Molecular Identification of the False Negative Mycoplasma Isolates from Bovine Mastitis Infections

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The main goal of this study is the application of polymerase chain reaction in dairy farm for diagnosis of the false negative Mycoplasma isolates from bovine mastitis. A total number of 350 and 90 milk samples were collected from udder quarters of 180 and 30 cows and buffaloes, respectively and examined by standard microbiological techniques as well as polymerase chain reaction (PCR). Mycoplasmas were isolated in percentages of 7.14%, 8% from subclinically mastitic cows and buffaloes, respectively and in percentages of 11.85%, 10% from clinically mastitic cows and buffaloes, respectively. Typing of Mycoplasma revealed 6 strains (24%) Mycoplasma bovis and 10 strains (40%) Mycoplasma bovigenitalium, 7 strains (28%) Mycoplasma arginini while the other two strains (8%) were Mycoplasma bovirhinis. Isolation of Mycoplasma from udder tissue in cows and buffaloes were in a percentage of 10% in cows while no Mycoplasma isolates were obtained from buffaloes' udder tissues. Application of PCR on these isolates and milk samples revealed 100% sensitivity and specificity. The PCR is simple, rapid, highly specific and accurate test for identification of Mycoplasma bovis and Mycoplasma bovigenitalium. In addition, the negative samples for Mycoplasmas can be detected even if the samples or the broth cultures were contaminated with bacteria.

Key words: Bovine mastitis, False negative Mycoplasma, PCR technique, Standard microbiological techniques.

Mycoplasma species are members of Mollicutes known to be able to cause clinical and subclinical mastitis in dairy cattle.1 In small ruminants, diseases induced by Mycoplasmas include respiratory diseases, mastitis, arthritis, genital diseases and eye lesions. The most important diseases in such animals are contagious caprine pleuropneumonia and contagious agalactia.2 Numerous species of Mycoplasma have been reported to be associated with mastitis in dairy cattle. Among all reported cases, Mycoplasma bovis, M. californicum, M. alkalescens, M. bovigenitalium and M. canadense are the species most commonly associated with bovine mastitis.3 Mycoplasma is highly contagious pathogen, and can be easily transmitted from cow-to-cow.3 Treatment is usually unsuccessful; therefore, control of mycoplasma mastitis by rapid

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detection and culling of the infected animals which act as a reservoir for infection is very essential for controlling the infections with this organism. 

Mycoplasmas are distinguished phenotypically from other bacteria by their minute size (125-150 millimicron) and total lack of a cell wall which explains many of the unique properties of the Mycoplasmas, such as sensitivity to osmotic shock and detergents, resistance to penicillin, and formation of peculiar fried-eggs shape colonies. Mycoplasma species are difficult to be isolated which makes diagnosis of infection challenging, because specialized media and incubation for several days in 10% CO$_2$ are required, many laboratories may not routinely culture milk for mycoplasma organisms. Additionally, cows with mycoplasma mastitis may shed the pathogen intermittently and shedding of Mycoplasma species into milk of infected cows may below the threshold of detection by standard culture methods. Moreover, Identification of $M.$ agalactiae and $M.$ bovis by immunofluorescence was laborious and time-consuming. Furthermore, $M.$ agalactiae and $M.$ bovis possess a particular ability to modify the phase and/or size of the membrane surface proteins, allowing escape of the host’s. To overcome the problems of the standard culture techniques, such as time of culture and loss of viable organisms due to storage, many PCR-based techniques have been developed for rapid detection of mycoplasma mastitis. Most of these studies have focused only on the identification of $M.$ bovis in milk samples.

Genus differentiations of Mycoplasma and Acholeplasma species can be performed by the nested PCR technique. The nested PCR products are digested using a restriction enzyme where species unique DNA fragment patterns can be used to distinguish Mycoplasma species. Real-time PCR technique has been used in clinical microbiology. It is a promising tool with an excellent sensitivity and specificity, very fast and suitable for high throughput of samples with an inherent quantitative ability. Development of a real-time PCR technique to detect major mycoplasma mastitis causing pathogens directly from milk samples may be a superior choice for the diagnosis of this disease given its abilities to rapidly determine, quantify, and distinguish Mycoplasma species, but not all laboratories could able to perform it due to the cost of the instruments. Consequently, the main goal of this study is the application of polymerase chain reaction in dairy farm for diagnosis of the false negative Mycoplasma isolates from bovine mastitis.

