Comparison between Molecular and Classical Techniques for Identification of *Mycoplasma* species Isolated from Mastitic Ruminants.

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Abstract: 165 cows and 19 buffaloes were examined to detect the *Mycoplasma* mastitis, the result revealed that 114 (69.59%) and 6 (31,57%) were clinically mastitic cows and buffaloes respectively while 51 (30.9%) and 13(68.42%) were apparently healthy cows and buffaloes respectively .On examining the apparently healthy cows and buffaloes, the result were 67 (32.84%) and 18 (34.61%) from subclinically mastitic cows and buffaloes respectively while 137(67.15%) and 34 (65.38%) fro apparently completely healthy. *Mycoplasma* were isolated in percentages of 8.9%, 5.5% from subclinically mastitic cow and buffaloes respectively and in percentages of 12.97%, 12.5% from clinically mastitic cows and buffaloes respectively. *M. bovis* was isolated from 8 (32%) and M. bovigenitalium was in percentage of 7 (28%) and the unidentified *Mycoplasma* was 10 (40%). Isolation of *Mycoplasma* from udder tissue in cows and buffaloes were in percentage of 2 (28.5%) in cows while no *Mycoplasma* isolates were obtained from buffaloes udder tissues. Application of PCR technique on these isolates and some negative samples, these were positive with percentage 100%. On the other hand, 192 sheep and 118 goats were examined. We found that in percentage of 82 (42.7%) and 43 (36.44) from sheep and goats respectively were clinically mastitic. Isolation of *Mycoplasma* was in percentage of 11 (13.41%) and 17 (39.53%) of sheep and goat respectively. Identification of these isolates revealed 8 (29%) was *M. agalactia* isolates and 20 (71%) was unidentified *Mycoplasma* spp. Application of PCR technique on *M. agalactia* isolates which identified by traditional techniques by use specific primers to *M. agalactia* revealed negative results but on using the primer specific to *M. bovis* to the same isolates, it was positive to all isolates 8 (100%).

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1. Introduction:

*Mycoplasma*s can cause many diseases in most species of the animals including human. In small ruminants, they can cause respiratory diseases, mastitis, arthritis, genital diseases and eye lesions. The most important of these diseases are Contagious Caprine Pleuropneumonia (CCPP) and Contagious Agalactia (CA) which are designated by the Office of International Epizooties as list B diseases because of their economic impact on livestock (Nicholas, 2002).

*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 (Sabry, 2004). *Mycoplasma*s are pleomorphic. They can easily change their shape and may appear as pear-shaped or circular with characteristic "fried egg" shaped colonies.

Mycoplasma bovine, ovine and caprine mastitis are a highly contagious disease that results in milk loss and culling of infected animals(Cree, 2002). Bradley et al. (2007) felt that the current literature did not warrant the widespread screening of mastitis cases for 'exotic' diagnoses, recommending that practitioners keep an open mind in the event of difficult to explain mastitis outbreaks and failures to respond to treatment

Because of their importance in veterinary medicine, and since infection spreads quickly once it established in a herd, it is very important that specific and rapid diagnostic procedures are developed for their detections. 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 immunodefence (Behrens. *et al*., 1994; Glew .*et al*., 2000).

The use of PCR made the identification of *M. bovis and M. agalactia* quicker compared to the conventional culture methods. In addition the

*Mycoplasma*s can be detected even if the organs or the broth cultures were contaminated with bacteria. (Cardoso *et al*., 2000 and Hirose *et al*., 2001). 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 (Kirk and Lauerman, 1994).

Incorrect identification by conventional diagnostic methods was recertified by PCR. Isolates from non-typical hosts, i.e. three *M. bovis* strains from small ruminants and two *M. agalactiae* strains from cattle, were characterized by sequencing the 16S and part of the 23S ribosomal RNA genes (Bashiruddin . *et al*., 2005a).

Consequently, this work was planned to clear out the comparison between classical methods and PCR technique in diagnosis of the false negative *Mycoplasma* isolates.

2. Materials and methods

Samples

A total number of 335 and 60 milk samples were collected from udder quarters of examined cows and buffaloes respectively. One hundred and thirty one milk samples were collected from114 clinically mastitic cows which had clinical signs of abnormal secretions of mammary glands containing clots or flakes, with udders showing swelling and hardness and 204 milk samples were collected from 51 apparent healthy one detected by palpation of udder and were subjected to California Mastitis Test (CMT) to detect subclinical mastitis. While a number of 8 milk samples were collected from 6 clinically mastitic buffaloes and 52 from 13 apparent healthy one. On the other hand a total number of 192 milk samples were collected from 82 mastitic and 110 apparent healthy ewes while a number of 118 milk samples were collected from 43 mastitic and 75 apparent healthy goats.

