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Molecular Typing of Mycoplasma Species Recovered from Bovine Mastitis

[1]Kamelia M. Osman, [2]K.A. Abd El-Razik, [1]E.E. Barbar, [3]Dina Y.H. ELShafey and [2]Amany A. Arafa

[1]Departement of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[2]Department of Animal Reproduction and AI, National Researche Centre, Giza, Egypt
[3]Department of Mycoplasma, Animal Health Research Institute, Dokki, Giza, Egypt

Abstract: Mycoplasma bovine mastitis is a highly contagious disease that results in milk loss and culling of infected animals. Therefore the aim of this work was to focus on the diagnosis of masitic mycoplasma including California Mastitic Test (CMT), indirect ELISA (iELISA), Polymerase Chain Reaction (PCR) and bacteriological isolation. Beside emphathize the importance of Mycoplasma in subclinical mastitis. A total of 236 apparently normal quarter milk samples were examined for mastitis using CMT.The incidence of subclinical mastitis was 32.62 and 26.25% in cows and buffaloes, respectively. Examination of cows and buffaloes for *Mycoplasma* revealed 9.09 and 0% from subclinical mastitic animals and 14.73 and 14.29%, from clinically mastitic animals, respectively. Identification of the isolated *Mycoplasma* revealed more than one species. The most important was *M. bovigenitalium* (40.74% from the total isolates), then *M.arginini* (37.04%), *M. bovis (18.52*%) and. *M.bovirihinis* (3.70%). Examination of 46 and 6 udder tissue samples from buffaloes and cows for Mycoplasma revealed 0 and 3.33% *M. arginini*, respectively. A serological study of 45 milk samples by ELISA test revealed 22.22% positive samples to *M. bovis* and 37.77% samples to *M.bovigenitalium* while examination of 10 serum samples revealed 20% as *M. bovis*. Application of the PCR specific to *M. bovis* for the total isolates recovered from mastitic milk samples revealed 18.52% as *M. bovis*. Finally there were significant increase in the levels of lysozyme and nitric oxide in subclinical and clinical Mycoplasma positive samples compared with negative and normal samples.

Key words: Mastitis · Mycoplasma · Isolation · PCR · Immunology

INTRODUCTION

Mycoplasma bovine mastitis is a highly contagious disease that results in milk loss and culling of infected animals. Several species of *Mycoplasma* have been associated with mastitis (*M. bovis, M. californicum, M. canadense, M. bovigenitalium, M. alkalescens, M. arginini, M. bovihirnis* and *M. dispar*) [1].

Like many other mycoplasmas*, M. bovis* can be found as a commensal in the respiratory and genital tracts. It is a frequent cause of severe mastitis, arthritis and less frequently genital infections. It is estimated to cost the US dairy industry over US $100 million annually, with infection rates of up to 70 per cent in some herds [2]. Individual cows may have persistent, ongoing mastitis problem and various treatment products prove ineffective [3].

Laboratory diagnosis of *Mycoplasma* species using conventional methods are usually difficult and time consuming. Therefore, other techniques such as the PCR are generally preferred [4,5]. In addition the mycoplasmas can be detected even if the organs or the broth cultures are contaminated with bacteria. A good target is for example the 16S ribosomal RNA gene [6]. Specific nested PCR had been developed, in which the preservative-treated milk samples can also be examined [7].

The present investigation was planned to throw light on application of CMT for detection of subclinical mastitis among the apparently healthy cows and buffaloes. Special interest was given for isolation of *Mycoplasma* species from milk samples and udder specimens of subclinical and clinical mastitic animals Also, identification of the isolated strains of *Mycoplasma* species by the traditional methods, estimation of the concentration of lysozyme and nitric oxide in subclinical and clinical mastitic milk samples and molecular typing of *Mycoplasma* strains using PCR and rapid detection of *Mycoplasma* in mastitic milk by ELISA were other targets.

