Prevalence of *Staphylococcus aureus* Subclinical Mastitis in Dairy Buffaloes Farms

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Abstract

A total of 239 buffaloes of different lactation seasons from two milk-producing buffalo's farms at Assiut Governorate, Upper Egypt, were monthly tested by California mastitis test during one lactation season for the presence of subclinical mastitis. The prevalence of subclinical mastitis ranged from 11.11 % to 37.21 % based on the number of lactation seasons. The linear relationship between number of lactations and prevalence of subclinical mastitis was studied and showed a strong negative correlation (P < 0.01, r = -0.91). The prevalence of subclinical mastitis of investigated farms was dramatically increased (P < 0.05) during the first three lactation seasons and then gradually decreased by increasing the number of lactations (P < 0.01). Milk samples from subclinical mastitic buffaloes were examined bacteriologically; 77.27% were culturally positive. *Staphylococcus aureus (S. aureus)* was the predominant mastitis pathogen followed by *Streptococcus agalactiae* and *Escherichia coli*. The culturally negative samples examined by PCR technique using species-specific primer, indicated that 80% of tested animals were positive to *S. aureus*. Referring to the false negative results on the conventional culturing procedures with that microorganism, this may be misleading in case of control measures of mastitis associated with *S. aureus* on farms, and single-time culturing procedures can be insufficient for definitive diagnosis. However, PCR as a rapid diagnostic tool for direct detection of *S. aureus* in milk is still valuable in comparison with compared to conventional culturing procedures.

Key words: *Staphylococcus aureus*, Subclinical Mastitis, Dairy Buffaloes, Assiut Governorate

Introduction

Subclinical mastitis, the asymptomatic inflammation of mammary tissue, is the most common form of mastitis. It is 15 to 40 times more common than clinical cases (Horner and Randles, 1995 and Khan and Muhammad, 2005). Mastitis prevalence was found to be influenced by the stage of lactation and anatomical abnormality of the udder, and some management aspects such as nutrition (Almaw et al., 2008). However, virtually all of the published information about the risk factors for mastitis refers to dairy breeds of cattle, and information about the condition in buffaloes is scarce. Though a high
probability exists that these identified risk factors may also be observed in this species, the degree of influence of these factors is still unknown (Salvador et al, 2012).

*S. aureus* is a notorious pathogen responsible for contagious mastitis in dairy buffaloes causing serious economical losses during lactation seasons (Zaitoun, 2008). Declining daily milk yield, undesirable milk with high somatic cell count appear to be the most common cause of losses associated with *S. aureus* mastitis (SAM). In addition, therapeutic treatment of SAM during lactation seasons is usually unsuccessful or with poor success and in some cases is deceptive (Jubb et al., 2007). This may induce a favorable chance for spread of infection among the producing animals; subsequently increasing the economic losses of SAM particularly in small-scale farming with sublevel of sanitary measures leading to a relatively high culling rate. Consequently, reliable method for the identification of *S. aureus* from mastitis milk is therefore crucial for preventing the spread of infection and for control of SAM.

The aim of the present study was to study the prevalence rate of subclinical mastitis in dairy buffalo farms located at Assiut Governorate, the most common mastitis pathogens that affect the level of milk production using conventional culturing procedure as well as molecular procedures and to test whether there is a correlation between the lactation seasons and prevalence of subclinical mastitis.

**Materials and Methods**

**Animals**

A total of 239 apparently healthy buffaloes from 2 different private farms at different lactation seasons at Assiut Governorate, Egypt were subjected to the study during one lactation season.

**Samples**

These buffaloes were subjected to examination with the California mastitis test (CMT): About 3 ml milk sample from each quarter was drawn in each of the 4 shallow cups in the CMT paddle (US PAT NO. 2935384) then approximately equal volume of 3 ml of the commercial available CMT reagents (Original Schalm CMT Technivet, 4 industry road, Box 189 Brunswick, Maine 04011, USA) was added to each cup and mixed together through swirling the paddle in a circular motion for few seconds. Only California mastitis test-positive samples were collected and transported to the laboratory on ice.

**Bacteriological Analysis**

Each positive CMT milk sample was collected after cleaning the teats, discarding a few streams of milk, and scrubbing the teat ends with cotton balls moistened with 70% alcohol. The four-quarter milk samples of each individual animal were pooled as one sample to minimize the costs of analysis of these samples.
All samples were incubated at 37°C on 5% sheep Colombia blood agar plates. The bacterial genus was determined according to Collins et al. (1991).

