Prevalence of *Mycoplasma bovis* in bovine clinical mastitis milk in Egypt

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**ABSTRACT**

Bovine mastitis caused by *Mycoplasma bovis* represents a major problem for dairy industry all over the world. Although *Mycoplasma species* were identified in Egypt decades ago, the prevalence of *Mycoplasma bovis* mastitis is not frequently investigated. The current study was designed to monitor the prevalence of *Mycoplasma bovis* in clinical bovine mastitis milk in Egypt. Clinical mastitis milk samples (n=703) were collected between 2016 to 2019 from different dairy farms located in different governorates in Egypt including, Giza (227), Alexandria (357), Dakahlia (78), Buhayrah (27), Ismailia (14) and presented for *Mycoplasma* isolation using conventional cultural method followed by molecular identification of *Mycoplasma bovis* using PCR targeting *mb-mp 81* gene of *M. bovis*. From the examined samples (n=703), Sixty-three (8.96%) were positive for *Mycoplasma* isolation. Among the 63 *Mycoplasma* isolates, 53 were identified by PCR as *Mycoplasma bovis* representing 84.12% of the recovered *Mycoplasma* isolates (n=63) and 7.53% from the total examined mastitis milk samples (n=703). From the 53 *Mycoplasma bovis* isolates, 17/227 (7.48%) were obtained from Giza, 13/357 (3.64%) from Alexandria, 18/78 (23.07%) from Dakahlia, 1/27 (3.70%) from Al-Buhayrah and 4/14 (28.57%) from Ismailia.

**Keywords:** *Mycoplasma bovis*, Mastitis, Milk, PCR, *mb-mp 81*, Egypt.


1. **INTRODUCTION**

*Mycoplasma bovis* is one of the serious cattle pathogens threatening animal welfare and the farming industry with capability of infecting cattle in all ages (Nicholas & Ayling, 2003). It had a worldwide distribution and associated with a variety of disease conditions, including mastitis, pneumonia, otitis media, arthritis and genital disorders (Parker et al., 2018). Indeed, *Mycoplasma bovis* is a main cause of mastitis problems in dairy cattle (Aebi et al., 2015). *Mycoplasma bovis* first record as a causative agent of sever mastitis outbreak was in 1961s in a commercial dairy herd in the USA and was named *Mycoplasma agalactiae var bovis* (Hale et al. 1962). Afterwards in the 1976s, *Mycoplasma agalactiae var bovis* was subsequently elevated to species level and named *Mycoplasma bovis* (Askaa and Erno, 1976). In Egypt, El-Ebeedy et al., (1985) and Eissa (1986) reported the first isolation of *Mycoplasma bovis* from bovine mastitis outbreaks, respectively. Since then *Mycoplasma bovis*, has persisted in Egyptian cattle herds. It belongs to genus *Mycoplasma*,
family *Mycoplasmataceae*, Class *Mollicutes* and characterized by the cell wall lackage, low G+C content (23-40%) and a limited genome size of 0.58-1.4 Mbp (Parte *et al*., 2011; Brown *et al*., 2015). In vitro cultivation of *Mycoplasma* is difficult due to their limited biosynthetic activity; therefore, complex media supplemented with cholesterol, serum and DNA is used for the in vitro growth of *Mycoplasma* micro-colonies with the characteristic “fried eggs” appearance, which are visible via stereomicroscope (Razin *et al*., 1998; Quinn *et al*., 2013; Calcutt *et al*., 2018).

Mastitis caused by *Mycoplasma bovis* results in economic losses from reduction in milk production, reduced quality of milk, diagnosis and treatment costs, deaths and culling losses (Kauf *et al*., 2007; Maunsell *et al*., 2011). In the USA, the financial losses triggered by *Mycoplasma bovis* mastitis were around $108 million per year with morbidity rates in approximate to 70% of a herd (Rosengarten and Citti, 1999). *Mycoplasma* mastitis characterized by several milk changes including, watery secretion with sandy flakes, yellowish brown secretions, purulent milk with cottage cheesy appearance (Bushnell, 1984). *Mycoplasma* mastitis might be transmitted via several routes, including dissemination of infection during milking time, importation of infected animals from outside the herd, internal transmission from extamammary *Mycoplasma* infection and presence of asymptomatic carrier to mastitis (Fox *et al*., 2005). Since, *Mycoplasma* mastitis is largely incurable by antimicrobial drugs with ineffectiveness of experimental vaccines against *Mycoplasma* mastitis, segregation and culling of infected animals is the core of *Mycoplasma* mastitis control strategy (Ross, 1993; Maunsell *et al*., 2011 and Fox, *et al*., 2005). Although *Mycoplasma bovis* was identified in Egypt decades ago, the prevalence of *Mycoplasma bovis* mastitis is not frequently investigated, as *Mycoplasma* is overlooked in many laboratories in mastitis cases owing to the lack of specialized techniques and required facilities needed for *Mycoplasma* detection, in addition to the difficulty of the in-vitro cultivation of *Mycoplasma*. This study was designed to monitor the prevalence of *Mycoplasma bovis* in bovine mastitis milk in different governorates in Egypt. A through bacteriological and molecular investigation of bovine mastitis milk from different dairy herds.