**MATERIALS AND METHODS**

**Samples**

Milk samples were collected from 180 cows and 30 buffaloes reared at El Menofia and Sharkia governorate, Egypt during the period from 1st October 2009 to 1st October 2011 under possible hygienic conditions as outlined by Koskinen et al. $^{9}$ 135 milk samples were collected from 120 clinically mastitic cows with abnormal secretions of mammary glands including clots or flakes in addition to udders swelling and hardness. 215 milk samples were collected from 60 apparently healthy cows which detected by palpation of udder and were subjected to California Mastitis Test (CMT) $^{3}$ to detect subclinical mastitis. Twenty milk samples were collected from 10 clinically mastitic buffaloes and 70 from 20 apparently healthy ones.

**Isolation and identification of Mycoplasma**

Isolation and identification of Mycoplasma from udder tissue and milk samples as well as differentiation between Mycoplasma and Acholeplasma isolates using the Digitonin sensitivity test were carried out according to Quinn et al. $^{11}$

**Extraction of DNA**

The DNA of the standards strains and of the other bacterial isolates yielded from bacteriological examination as well as from the milk samples were extracted by hexadecyl trimethyl ammonium bromide (CTAB) according to Moussa and Shibl. $^{12}$

**PCR primers**

For molecular detection of Mycoplasma isolates using PCR, two sets of primers were used; the first one targeting the $M.$ bovis $^{13}$, Mb. F1: 52 - CCA GCT CAC CCT TA T ACA T -32 ) and MbR1: 52 - TGA ATC ACC ATT TAG ACC G-32 ) and amplify442 bp fragments. The second one targeting $M.$ bovigenitalium $^{13}$ MMBr-MN (52 - ACC ATG GGA GCT GGT AA T-32 ) and MMBr-MN-927 (52 - TTC TTA CTT CTA AAG TA T-32 ) and amplify 928 bp fragments.

**PCR design and amplification**

PCR reaction mixes (25 il) consisted of 1U
*Taq* DNA polymerase (Roche), 1× PCR buffer, 2 mM MgCl₂, 1.25 mM of each deoxynucleotide triphosphate and 25 pmol of each primer. Reactions were performed in an automated DNA thermal cycler (GeneAmp 9700, Applied Biosystems). Samples were subjected to 30 cycles of amplification. An amplification cycle consisted of denaturation for 1 min at 94 °C, primer annealing to the template at 55 °C for 1 min and primer extension at 72 °C for 1 min. Finally 10 min incubation was performed at 72 °C. The PCR products were visualized by agarose gel electrophoresis, according to Moussa and Shibl. Calculation of the fragment size was performed at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov) using NCBI BLAST software.

**RESULTS AND DISCUSSION**

*Mycoplasma* is a highly contagious pathogen, and can be easily transmitted from cow-to-cow. Recently, Bradley et al. have reported cases of *M. bovis* mastitis in dairy herds and recommended that veterinarians should consider *Mycoplasmas* where there is unresponsive mastitis, particularly in view of the fact that at least 11% of cases of mastitis go undiagnosed. Furthermore, Bradley et al. reported rather surprisingly that 26.5% of clinical mastitic samples and 38.6% of subclinical cases produced no bacterial growth. Therefore, the current study is aimed to apply the PCR in dairy farm for diagnosis of the false negative *Mycoplasma* isolates from bovine mastitis.

Out of 185 examined cows 120 (66.67%) cow were affected with mastitis as shown in Table 2. On the other hand the mastitic buffaloes were 10 out of 30 (33.33%) examined buffaloes. Out of 215 apparently normal quarter milk samples collected from 60 apparently healthy cows, subclinical mastitis reached 70 with an incidence of (32.56%), and 145 were negative for CMT with an incidence of (67.44%). On the other hand out of

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of milk samples</th>
<th>No. of animals</th>
<th>Clinically mastitic</th>
<th>Apparently healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>350</td>
<td>180</td>
<td>135</td>
<td>120</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>90</td>
<td>30</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1. Milk samples collected from different animal species

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Udder status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apparently healthy</td>
<td>Mastitic</td>
</tr>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>Cows</td>
<td>60 33.33%</td>
<td>120 66.67%</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>20 66.67%</td>
<td>10 33.33%</td>
</tr>
</tbody>
</table>

Table 2. Incidence of clinical mastitis among the examined lactating cows and buffaloes.