A total number of 80 udder tissues were collected belonged to cows, buffaloes, ewes and goat with numbers 10, 36, 13 and 20 respectively.

Cultivation of *Mycoplasma*: (Razin and Tully, 1983)

For udder tissues:A sample of the udder tissue was seared with a hot spatula to reduce surface contamination and about 0.5 g of the tissue was aseptically removed into a sterile mortar, cut into small pieces by a sterile scissor and grinned with sterile sand, after which 5 ml of broth medium was added.

A part of the mixture was directly plated (Plat 0) was made and about 0.2- 0.3ml was transferred into the broth (Broth 0). By the 3rd day plate (0) and broth (0) were transferred into PPLO plate (1) and broth (1) . On the sixth day, another plating was tried (Plate 3) beside an indirect plating (Plate 2) from the original broth on the 9th day. From Broth (1) an inoculum was made into another broth tube (Broth 2) from which a last plating (Plate 4) was made. The agar plates were inoculated at 37°C under reduced oxygen tension in a CO_2 incubator (5-10% CO_2). The plates were examined for suspected colonies after 48 hours under a stereomicroscope using oblique light and then daily up to 7- 10 days.

For milk samples:

About 1ml of a well mixed milk sample was inoculated in 5ml broth, and a part of the mixture was directly plated (Plat 0) was made and about 0.2-0.3ml was transferred into the broth (Broth 0). By the 3rd day plate (0) and broth (0) were transferred into PPLO plate (1) and broth (1). On the sixth day, another plating was tried (Plate 3) beside an indirect plating (Plate 2) from the original broth on the 9th day. From Broth (1) an inoculum was made into another broth tube (Broth 2) from which a last plating (Plate 4) was made. The agar plates were inoculated at 37°C under reduced oxygen tension in a CO_2 incubator (5-10% CO_2). The plates were examined for suspected colonies after 48 hours under a stereomicroscope using oblique light and then on every other day up to 7- 10 days. Filtration with a syringe filter was used to overcome contaminated samples or fatty samples.

Differentiation between *Mycoplasma* and *Acholeplasma* isolates using the Digitonin sensitivity test (Erno and Stipkovits, 1973 a, b and Freundt, 1973).

Filter paper discs containing 0.02 ml of a 1.5% ethanol solution of digitonin were placed on plates inoculated by the running drop technique with 0.1 ml of cultures. The plates were incubated at 37°C in a moist CO_2 incubator for 3 days, and then examined for the development of inhibition zones around the discs. *Mycoplasma* is digitonin sensitive, while *Acholeplasma* is digitonin resistant.

Biochemical characterization (Erno and Stipkovits, 1973a, b).

Stereotyping of *Mycoplasma* by Growth Inhibition Test (GIT) (Clyde et al., 1984)

Filter paper discs soaked in 20 ul of *Mycoplasma agalactia, Mycoplasma bovigenitalium* and *Mycoplasma bovis* antisera were placed on the inoculated plates by the running drop technique. The plates were incubated at 37°C in CO_2 incubator for 3-7 days. The interpretation was made by observing the zone of inhibition around the antisera discs.

Extraction of DNA by Chemical method using Phenol, *Chlorophorm, Isoamyl:* (Ausubel et al., 2003)

The centrifuged colony pellets were resuspended in 200 µl sterile distilled water to which 200 µl of lysis buffer was added. The mixture was vortexed efficiently then placed in a boiling water bath for 5 minutes. Equal volume of phenol/choloroform/isoamyl alcohol (25:24:1) was added and mixed by vortex then centrifuged at 12.000 rpm for10 minutes. After centrifugation, 3 layers were separated (an aqueous layer containing the DNA, a creamy layer containing the proteinous material, a rosy yellow layer containing phenol). The aqueous layer was transferred to a fresh tube at which an equal volume of phenol/ choloroform/isoamyl alcohol (25:24:1) was added and mixed by vortex then centrifuged at 12.000 rpm for 10 minutes, this step was repeated till the middle proteinous layer disappeared. The aqueous layer was transferred to a fresh tube with the addition of equal volume of choloroform/isoamyl alcohol (24:1) and mixed by vortex then centrifuged at 12.000 rpm for 10 minutes. The aqueous layer was transferred to a fresh tube with an equal volume of isopropanol was added and mixed gently. After storage at -20^0C for 1 hour, the DNA was pelleted at 12.000 rpm for 20 minutes, followed by washing with 70% ethanol and recentrifugation at 12.000 rpm for 10 minutes. The DNA pellet was dried and resuspended in 50µ l deionized distilled water.