Corresponding Author: Khaled A. Abd Elrazik, Department of Animal Reproduction and AI, National Researche Centre, Postal code: 12622, Dokki, Giza, Egypt

MATERIALS AND METHODS

Collection of Samples

Milk Samples: Milk samples were collected from 170 cows and 26 buffaloes reared at El-Nobaria area of Alexandria governorate during the period from 7/2005 to 7/2006 under possible hygienic conditions as outlined by [8]. Each milk sample was divided into 2 parts, each in a separate sterile McCartney bottle. One was for bacteriological examination and the second was used to separate milk serum by centrifugation at 6000xg for 15 minutes. The extracted milk serum was stored at - 70°C until assayed for lysozyme and nitric oxide.

udder Tissue Samples and Blood Samples: Samples were collected from slaughtered cows and buffaloes at the abattoir [8].

Examination of Milk Samples: Milk samples were examined using the California mastitis test (CMT) according to [9] followed by cultivation of *Mycoplasma* from udder tissue and milk samples [10], followed by purification of the isolates [11], differentiation between *Mycoplasma* and *Acholeplasma* isolates using the Digitonin sensitivity test [12]. Biochemical characterization using glucose fermentation and arginine hydrolysis tests [13, 14], Urease [15] were carried out.

Sereotyping of *Mycoplasma*: Sereotyping of *Mycoplasma* was carried out by growth Inhibition test (GIT) according to [16].

Estimation of the Concentration of Nitric Oxide and Lysozyme in Mastitic Milk Samples: a total of 47 milk samples were selected from the total examined cows and classified into 5 groups: Control non-mastitic animals (n=10), Clinical *Mycoplasma* negative mastitic milk samples (n=10), Subclinical *Mycoplasma* negative mastitic milk samples (n=10), Clinical *Mycoplasma* positive mastitic milk samples (n=10), Subclinical *Mycoplasma* positive mastitic milk samples (n=7). Measurement of nitric oxide in milk samples was performed according to [17] and Detection of lysozyme was carried out according to [18].

PCR: Extraction of DNA from the bacterial pellets was according to [19] and running of PCR was according to [20]. The amplified product size was equals to 442bp.

Enzyme Linked Immunosorbent Assay Protocol (i-ELISA): Using Whole cell antigen according to [21] and proceeded according to [22].The samples were read at wave length of 405nm.

Statistical Analysis: Statistical analysis were carried out according to [23].

RESULTS

Incidence of Subclinical and Clinical Mastitis: As shown in Table 1 and 2, the incidence of subclinical mastitis was 32.62 and 26.25% in cows and buffaloes, respectively.

The incidence of mastitis including subclinical mastitis was higher in cattle than buffaloes.

Table 3 showed that the incidence of *Mycoplasma* mastitis was common in one quarter in cattle and buffaloes, while it is rare in all quarters in cattle and was not seen in buffaloes.

Incidence of *Mycoplasma* Species: Table 4 indicates that in the subclinical stage the total recovered *Mycoplasma* species isolates were 7 (9.1%) from the cows while no *Mycoplasma* species isolates were recovered from the buffaloes.

On the other hand, the incidence of *Mycoplasma* species isolates that were isolated from the clinically affected quarters milk samples of cows and buffaloes were 19 (14.73%) and one (14.29%), respectively.

Higher incidence of mycoplasma species were isolated from subclinical mastitis in cattle than in buffaloes.

Biochemical and Serological Identification of *Mycoplasma* Isolates: Table 5 illustrates that *M. bovigenitalium* were isolated in high incidence (40.74%), followed by *M. arginini* (37.04%), *M. bovis* (18.52%) and *M. bovirihinis* (3.70 %) from mastitic milk samples. On the other hand, Table 6 illustrated that *M. arginini* isolates were the only *Mycoplasma* species isolated from the udder of cows with a percentage of 33.3%.

Lysozyme and Nitric Oxide Concentrations in the Mastitic Milk of Cows: Table 7 indicates that the concentration of the lysozyme was significantly ($P<0.01$) higher in case of the Mycoplasma clinically mastitic group Moreover, the subclinical *Mycoplasma*

Table 1: Incidence of mastitis among the examined lactating cows and buffaloes

		Apparently normal animals		Clinically mastitic animals	
Animal species	No. of examined lactating animals	No.	%	No.	%
Cows	170	59	34.70	111	65.29
Buffaloes	26	20	76.92	6	23.07

Table 2: Incidence of the subclinical mastitis among the examined apparently normal animals as detected by CMT

		Subclinical mastitic quarters milk samples		Normal quarters milk samples	
Animal species	No. of apparently normal quarters milk samples	No.	%	No.	%
Cows	236	77	32.62	159	67.37
Buffaloes	80	21	26.25	59	73.75

