microorganisms isolated from milk samples positive for mastitis in ewes and does

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Ewes</th>
<th>%*</th>
<th>No.</th>
<th>%*</th>
<th>Does</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>21</td>
<td>38.9</td>
<td>26</td>
<td>46.4</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>24</td>
<td>44.4</td>
<td>15</td>
<td>26.8</td>
<td></td>
</tr>
<tr>
<td>Streptococcus spp</td>
<td>15</td>
<td>27.8</td>
<td>14</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Coagulase negative Staphylococci</td>
<td>14</td>
<td>26.0</td>
<td>11</td>
<td>19.6</td>
<td></td>
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<tr>
<td>Citrobacter spp.</td>
<td>2</td>
<td>3.7</td>
<td>3</td>
<td>5.4</td>
<td></td>
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<tr>
<td>Enterobacter spp.</td>
<td>1</td>
<td>1.9</td>
<td>2</td>
<td>3.6</td>
<td></td>
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</tbody>
</table>

*in relation to bacteriologically positive milk samples

Table 5: Relation between Somatic Cell Count (× 10³ cell/ml) and etiological agent isolated from milk of subclinically infected animals

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Ewes</th>
<th>SCC (GM± SEM)</th>
<th>No.</th>
<th>%**</th>
<th>SCC (GM± SEM)</th>
<th>No.</th>
<th>%**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major pathogens:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>28</td>
<td>51.9</td>
<td>410.6±53.4</td>
<td>34</td>
<td>60.7</td>
<td>471.5±54.2</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>10</td>
<td>18.5</td>
<td>504.9±115.9</td>
<td>19</td>
<td>33.9</td>
<td>569.4±82.9</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>10</td>
<td>18.5</td>
<td>415.6±175.3</td>
<td>5</td>
<td>8.9</td>
<td>470.8±148.4</td>
<td></td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>8</td>
<td>14.8</td>
<td>312.3±44.5</td>
<td>10</td>
<td>16.1</td>
<td>329.8±48.2</td>
<td></td>
</tr>
<tr>
<td>Minor pathogens:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulase negative Staphylococci</td>
<td>9</td>
<td>16.7</td>
<td>236.9±26.4</td>
<td>7</td>
<td>12.5</td>
<td>251.2±43.3</td>
<td></td>
</tr>
<tr>
<td>Mixed infection</td>
<td>17</td>
<td>31.9</td>
<td>645.6±74.9</td>
<td>15</td>
<td>26.8</td>
<td>608.5±108.8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>431.9±38.7</td>
<td>56</td>
<td>466.7±45.3</td>
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</table>

** % of infected udder halves.
GM: geometric mean, SEM: Standard error of mean.
Significant difference between SCC of major pathogens and minor pathogens (P value < 0.05).

In the current study, Streptococci species were recovered from 27.8% of ewes’ positive samples. This result is in contrary with numerous studies which reported lower isolation rates of 7.4% [7] and 15.2% [30] in Egypt and 12% in Brazil [31].

In goats, Streptococci were isolated from 25% of positive samples. This rate was higher than that previously recorded in Egypt [30] and lower than that (56%) stated in Botswana [32].

Coagulase negative Staphylococci in the present study represent the lower isolation rate than pathogens aureus, E. coli and Streptococci. It represents 26% and 19.6% of ewes and does positive samples, respectively. These results are in consistence with several studies which recorded that the most prevalent pathogens of the mammary gland in subclinical mastitic sheep and goats were Coagulase negative Staphylococci and its isolation rate ranges from 45% to 48% ovine positive milk samples as recorded in Poland [1] and USA [33], respectively. However, it ranges from 60% to 80.7% in caprine milk samples in Poland and United Kingdom [1,34].

The least identified pathogens from ewes and does positive samples were Citrobacter species and Enterobacter. Their isolation rates were (3.7% Vs 1.9%) in ewes’ positive samples and (5.4% Vs 3.6%) in does’ positive samples. In addition, Enterococcus fecalis was recovered from one doe milk sample (1.8%).