**Polymerase Chain Reaction (PCR)**

Chromosomal DNA from culture negative samples was extracted by using Dneasy Tissue Kit (QIAamp® DNA Mini Kit) with some modifications. The extracted DNA was used as a template for PCR amplification. Positive control containing *S. aureus* (Zaitoun, 2008) and negative control containing Dnase free water were included in each experiment. PCR amplifications were performed with a pair of primers specific for *S. aureus*, synthesized from the previously published sequences (Kuźma et al., 2003):

- primer 1: 5’-GCG ATT GAT GGT GAT ACG GTT-3’,
- primer 2: 5’-AGC CAA GCC TTG ACG AAC TAA AGC-3’.

The PCR cycles consisted of pre-heating at 95°C for 10 min, denaturation at 94°C for 1 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1.5 min. The amplification was performed for 37 cycles with a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing 0.5 mg of ethidium bromide per ml. The sizes of the amplification products 270 bp were estimated by comparison with a 100bp DNA step ladder (Kuźma et al., 2003).

**Statistical Analysis**

Statistical analysis was done according to Chatfield (1970).

**Results**

Prevalence of subclinical mastitis using CMT - The prevalence of subclinical mastitis ranged from 11.11% to 37.21% based on the number of lactation seasons. Prevalence rates of subclinical mastitis of the investigated buffaloes at different lactation periods were shown in Table 1. A linear relationship between the number of lactations and prevalence of subclinical mastitis of the examined animal is diagrammed in Fig. 1. The regression line with standardized coefficient (r = -.81) and ANOVA for confidence are done in Fig. 1 and Table 2, respectively.

**Prevalence of *S. aureus* subclinical mastitis**

Microbiological examination of the 66 CMT positive samples was positive for bacterial culture in 51 samples (77.27%), while 15 samples were negative. Out of the 51 culture positive samples, 96 isolates
were detected, *S. aureus* represented 78.12%, *Streptococcus agalactiae* represented 14.58%, *E. coli* represented 6.25%. 1.04% of cultures was unidentified Gram-negative bacteria. Frequency of isolation of the mastitis pathogens from the collected milk samples was summarized in Fig. 2.

The culturally negative samples (15 samples) were tested by PCR technique that indicated 12 samples were positive giving the specific band for *S. aureus* at 270 bp, while the remainder were negative (Fig. 3).

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**Fig. 1:** Linear relationship (regression) between the number of lactation seasons and prevalence (% affected) of subclinical mastitis of dairy buffaloes

**Fig. 2:** Frequency isolation of the mastitis pathogens (96 strains) from sub clinically mastitis cases (66 cases) of the examined dairy buffaloes
Fig. 3: PCR products of milk DNA samples showing the positive samples containing S. aureus at 270bp (M= DNA marker, 1-10= positive samples)

Table 1: Prevalence rate of subclinical mastitis of dairy buffaloes at different lactation seasons and values of Regression formula.

<table>
<thead>
<tr>
<th>Lactation season</th>
<th>No. of tested animals with CMT</th>
<th>No. of positive animals with SCM</th>
<th>Prevalence rate</th>
</tr>
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<tbody>
<tr>
<td>1st</td>
<td>12</td>
<td>3</td>
<td>0.25 (25.00)a</td>
</tr>
<tr>
<td>2nd</td>
<td>22</td>
<td>7</td>
<td>0.32 (31.82)</td>
</tr>
<tr>
<td>3rd</td>
<td>43</td>
<td>16</td>
<td>0.37 (37.21)</td>
</tr>
<tr>
<td>4th</td>
<td>39</td>
<td>13</td>
<td>0.33 (33.33)</td>
</tr>
<tr>
<td>5th</td>
<td>26</td>
<td>7</td>
<td>0.27 (26.92)</td>
</tr>
<tr>
<td>6th</td>
<td>27</td>
<td>6</td>
<td>0.22 (22.22)</td>
</tr>
<tr>
<td>7th</td>
<td>30</td>
<td>7</td>
<td>0.23 (23.33)</td>
</tr>
<tr>
<td>8th</td>
<td>19</td>
<td>4</td>
<td>0.21 (21.05)</td>
</tr>
<tr>
<td>9th</td>
<td>12</td>
<td>2</td>
<td>0.17 (16.67)</td>
</tr>
<tr>
<td>&gt;9th</td>
<td>9</td>
<td>1</td>
<td>0.11 (11.11)</td>
</tr>
<tr>
<td>Total</td>
<td>239</td>
<td>66*</td>
<td>27.61 %</td>
</tr>
</tbody>
</table>

CMT: California Mastitis Test  
SCM: Subclinical mastitis  
a: The numbers between parentheses in this column are the percentage of infection with subclinical mastitis of buffaloes per each lactation season  
*: Of these positive cases, 77.27% were bacteriologically positive by conventional culturing procedure, 96 strains were recovered (Fig 3), and 22.73% were culturally negative.