% was calculated according to the total number of the examined apparently normal milk samples

Table 3: The distribution of infected quarters in the subclinically mastitic animals

	Subclinically mastitic cows		Subclinically mastitic buffaloes	
Number of affected quarters	No.	%	No.	%
One quarter	48	81.35	19	95.00
Two quarters	5	8.47	1	5.00
Three quarters	5	8.47	0	0.00
Four quarters	1	1.69	0	0.00
Total	59	99.98	20	100.00

% was calculated according to the total number of the examined apparently normal milk samples

Table 4: Incidence of *Mycoplasma* species recovered from subclinical and clinical mastitic milk samples

	On quarter milk sample bases					
	Subclinically mastitic			Clinically mastitic		
Animals	No. of examinedQMS	No. of positive QMS	%	No. of examined QMS	No. of positive QMS	%
Cows	77	7	9.10	129	19	14.73
Buffaloes	21	0	0.00	7	1	14.29

QMS= Number of quarters milk samples

% was calculated according to the total number (No.) of examined quarter milk samples

Table 5: Biochemical and serological identification of 27 *Mycoplasma* isolates recovered from mastitic milk samples

Types of *Mycoplasma* isolates	D.S	U.A	G.F	A.H	No.of +ve* isolates	%
M. bovis	+	-	-	-	5	18.52
M. bovigenitalium	+	-	-	-	11	40.74
M. arginini	+	-	-	+	10	37.04
M. bovirihinis	+	-	+	-	1	3.70
Total					27	100.00

D.S. = Digitonin sensitivity. U.A. = Urease activity.

G.F. = Glucose fermentation. A.H. = Arginine hydrolysis.

+ve* number of isolates positive to specific antisera by Growth inhibition test.

Table 6: Biochemical and serological identification of 2 *Mycoplasma* isolates recovered from udder tissues

Animal species	Number of examined udder tissue samples	D.S	U.A	G.F	A.H	No.of +ve* isolates	%
Cows	6	+	-	-	+	2	33.30
Buffaloes	46						0.00
Total	52						3.80

D.S. = Digitonin sensitivity. U.A. = Urease activity. G.F. = Glucose fermentation. A.H. = Arginine hydrolysis.

+ve* number of isolates positive to specific antisera by Growth inhibition test.

Table 7: Lysozyme and nitric oxide concentrations in the mastitic cow's milk samples (Mean + S.D.)

Group	Nitric oxide (µMol)	Lysozyme (µg/ml)
Control non-mastitic animals (n=10)	5.566^{a}±1.28	96.341^{a}±49.92
Clinically negative for *Mycoplasma* (n = 10)	10.620^{c}±0.31	144.396^{b}±0.24
Subclinically negative for *Mycoplasma* (n = 10)	9.320^{b}±0.20	107.254^{a}±0.21
Clinically positive for *Mycoplasma* (n = 10)	12.813^{d}±0.15	199.211^{d}±22.96
Subclinically positive for *Mycoplasma* (n = 7)	13.428^{e}±0.33	171.562^{c}±0.30

Different superscripts a, b and c are significantly different at P> 0.01

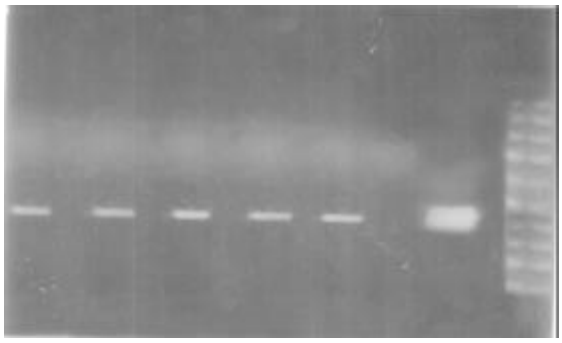

Photo 1: Agarose gel electrophoresis showing amplification of the 442 bp fragment of *M. bovis* DNA, lanes 4-8. Lane 1 the 100 bp DNA ladder. Lane 2, positive control (*M. bovis* reference strainDNA) and lane 3 showing no amplification of *M. boviginitalium* reference strainDNA