Running of PCR: (Riffon et al., 2001)

The amplified reactions were performed in 50 µl volumes in micro amplification tubes (PCR tubes). The reaction mixture consisted of 10 µl (200 ng) of extracted DNA template from bacterial cultures, 5 µl 10x PCR buffer, 1 µl dNTPs (40 µM), 1 µl Ampli Taq DNA polymerase, 1 µl (50 pmol) from each primer pairs (each primer pair was used separately) and the volume of the reaction mixture was completed to 50 µl using deionized distilled water and the thermal cycler was adjusted as follows: For *M.bovis* initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing step at 52°C for 1 minute and extension at 72°C for 150 seconds. A final extension step was done at 72°C for 5 minutes. The PCR products were stored in the thermal cycler at 4°C until they were collected. The amplified product size equals to 227bp for *M. bovis* and loads 10 µl from PCR products.

For *M. agalactia* : initial denaturation at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing step at 57°C for 60 seconds and extension at 65°C for 60 seconds. A final extension step was done at 65°C for 10 minutes. The PCR products were stored in the thermal cycler at 4°C until they were collected.

Screening of PCR products by agarose gel electrophoresis (Sambrook et al., 1989):

The PCR products were electrophoresed in 2% agarose gel using Tris-borate EDTA buffer. The gel containing separated DNA was stained with ethidium bromide and examined under short wave UV transilluminator; Standard marker containing known fragments of DNA either 100 bp or 250 bp ladders was used.

Oligonucleotide primers used for amplification of DNA recovered from *Mycoplasma bovis* isolates:

The PCR amplicone was a part of *M. bovis* DNA sequence, with the following primer sequences these primers amplify a 227 bp fragment. (Yassin et al., 2004).

Forward
5\ GCA ATA TCA TAG CGG CGA AT 3\
Reverse
5\ TCT CAA CCC CGC TAA ACA TC 3\

Oligonucleotide primers used for amplification of DNA recovered from *Mycoplasma agalactia* isolates:The PCR amplicone was a part of *M. agalactia* DNA sequence, with the following primer sequences: these primers amplify a 375bp fragment. (Tola et al., 1996).

Forward
5\AAA GGT GCT TGA GAA ATG GC3\
Reverse
5\GTT GCA GAA GAA AGT CCA ATCA3\

3. Results and Discussion

From the results presented in Table (1) the mastitic cows were 114 out of examined 165 in a percentage of 69.1%. On the other hand the mastitic buffaloes were 6 out of 19 in a percentage of 31.6%, these results were in agreement with those reported by Osman *et al.* (2009). While results in table (2) represent that, out of 204 apparently normal quarters milk samples collected from 51 apparently healthy cows, subclinical mastitis reached 67 with an incidence of (32.84%), and 137 were negative for CMT with an incidence of (67.16%), On the other hand out of 52 apparently normal quarters milk samples of buffaloes, 18 were sub clinically mastitis with an incidence of (34.61%).These results nearly similar with those obtained by Kamelia *et al.* (2008)and Bachaya *et al*. (2005), who reported subclinical mastitis in 32.62 and 26.25% of cows and buffaloes, respectively.

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

Udder status / Species	Apparently healthy		Mastitic		Total
	No.	Percentage (%)	No.	Percentage (%)	
Cows	51	30.9%	114	69.1%	165
Buffaloes	13	68.4%	6	31.6%	19

Table (2): Incidence of the subclinical mastitis among the apparently normal quarters cow and buffaloes as detected by CMT.

Animal species	Subclinically mastitic quarters		Normal quarters		Total
	No.	Percentage (%)	No.	Percentage (%)	
Cows	67	32.8	137	67.2	204
Buffaloes	18	34.6	34	65.4	52

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

Results in table (3) demonstrated that 82 out of 192 examined ewes and 43 out of 118 examined goats were clinically mastitic (42.7% and 36.4% respectively).These results were in agreement with Iqbal *et al*. (2004) .

Table (3): Incidence of clinical mastitis and apparently normal sheep and goats.

Udder / Species	Apparently healthy		Mastitic		Total
	No.	Percentage (%)	No.	Percentage (%)	
Sheep	110	57.3%	82	42.7%	192
Goat	75	63.6%	43	36.4%	118

Table (4) illustrated the subclinical stage the total recovered *Mycoplasma* isolates were 6 (8.9%) from the cows while 1 (5.5%) *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 17 (12.97%) and one (12.5%) respectively, a similar results obtained by Gonzalez and Wilson (2003).

Table (4): Incidence of *Mycoplasma* in subclinically and clinically mastitic cows and buffaloes (Quarter milk samples).