In Egypt, Enterobacter species were previously recovered from 1.5% and 4.06% of subclinically mastitic milk of sheep and goats, respectively [30].

The intensity of cellular immune defense could be indicated by Somatic cell count and it represents a marker of the sanitary status of the mammary gland as it increases during the course of intramammary infection as a result of migration of leukocytes from the blood towards the mammary gland [35]. As shown in
Table (5) the results reveal that the mean Somatic Cell Count in milk samples of subclinically infected ewes with major and minor pathogens was $410.6 \pm 53.4 \times 10^3$ and $236.9 \pm 26.4 \times 10^3$ cell/mL, respectively. While, in goats it was $471.5 \pm 54.2 \times 10^3$ and $251.2 \pm 43.3 \times 10^3$ cell/mL, in those infected with major and minor pathogens, respectively. These results showed that the mean SCC of the udder halves infected with major pathogens was significantly ($P < 0.05$) higher than those infected with minor pathogens. This finding is consistent with Alekish et al. [22] in Jordan who found that most of the milk samples of subclinically infected ewes with S. aureus as a major pathogen had SCC more than $1 \times 10^6$ cell/mL. While, coagulase negative staphylococci as minor pathogens showed moderate increase in SCC ($250 \times 10^3 - 1 \times 10^6$ cell mL). In goats, Anniss and McDougall [36] found that the SCC of the glands infected with a major pathogen was significantly ($P < 0.05$) higher than glands with a minor pathogen infection ($800 \times 10^3$ and $481 \times 10^3$ cell/mL), respectively. The higher SCC of milk samples from animals infected with a major pathogen might be attributed to its pathogenicity that triggers the immune system. Coagulase negative Staphylococci are less pathogenic for the mammary gland of domestic ruminants; therefore, they produce persistent subclinical mastitis with less stimulation of immune system and then modest increase in SCC. Hence, the increase in the SCC might depend on the host immune system at the time of the infection in addition to the pathogenicity of the isolated organism [22]. Knowing such correlation between the SCC and the isolates is necessary for predicting the target pathogen when screening for the presence of mastitis and planning a control program.

The results of the molecular identification of the suspected isolates reveal that the investigated specific gene for each pathogen was confirmed in all 26 isolates examined. Thus, indicating the high specificity and sensitivity of PCR assay in the detection of pathogens.

**Conclusion**

The higher prevalence of subclinical mastitis among small ruminants alarming that attention should be directed toward such form of mastitis, Somatic cell count and intramammary infection are significantly associated with bacterial isolates, S. aureus and E. coli are identified as the predominant etiologic agents of SCM among small ruminants.

**Conflict of interest**

The authors declare no conflict of interest.

**References**


الخصائص الواردة في الجدول 

<table>
<thead>
<tr>
<th>السبب البكتيري</th>
<th>النتائج</th>
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<tbody>
<tr>
<td>الميكروب البوليولاي</td>
<td>40.7%</td>
</tr>
<tr>
<td>الميكروب الأبلائي</td>
<td>48.1%</td>
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<tr>
<td>الميكروب البوليولاي</td>
<td>40.7%</td>
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<tr>
<td>الميكروب البوليولاي</td>
<td>48.1%</td>
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</tbody>
</table>

تظهر النتائج أن الميكروب البوليولاي كان السبب الأكثر شيوعاً، إذ تم اكتشافه في 40.7% من الحالات. التحليل المكتسب للأ nghiệm والماعز من الاعتقاد أن هذا السبب كان السبب الأكثر شيوعاً في الاصابة بأمراض الضرع. يمكن أن يكون هذا السبب أكثر شيوعاً بسبب الانتشار الكبير للبكتيريا في المناطق الحضرية والريفية، مما يزيد من سوء الظروف المعيشية للحيوانات. في النهاية، يوصى بالتشخيص المبكر والعلاج المبكر لأمراض الضرع لمنع الانتشار والوقاية من الإحباط والتلف三亚.