Table 2: ANOVA for confidence of linear regression.

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<th>SS</th>
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<td>Regression</td>
<td>0.036</td>
<td>1</td>
<td>0.36</td>
<td>15.53**</td>
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<tr>
<td>Residual</td>
<td>0.16</td>
<td>8</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.55</td>
<td>9</td>
<td>-</td>
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**: Highly significance (P < 0.01)  
Calculated $F = 52.15$  
Tabulated $F_{0.05} = 4.06$  
Tabulated $F_{0.01} = 6.61$
Discussion

Our results indicated that the overall prevalence of subclinical mastitis of dairy buffaloes (27.61%) is slightly lower than reported previously by Akl (1988), Ibrahim (1990), Mahmoud (1990) and Osman (1996), they indicated that the prevalence of subclinical mastitis in dairy buffaloes located in different Governorates of Egypt was 43.70%, 31.00%, 34.30% and 35.30% respectively. The highest incidence of buffalo subclinical mastitis was reported by Abdel Ghani (2005) who reported that 67.5% of the examined buffaloes in Assiut Governorate were significantly infected. Conversely, the lowest prevalence of subclinical mastitis of dairy buffaloes was reported by Khalil et al. (1996). They indicated that 6.67% of the tested animals in Bani-Suef Governorate were infected by subclinical mastitis. Similarly, Ahmed et al. (2008) revealed that the prevalence of subclinical mastitis of dairy buffaloes kept in small holder farms at Sharkia Governorate was 9.69%. Variations may be attributed to several reasons including population density of animals, variances of hygienic conditions, milking sanitations control program of each locality as encountered by Edmondson and Bramely (2004) and Haltia et al. (2006).

The prevalence of mastitis fluctuates from one area to another and from farm to farm depending on the hygienic measures and milking sanitation as well as the healthy environment around the dairy animal (Zaitoun and Manaa, 1992 and Abdelhameed and Sharaf, 2009).

In the present study, the correlation between the lactation numbers of the dairy buffaloes and the prevalence of subclinical mastitis was statistically analysed, and showed that the prevalence of subclinical mastitis is primarily increased by increasing the lactation numbers until the third lactation season (Peak infection rate 37.21%). Thereafter, it gradually decreases from 33.33% to 11.11% showing a negative correlation. This negative correlation may be caused by a build-up of acquired immunity by subsequent lactations (Zaitoun 2008) where the author concluded that there were reverse correlation between the prevalence of mastitis and the lactation seasons of the River Nile buffaloes. However, other opinions by El-Bayomi and Mahmoud (1987), Ibrahim (1990) and Osman (1996) suggested that the prevalence of subclinical mastitis increased by increasing the age of the animal depending on the remarkable physiological alterations in the morphological features of the teat of buffaloes, where teat of the aged buffalo is more pendulous than in young ones. Therefore, the risk of exposure to abrasions or wounds is increased and subsequently leading to the increase of infection rate.

Bacteriological examination of milk samples from the current study showed that *S. aureus* is the predominant isolate. This may support the veterinary importance of *S. aureus* as a major cause of subclinical mastitis of dairy buffaloes. Tollersrud et al. (2006), Ali et al. (2008), Zaitoun (2008) and
Abdelhameed and Sharaf (2009) concluded that *S. aureus* is an extremely important etiologic agent responsible for serious intra-mammary infection of all dairy ruminants including buffaloes.

**Conclusion**

The frequency distribution of the isolated mastitis pathogens showed that 78.12% was *S. aureus*. Out of the 15 negative samples by bacteriological examination, 12 milk samples were positive to *S. aureus* by PCR. PCR examination is crucial as the false negative results can be misleading and can result in erroneous decisions in dairy farms which would increase the chances of spread of infection to other healthy animals.

**References**