2. Materials and methods

2.1. Collection of samples:
A total of 703 clinical mastitis milk samples were collected from different dairy farms located in different governorates in Egypt including, Giza (227), Alexandria (357), Dakahlia (78), Buhayrah (27), Ismailia (14). Samples were collected under aseptic condition from cattle suffering from mastitis with changes in milk characters as bloody and chocolate milk with consistency ranging from watery to thick and colostrum like then submitted to *Mycoplasma* isolation using conventional cultural technique followed by molecular identification of *Mycoplasma bovis* using PCR targeting *mb-mp 81* gene of *Mycoplasma bovis*.

2.2. Isolation of Mycoplasma from mastitis milk using conventional cultural method:
It was performed according to Nicholas *et al*., (2008), Hazelton *et al*., (2018). *Mycoplasma* isolation from milk samples was done by using, *Mycoplasma* agar (Oxoid CM0401) and broth (Oxoid CM0403) supplemented with *Mycoplasma* selective supplement G (Oxoid SR0059) and 0.2% w/v deoxyribonucleic acid sodium salt from calf thymus (Sigma-Aldrich D1501). 0.1 ml of milk was inoculated into 5 ml *Mycoplasma* broth followed by incubation for 7 days at 37º c in a candle jar with elevated CO2 levels, and examined for growth daily then subculturing is done into broth and plates.
Plates were examined using stereomicroscope to detect the characteristic fried egg colonies. Suspected samples were subcultured three times before being rejected as negative samples.

2.3. Biochemical identification of Mycoplasma bovis isolates recovered from examined milk samples:

It was performed according to Freundt et al. (1973), Erno and Stepkovits (1973) by application of Digitonin sensitivity test, Glucose fermentation test and Arginine hydrolysis test.

2.4. Molecular identification of Mycoplasma bovis isolates:

Positive culture isolates were submitted to Mycoplasma bovis specific PCR using forward primer mb-mp 81 F 5’-TATTGGATCAACTGCTGGAT-3’ and reverse primer mb-m 81 R 5’-AGATGCTCCACTTATCTTAG-3’ targeting mb-mp 81 gene with 447 bp amplicon size (Foddai et al., 2005). Reference strains Mycoplasma bovis NCTC 10131 and reference strain Mycoplasma bovigenitalium NCTC 10122 were used as control positive and control negative, respectively.

2.4.1. Extraction of DNA from Mycoplasma isolates:

Extraction of DNA from Mycoplasma isolates was done by using boiling method according to Queipo-Ortuno et al., (2007) with the following modifications; one ml of Mycoplasma broth culture was centrifuged at 12,000 rpm for 10 minutes. Supernatant was discarded and pellet was washed twice by using 1x tris EDTA (TE) buffer at 10,000 rpm then supernatant was discarded. 100 µl 1x TE buffer was added to the pellet followed by boiling in a heat block for 20 minutes then cooling at -20°C freeze for 10 minutes followed by centrifugation at 12,000 rpm for 10 minutes then supernatant was collected into a new microcentrifuge tube and stored at -20°C for use.

2.4.2 Amplification and cycling protocol of PCR:

It was performed according to specific author Foddai et al., (2005) and Dream Taq green master mix (Thermo scientific™) kit code No. K1081. PCR amplification was carried out on a T100 thermal cycler (Bio-rad) in a total reaction volume of 20 µl containing 10 µl dream Taq green master mix (Thermo scientific™, K1081), 0.5 µl of each forward and reverse primers, 5µl Nuclease free molecular biology grade water and 4 µl test DNA at thermal profile of 1 cycle of 94°C for 4 min; 30 cycles of 94°C for 60 s, 54°C for 60 s, 72°C for 60 s; 1 cycle of 72°C for 10 min; and a final hold at 4°C until stop.

2.4.3. Detection of PCR products:

It was performed according to Sambrook et al., (1989). Amplicons were detected by electrophoresis on 2% agarose gel stained by ethidium bromide and examined by gel documentation system (Bio-Rad).