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Subclinically mastitic quarters</th>
<th>Normal quarters</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
</tr>
<tr>
<td>Cows</td>
<td>70 32.56%</td>
<td>145 67.44%</td>
<td>215</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>25 35.71%</td>
<td>45 64.29%</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 3. Incidence of subclinical mastitis among cows and buffaloes as detected by CMT

% was calculated according to the total number of the examined apparently normal milk samples.
70 apparently normal quarters milk samples of buffaloes, 25 were subclinically mastitic with an incidence of (35.71%) as shown in Table (3), these results were in agreement with those reported by Osman et al.16

Moreover, the incidence of subclinical mastitis in cows and buffaloes were (32.56%) and (35.71%), respectively as shown in Table (3). These results were nearly similar to those obtained by Kamelia et al.17 and Bachaya et al.18, they reported subclinical mastitis in 32.62 and 26.25% of cows and buffaloes, respectively.

*Mycoplasma* isolates were recovered from 5 (7.14%) subclinically mastitic cows, while only two *Mycoplasma* isolate (8%) was recovered from subclinically mastitic buffaloes as shown in Table 4.

**Table 4. Incidence of Mycoplasma in subclinically and clinically mastitic cows’ and buffaloes’ quarter milk samples**

<table>
<thead>
<tr>
<th>Species</th>
<th>Subclinically mastitic</th>
<th>Clinically mastitic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Examined</td>
<td>Positive QMS</td>
</tr>
<tr>
<td>Cows</td>
<td>70</td>
<td>7.14</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>25</td>
<td>8</td>
</tr>
</tbody>
</table>

QMS= Quarters Milk Samples
% was calculated according to the total number (No.) of examined quarter milk samples.

**Table 5. Biochemical and serological identification of *Mycoplasma* isolates recovered from mastitic cows and buffaloes**

<table>
<thead>
<tr>
<th>Types of Mycoplasma isolates</th>
<th>D.S.</th>
<th>U.A.</th>
<th>G.F.</th>
<th>A.H.</th>
<th>Positive isolates*(GIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6 24</td>
</tr>
<tr>
<td><em>M. bovigenitalium</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 40</td>
</tr>
<tr>
<td><em>M. arginini</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>7 28</td>
</tr>
<tr>
<td><em>M. bovirhinis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>2 8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25 100</td>
</tr>
</tbody>
</table>


**Table 6. Biochemical and serological identification of *Mycoplasma* isolates recovered from udder tissues of cows and buffaloes**

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of examined udder tissue samples</th>
<th>D.S</th>
<th>U.A.</th>
<th>G.F.</th>
<th>A.H.</th>
<th>Positive isolates*(GIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>30</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 10</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>30</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0 0</td>
</tr>
</tbody>
</table>


Positive number of isolates positive to specific antisera by Growth inhibition test (GIT).
Table (4). On the other hand, the incidence of *Mycoplasma* in clinically affected quarter milk samples of cows and buffaloes were 16 (11.85%) and two (10%) respectively.

Table (5) showed the results of biochemical and serological identification of *Mycoplasma* species isolated from the examined cows and buffaloes. *M. bovis* was isolated in a percentage of 24% while (40%) of the isolates were *M. bovigenitalium*, (28%) of the isolates were *M. arginini*, while the other (8%) were identified as *M. bovirhinis*.

Isolation of *Mycoplasma* from udder tissue in cows and buffaloes were in a percentage of 10% in cows while no *Mycoplasma* isolates were obtained from buffaloes’ udder tissues as shown in Table (6). A similar results obtained by Gonzalez and Wilson3. *M. bovis* was isolated in a percentage of 24% while (40%) of the isolates were *M. bovigenitalium* and (28%) of the isolates were *M. arginini*, while the other (8%) were identified as *M. bovirhinis*. These results agreed with that of Biddle et al.4 and Kamelia et al17.

PCR could detect all the bacteriologically positive samples for *M. bovigenitalium* and amplification of 442 bp fragments specific for *M. bovis* were observed as shown in Figure (1), moreover, PCR could detect another 3 milk samples negative with standard bacteriological examination for *M. bovis* and amplification of 442 bp fragments were observed as shown in Figure (1) with higher sensitivity of the PCR in the detection of the positive samples with *Mycoplasmas*, our results confirm the conclusion of many authors.7-10 Moreover, PCR could detect 3 milk samples negative with standard bacteriological examination for *M. bovis* and amplification of 442 bp fragments were observed as shown in Figure (1). Our results
indicated the ability of the PCR to detect the false negative samples.9,10

PCR could detect also all the bacteriologically positive samples for M. bovigenitalium and amplification of 928 bp fragments specific for M. bovigenitalium were observed as shown in Figure (2), moreover, PCR could detect another 2 milk samples negative with standard bacteriological examination for M. bovis and amplification of 928 bp fragments were observed as shown in Figure (2). Our results also confirm the higher sensitivity and specificity of the PCR in the detection of Mycoplasma mastitis and identification of false negative samples.8,10

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