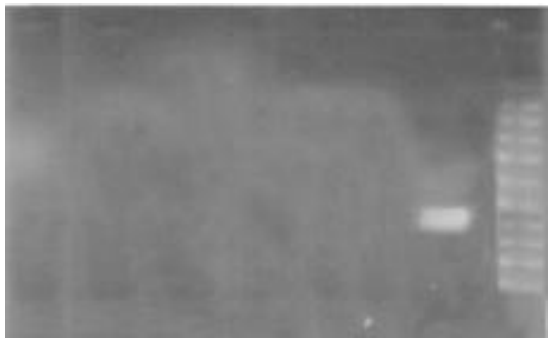

Photo 2: Agarose gel electrophoresis showing amplification of the 442 bp fragment of of *M. bovis* reference strain DNA (lane 2). Lane 3 showing no amplification of *M. boviginitalium* rference strainDNA. Lane 1 showing the 100 bp DNA ladder. Lanes 4-13 showing no amplification of the extracted DNA of other *Mycoplasma* isolates

positive group recorded a significant increase of lysozyme level in comparison with the non-mastitic and subclinical mastitic

***Mycoplasma* Negative Groups:** The nitric oxide concentration was significantly higher in the subclinically and clinically *Mycoplasma* positive mastitic groups than in the non-mastitic, subclinically and clinically mastitic *Mycoplasma* negative groups.

PCR: PCR was used for amplification of 442-bp fragment of *M. bovis* from the extracted DNA of *Mycoplasma* species isolates. The specificity of the oligonucleotide primers was confirmed by the positive amplification of the 442 bp fragment from the extracted DNA of *M. bovis*. On the other hand, no amplification was observed with the extracted DNA of *M. boviginitalium* standard strain as shown in photo 1.

The results observed also in photo 2 Revealed positive amplification of 442 bp fragment of *M. bovis* from the extracted DNA of the standard strain of *M. bovis* lane 2 while lanes 4 to 13 showed negative amplification of the 442 bp fragment of *M. bovis* from the extracted DNA of other *Mycoplasma* isolates. Lane 1 showed the 100 bp ladder.

Table 8: Serological detection of *M. bovis* and *M. bovigenitalium* in milk whey and serum samples by the indirect ELISA

		ELISA used to detect antibodies against			
		M. bovis		*M. bovigenitalium*	
Samples	No. of examined samples	+ve	%	+ve	%
Milk whey	45	10	22.22	17	37.77
Serum	10	2	20.00	0	0.00

+ve= samples are considered positive when their O.D. (optical denisty) is more than double the negative control

Table 9: Comparison between the traditional methods and ELISA for the detection of *Mycoplasma* species in milk samples

			ELISA used to detect antibodies against							
			M. bovis		*M. bovigenitalium*		*M. bovis* & *M. bovigenitalium*			
No. of examined by milk samples Traditional method	Milk samples taken from cases of		No.	%	No.	%	No.	%	Total positive	%
Mycoplasma Positive	Subclinical	3	0	0.00	1	33.3	2	66.70	5	100.00
(n=5; 11.1%)	Clinical	2	1	50.00	0	0.0	1	50.00		
Mycoplasma Negative	Subclinical	22	1	4.50	5	22.7	4	18.20	15	37.50
(n=40; 88.9%)	Clinical	18	1	5.60	4	22.2	0	0.00		
Total (n=45)		45	3	6.70	10	22.2	7	15.60	20	44.40

ELISA: Table 8 indicates that in the detection of the antibodies against *M. bovis* and *M. bovigenitalium* in the 45 milk whey samples examined. 10 (22.2%) samples were positive for *M. bovis* and 17 (37.7%) samples were positive for *M. bovigenitalium*. On the other hand, the detection of the antibodies in serum samples were positive in 2 (20%) samples only against *M. bovis* from the 10 examined samples.

Table 9 showed that all the Mycoplasma bacteriologically positive whey samples (5) were also ELISA positive (1 *M. bovis*, 1 *M. Bovigenitalium*, 3 mixed infection with both).

The indirect ELISA detected 15 (37.5%) positive samples for *Mycoplasma* differentiated into: *M. bovis* 2 samples (5%), *M. bovigenitalium* 9 samples (22.5%) and a mixed infection of *M. bovis* and *M. bovigenitalium* 4 samples (10%).