3. RESULTS

3.1 Isolation of Mycoplasma from Mastitis milk samples:

Sixty-three Mycoplasma isolates were recovered from 703 mastitis milk samples in a percentage of 8.96% (Figure 1) with the isolates showing the characteristic fried egg colonies on Mycoplasma agar (Figure 2). Out of the 63 Mycoplasma isolates, 23/227 (10.13%) were obtained from Giza, 17/357 (4.76%) from Alexandria, 18/78 (23.07%) from Dakahlia, 1/27 (3.70%) from Al-Buhayrah and 4/14 (28.57%) from Ismailia (Table 1, Figure 3).

3.2. Biochemical identification of Mycoplasma isolates recovered from mastitis milk samples:

All examined Mycoplasma isolates were digitonin sensitive with >5 mm zones of growth inhibition in digitonin disc diffusion assay. Furthermore, Isolates were negative for
3.3. Molecular identification of Mycoplasma bovis by PCR targeting mb-mp 81 gene:

Out of the 63 Mycoplasma isolates, 53 were identified as Mycoplasma bovis by PCR targeting Mycoplasma bovis mb-mp 81 gene representing 84.12% of Mycoplasma isolates (n=63) and 7.53% from the total examined mastitis milk samples (n=703) (Figure 4).

3.4. Incidence of Mycoplasma bovis from different governorates in Egypt:

Fifty-three Mycoplasma bovis were recovered from 703 clinical mastitis milk samples collected from different dairy farms located in different governorates in Egypt as the following, 17/227 (7.48%) were obtained from Giza, 13/357 (3.64%) from Alexandria, 18/78 (23.07%) from Dakahlia, 1/27 (3.70%) from Al-Buhayrah and 4/14 (28.57%) from Ismailia (Table 2, Figure 5).

Table 1: Incidence of Mycoplasma spp. recovered from bovine mastitis milk samples from different governorates in Egypt.

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Number of samples</th>
<th>Number of positive samples</th>
<th>Percentage of positive samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giza</td>
<td>227</td>
<td>23</td>
<td>10.13</td>
</tr>
<tr>
<td>Alexandria</td>
<td>357</td>
<td>17</td>
<td>4.76</td>
</tr>
<tr>
<td>Dakahlia</td>
<td>78</td>
<td>18</td>
<td>23.07</td>
</tr>
<tr>
<td>Buhayrah</td>
<td>27</td>
<td>1</td>
<td>3.70</td>
</tr>
<tr>
<td>Ismailia</td>
<td>14</td>
<td>4</td>
<td>28.57</td>
</tr>
<tr>
<td>Total</td>
<td>703</td>
<td>63</td>
<td>8.96**</td>
</tr>
</tbody>
</table>

* Percentage in relation to number of examined samples in each row.

**Percentage in relation to Total number of examined samples (n=703).

Table 2: Incidence of Mycoplasma bovis isolated from bovine mastitis milk samples from different governorates in Egypt.

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Number of samples</th>
<th>Number of positive samples</th>
<th>Percentage of positive samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giza</td>
<td>227</td>
<td>17</td>
<td>7.48</td>
</tr>
<tr>
<td>Alexandria</td>
<td>357</td>
<td>13</td>
<td>3.64</td>
</tr>
<tr>
<td>Dakahlia</td>
<td>78</td>
<td>18</td>
<td>23.07</td>
</tr>
<tr>
<td>Buhayrah</td>
<td>27</td>
<td>1</td>
<td>3.70</td>
</tr>
<tr>
<td>Ismailia</td>
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<td>4</td>
<td>28.57</td>
</tr>
<tr>
<td>Total</td>
<td>703</td>
<td>53</td>
<td>7.53**</td>
</tr>
</tbody>
</table>
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**Fig. 1.** Incidence of *Mycoplasma spp.* isolated from bovine mastitis milk samples in Egypt.

**Fig. 2.** Fried egg appearance of *Mycoplasma* colonies on *Mycoplasma* agar.

**Fig. 3.** Incidence of *Mycoplasma spp* isolated from bovine mastitis milk samples from different governorates in Egypt.
Fig. 4. Agarose gel electrophoresis of PCR amplified products of *Mycoplasma bovis mb-mp 81* gene from isolates. Lane M: Marker (GeneRuler100 bp DNA ladder, Thermo scientific™), Lane 1: Positive control (*Mycoplasma bovis* NCTC 10131), Lane 2: negative control (*Mycoplasma bovigenitalium* NCTC 10122), Lanes 4-11, 13: Positive for mb-mp 81 gene with 447 bp amplicon, lanes 3, 12: negative mb-mp81.

