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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 bovirihinis*. 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 revealed100% 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.

*Mycoplasm*a 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.[1] *Mycoplasma*s 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 *Mycoplasma*s, 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[1], 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.[4] 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. [5, 6] 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.[7-10] 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. 215milk 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 TAT 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] MMBsr-MN (52 - ACC ATG GGA GCT GGT AAT-32) and MBmr-MN-927 (52 - TTC TTA CTT CTA AAG TAT-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 $MgCl_2$, 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 t to Moussa and Shibl.[12]

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 highly contagious pathogen, and can be easily transmitted from cow-to-cow.[3] Recently, Bradley et al.[14] 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.[15] Furthermore, Bradley et al.[14] 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 1. Milk samples collected from different animal species

Animal species	No. of milk samples	No. of animals	Clinically mastitic		Apparently healthy	
			Milk samples	No. of animals	Milk samples	No. of animals
Cows	350	180	135	120	215	60
Buffaloes	90	30	20	10	70	20

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

Animal species	Udder status				Total
	Apparently healthy		Mastitic		
	No.	(%)	No.	(%)	
Cows	60	33.33%	120	66.67%	180
Buffaloes	20	66.67%	10	33.33%	30

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

Animal species	Subclinically mastitic quarters		Normal quarters		Total
	No.	(%)	No.	(%)	
Cows	70	32.56	145	67.44	215
Buffaloes	25	35.71	45	64.29	70

% 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. Incidence of *Mycoplasma* in subclinically and clinically mastitic cows' and buffaloes' quarter milk samples

Species	Quarter status					
	Subclinically mastitic			Clinically mastitic		
	Examined	Positive QMS		Examined	Positive QMS	
	QMS	No.	%	QMS	No.	%
Cows	70	5	7.14	135	16	11.85
Buffaloes	25	2	8	20	2	10

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

Types of *Mycoplasma* isolates	D.S	U.A	G.F.	A.H.	Positive isolates*(GIT)	
					No.	%
M. bovis	+	-	-	-	6	24
M. bovigenitalium	+	-	-	-	10	40
M. arginini	+	-	-	+	7	28
M. bovirihinis	+	-	+	-	2	8
Total		25	100			

D.S. = Digitonin sensitivity. U.A. = Urease activity.
G.F. = Glucose fermentation. A.H. = Arginin hydrolysis
Positive isolates: number of isolates positive to specific antisera by Growth inhibition test (GIT).

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

Animal species	No. of examined udder tissue samples	D.S	U.A	G.F.	A.H.	Positive isolates*	
						No.	%
Cows	30	+	-	-	-	3	10
Buffaloes	30					0	0

D.S. = Digitonin sensitivity. U.A. = Urease activity.
G.F. = Glucose fermentation. A.H. = Arginin hydrolysis
Positive number of isolates positive to specific antisera by Growth inhibition test.

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. bovirihinis*.

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 Wilson[3]. *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. bovirihinis*. These results agreed with that of Biddle et al.[4] and Kamelia et al[17].

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

Fig. 1. Agarose gel electrophoresis showing amplification of 928 bp fragments of specific for *M. bovigenitalium*

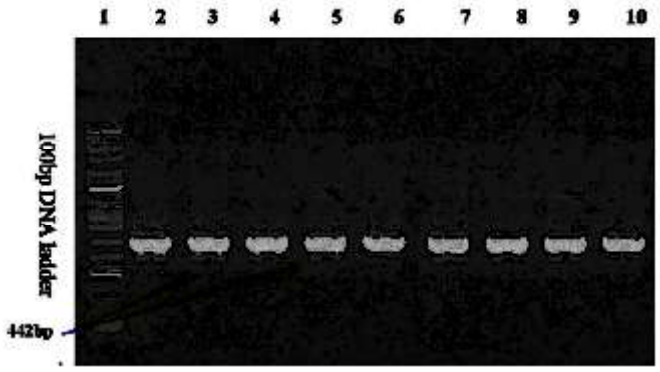

Fig. 2. Agarose gel electrophoresis showing amplification of 442 base pair fragments of which is conserved in *Mycoplasma bovis*

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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