DISCUSSION

Mastitis caused by mycoplasmas, is mainly by *M. bovis*. The inflammation can be acute, chronic or subclinical. Most infections by *M. bovis* spread from cow to cow and from cow to calf. *Mycoplasma* mastitis is an endemic problem in large dairy herds as it causes considerable losses due to the decrease of milk production, diseases in calves and reproduction disorders [24].

Recently, [25] 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 [26]. Furthermore, [25] reported rather surprisingly that 26.5 % of clinical mastitic samples and 38.6 % of subclinical cases produced no bacterial growth.

Diagnosis of *Mycoplasma* infection in milk depends primarily upon microbiological culture of udder secretions. In the present investigation, *Mycoplasma* species were isolated from milk samples of subclinical and clinical mastitic cows in an incidence of 9.1 and 14.73%, respectively; while in buffaloes, the incidence was 0 and 14.29% in the subclinical and clinical mastitic animals, respectively. In the udder tissue, the isolation incidence of *Mycoplasma* species was found to be 33.30% in the cow and buffaloes, respectively.

In this study, the involvement of more than one quarter, sometimes all four, is considered to be a typical clinical characteristic of mycoplasma mastitis as reported by [27].

Microbiological testing is primarily used to diagnose *M. bovis* mastitis within individuals or herds [28]. Specialized growth media is necessary to culture *M. bovis* [29]. Cows can become intermittent shedders of *M. bovis*. It has been shown that as many as 40% of cows can shed <100 CFU/ml *M. bovis* in the milk [30]. Therefore, the risk of false negative test results to a herd can be problematic. Conversely, the risk of false positive test results is reduced in view of the fact that non-pathogenic *Mycoplasma* species rarely cause mastitis [31].

The diversity usually encountered in the rates of Mycoplasmas isolations throughout the world could be attributable to the various rates of *Mycoplasma* shedding related to stages of infection in infected udders [32] and the sensitivity of the bacteriologic procedure used to detect *Mycoplasma.* Large numbers of mycoplasmas are usually present in samples from clinical cases of mastitis, but only a few organisms may exist in samples from chronic or carrier cows [33]. Most mycoplasmas isolated from bulk tank milk and cow milk samples are pathogenic but some may be *Acholesplasma laidlawii*, a common nonpathogenic saprophytic contaminant frequently found in the dairy environment and the teat skin [33]. Therefore, speciation of *Mycoplasma*-like colonies is recommended. Digitonin inhibition of sterol metabolism by *Mycoplasma* was reported as a practical and easy method to discriminate between isolates of *Mycoplasma* and *A. laidlawii* from milk [34].

Several species of *Mycoplasma* can cause mastitis in dairy cows [1]. Disease detection at the herd level is usually made by isolation of mycoplasmas from either bulk tank milk or samples from cows with clinical mastitis. It is not uncommon to isolate mycoplasmas in mixed culture [35].

Recent US studies, suggested that 1 to 6% of the dairy herds had at least 1 cow with Mycoplasma-induced mastitis [31, 36, 37]. Sampling bulk tank milk only once may give an underestimation of the prevalence, due to intermittent shedding [31]; therefore, multiple sampling should be performed [38]. Sensitivity of a single culture of bulk milk samples for *Mycoplasma* spp. ranges from 33 to 59% [39]. The Mycoplasma spp. that were found in the present study were both pathogenic and can cause mastitis.

As the direct culture is the most effective and sensitive method for detecting mycoplasma, but it is also the most difficult and time consuming [40] and it cannot reliably detect some fastidious strains of *Mycoplasma*, making it less than 100% effective. Therefore, there

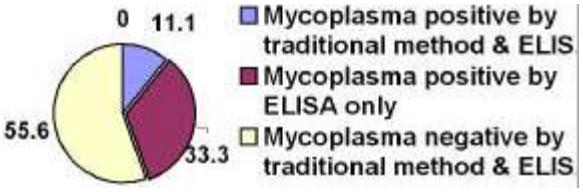

Fig. 1: A comparison on the efficacy of isolation of *Mycoplasma* spp. using the Traditional culture technique against the ELISA.