Fig. 5. Incidence of *Mycoplasma spp* isolated from bovine mastitis milk samples from different governorates in Egypt.

4. DISCUSSION

Mastitis, the dairy cattle’s most costly disease, remains an on-going issue for dairy industry (Barkema *et al*., 2009). Many *Mycoplasma* species exist but few are associated with mastitis, with more than 50% of *Mycoplasma* mastitis cases, caused by *Mycoplasma bovis* (Wen *et al*., 2019). In the current study, we monitor the prevalence of *Mycoplasma bovis* in bovine mastitis milk in Egypt through bacteriological and molecular investigation of bovine clinical mastitis milk from different dairy herds in different governorates in Egypt. The results revealed the recovery of sixty-three *Mycoplasma* isolates out of the 703 (8.96%) examined clinical mastitis milk with colonies giving the characteristic “fried egg appearance” which is attributed to the embedment of the central portion of the colony into the agar with a surrounding zone of surface growth (McVey *et al*., 2013). In addition, isolates were positive for digitonin sensitivity test with ≥ 5 zones of growth inhibition. This is attributed the interaction of Digitonin with sterol forming a complex, which interrupts the exogenous uptake of sterol by *Mycoplasma*, and so, killing of *Mycoplasma* (Boonyayatra 2012). Out of the sixty-three *Mycoplasma* isolates, 53 were identified as *Mycoplasma bovis* by PCR targeting mb-mp 81 gene of *Mycoplasma bovis* adapted from (Foddai *et al*., 2005) in a percentage of 7.53%. From the 53 *Mycoplasma bovis* isolates, 17/227 (7.48%) were obtained from Giza, 13/357 (3.64%) from Alexandria, 18/78 (23.07%) from Dakahlia, 1/27 (3.70%) from Al-Buhayrah and 4/14
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(28.57%) from Ismailia. Comparing with other results, Al-Farha *et al.*, (2017) reported slightly lower percentage 6.2% in South Australia with a percentage of 6.2%. While Arcangioli *et al.*, (2011) and Surýnek *et al.*, 2016 reported that *Mycoplasma bovis* was not isolated from the Southeast of France and the Czech Republic, respectively. Filioussis *et al.*, 2007 reported the isolation of *Mycoplasma bovis* in a slightly higher percentage 8.2% in Northern Greek. While Karahan *et al.*, (2010) reported significantly higher percentage 21.1% from eastern Turkey. In Egypt, Gad *et al.*, (1987), Abd El-Rahman and Saad (1993), Hassan and El Rashidy (2002), Hassan and Essmail (2004), Darwish *et al.*, (2015) reported higher percentages of *Mycoplasma bovis* isolation from clinical mastitis milk: 70.83%, 14.37%, 42.35%, 52% and 11.68% respectively. While El-Gamal *et al.*, (1999) obtained lower percentage 2.7%. This variation in the prevalence of *Mycoplasma bovis* upon local and global levels may be attributed to several causes, including herd sizes as explained by Arcangioli *et al.*, 2011; this previous publication attributed the sporadic nature of *Mycoplasma* mastitis in France to the small size of the herds together with the management practices applied within the herd. In addition, *Mycoplasma* can be transmitted by infected milk, milk clusters or milkers’ hands (Calcutt *et al.*, 2018), specially that infected animals might turn to a symptomatic shedders of *Mycoplasma* without showing any clinical signs. Moreover, the introduction of new animals from outside of the herd serves as a major risk factor for occurrence of *Mycoplasma* mastitis outbreaks (Punyapornwithaya *et al.*, 2010).

5. CONCLUSION

*Mycoplasma* mastitis caused by *Mycoplasma bovis* is a perilous problem for dairy industry throughout the world. Our research findings disclosed that *Mycoplasma bovis* mastitis is quite common in Egypt. Accordingly, Large-scale epidemiological investigations should frequently be carried out to withstand the prevalence of the *Mycoplasma bovis* mastitis infection in Egypt. In addition, restrict prevention and control strategies should be applied combined routine bacteriological examination for *Mycoplasma* spp. for the newly purchased animals prior to their introduction to the herd.

6. REFERENCES


Ashraf et al. (2019). BVMJ-36 (2): 57-65


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Karahan, M., Kalin, R., Atil, E. and Cetinkaya, B. (2010). Detection of *Mycoplasma bovis* in cattle with mastitis and respiratory problems in eastern Turkey. Veterinary Record, 166(26), 827–829