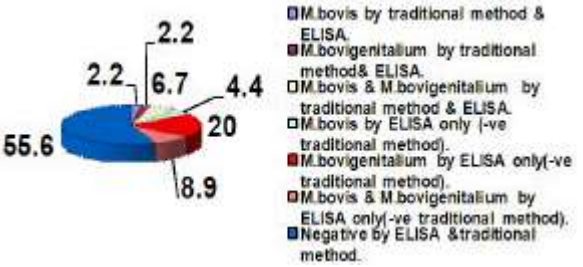

Fig. 2. A comparison on the efficacy of isolation and differentiating between the *Mycoplasma* spp. using the Traditional culture technique against the ELISA.

are a wide variety of indirect test methods available, including DNA probes, DNA fluorochrome staining, autoradiography, ELISA, immunofluorescence and specific biochemical assays. These tests are faster than direct culture and they can detect the fastidious, difficult to cultivate strains that are occasionally missed by direct culture. However they lack the sensitivity of direct culture, requiring much higher levels of contamination for detection. As a result, they have more frequent false negatives than direct culture methods, potentially leaving researchers who rely solely on a single indirect test with a false sense of security [41].

One of the indirect tests, the ELISA, used in general practice for the identification of *Mycoplasma* infection was used in the present investigation to be compared with the Traditional culture technique. From Figure (1), it could be noticed that, when the ELISA technique was used to detect the *Mycoplasma* in the negative samples as indicated in the results reached when the Traditional culture procedure was used as a preliminary step in diagnosis, the ELISA was able to detect *Mycoplasma* in 10 (45.45%) samples from the 22 subclinical negative samples. In addition, 5 (27.77%) other isolations were recorded from the 18 negative clinical samples. The ELISA was also able to increase the level of specific identification of the different species as seen in Figure 2.

These came in agreement with that of [42] that assays that assess the presence of *anti - M. bovis* circulating antibodies offer an improved alternative, because they can identify animals which have been infected within a large herd even in the absence of shedding organisms and it is comparable with that of [43] as monoclonal antibody blocking enzyme-linked immunosorbent assay (B-ELISA) developed to detect antibodies to Mycoplasma bovis in cattle sera proved to be a valuable and reproducible tool in the Sero-diagnosis of *M. bovis* infection in cattle.

Diagnosing *M. bovis* is most commonly done by culture and subsequent serology to identify individual species. However, the use of DNA-based tests promises to be faster, more sensitive and more specific. The detection of *Mycoplasma* by PCR is based on the *in vitro* amplification of the highly-conserved 16S rRNA gene [7, 44]. PCR can amplify the target DNA sequence by as much as five orders of magnitude, thus potentially solving the two largest problems dealing with *Mycoplasma*: early detection and low numbers of organisms in the clinical samples. Detection levels as low as 5 CFU/ml in milk samples have been reported [7]. A rapid and sensitive procedure was developed by [45] to detect Mycoplasma bovis from milk samples where Mycoplasma cells were collected by selective binding to a monoclonal antibody, thus separating them from milk proteins and facilitating DNA extraction.

This investigation was able to detect and confirm a positive amplification of the 442 bp fragment from the extracted DNA of the 5 isolates of *M. bovis*. This specificity was observed when no amplification was observed with the extracted DNA of *M. boviginitalium* standard strain and the other species of *Mycoplasma* as characterized by the biochemical and serological tests (Photos 1, 2).

One means of decreasing the adverse effects of mastitis is to modulate the mammary gland immune system to preserve mammary epithelium integrity and, hence, the amount of milk production. Many researchers [46, 47] have tried to identify key inflammatory mediators involved in mastitis to develop therapeutic strategies to reduce the inflammatory reaction and prevent tissue damage. The results of [48] raise the possibility that interfering with NO production during mastitis may help to prevent tissue damage. also [49] results indicated that important amounts of NO are released during mammary inflammation driven by mastitis. [48] Suggested a possible link between NO and bovine mastitis. NO acts as an inflammatory mediator in this disease. Our results clearly showed that significant amounts of NO are released during *Mycoplasma*-induced mastitis. This came in agreement with that of [50].

The significant increase in milk lysozyme activity as recorded in the present investigation obtained from either subclinically and/or clinically mastitic cows was quite evident. This increase in the lysozyme concentration in the milk of mastitic animals was observed by [51,52].

It was concluded that, as no diagnostic tool is 100% accurate, it is suggested that PCR, molecular probe and IgG, IgM and IgA antibodies against peptide-specific mycoplasma should all be performed to gain the most accurate result.

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