Quarter status / Species	Subclinically mastitic			Clinically mastitic		
	Examined QMS	Positive QMS		Examined QMS	Positive QMS	
		No.	%		No.	%
Cows	67	6	8.9	131	17	12.97
Buffaloes	18	1	5.5	8	1	12.5

QMS= Quarters Milk Samples

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

The results in table (5) revealed in the clinical stage the total number of *Mycoplasma* isolates were 11 (13.41%) from sheep while 17 (39.53%) *Mycoplasma* isolates were recovered from goats, and this agreed with Otlu, (1997).

Table (5): Incidence of *Mycoplasma* in clinically mastitis sheep and goats.

Quarter status / Species	Examined QMS	Positive QMS	
		No.	%
Sheep	82	11	13.41
Goat	43	17	39.53

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

Table (6) showed that *Mycoplasma bovis* isolates causing mastitis in cows and buffaloes were (32%) while these records decreased to (28%) in *Mycoplasma bovigenitalium and* unidentified *Mycoplasma* is 40% respectively these results agreed with that of Biddle *et al*., (2003) and disagreed with Kamelia *et al*.(2008) . On the other hand the results in table (7) illustrated *Mycoplasma agalactia* isolates causing mastitis were (29%) and unidentified *Mycoplasma* were (71%), these results were in agreement with Iqbal *et al*. (2004).

Table (6): Biochemical and serological identification of *Mycoplasma* isolates recovered from clinical mastitic and mastitic cows and buffaloes.

Types of *Mycoplasma* isolates	D.S	U.A	G.F	A.H	Positive isolates (GIT)	
					No.	%
M.bovis	+	-	-	-	8	32
M.bovigenitalium	+	-	-	-	7	28
unidentified *Mycoplasma*	+				10	40
Total					25	100

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

Table (7): Biochemical and serological identification of *Mycoplasma* isolates recovered from mastitic milk samples of sheep and goats.

Types of *Mycoplasma* isolates	D.S	U.A	G.F	A.H	Positive isolates (GIT)	
					No.	%
M.agalactia	+	-	-	-	8	29
Unidentified *Mycoplasma*	+				20	71
Total					28	100

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

Table (8):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	110	+	-	-	-	2	20
Buffaloes	36					0	0

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

Table (9): Results of the isolation of *Mycoplasmas* recovered from udder tissues of sheep and goats.

Animal species	No. of examined udder tissue samples	Positive isolates	
		No.	%
Sheep	13	0	0
goats	20	0	0

+ve* number of isolates positive to *Mycoplasma*

PCR and culture methods were applied for the identification of the *Mycoplasma* isolated from bovine milk, to 11 milk samples(10 + 1 reference sample (positive for both PCR and culture). The results showed that out of the 11 samples, only 8 samples were positive for culture while the remaining 3 were negative for culture. On the other hand all 11 samples were positive for PCR using *M.bovis* primers as illustrated in table (10).

Table (10): Results of PCR (*M.bovis*) and culture of 11 milk bovine samples:

Culture (*M. bovis*)	**PCR (*M. bovis*)**		**Total**
	Positive	Negative	
Positive	8	0	**8**
Negative	3	0	**3**
Total	**11**	**0**	**11**

On the other hand the eight *M. agalactia* isolates which identified by cultural and serological methods were negative by PCR using specific *M. agalactia* primers and use reference strain to *M. agalactia* while the same 8 isolates were positive by PCR using *M. bovis* primers as shown in table (11).

Table (11): Results of culture and PCR (*M.agalactia* and *M.bovis*) for 8 milk samples collected from sheep and goat:

Culture (*M.agalactia*)	**PCR (*M. agalactia*)**		**PCR (*M. bovis*)**	
	Positive	Negative	Positive	Negative
Positive	0	8	8	0
Negative	0	0	0	0

As shown in table (11) there is a clear relation between *M. bovis* and *M. agalactia*. However in the present study 8 *M. agalactia* isolates isolated from milk of sheep and goats and identified using traditional techniques and serology, on contrast the application of PCR to these *M. agalactia* isolates, using specific primers for *M. agalactia* revealed negative results,while on using *M. bovis* specific primers on the same isolates the results were positive for all isolates. According to the obtained results and the previous literatures in Egypt it is considered the first record to isolate *M. bovis* from sheep and goats milk, these results were in agreement with (Kumar and Singh, 1984; Chima *et al*., 1986 and Richard *et al.*, 1989) who succeeded to isolate *M. bovis* from sheep and goats.

The incorrect identification by conventional diagnostic methods was recertified by PCR. Bashiruddin et *al*., 2005a reported isolates from non-typical hosts, i.e. three *M. bovis* strains from small ruminants and two *M. agalactiae* strains from cattle, were characterized by sequencing the 16S and part of the 23S ribosomal RNA genes.

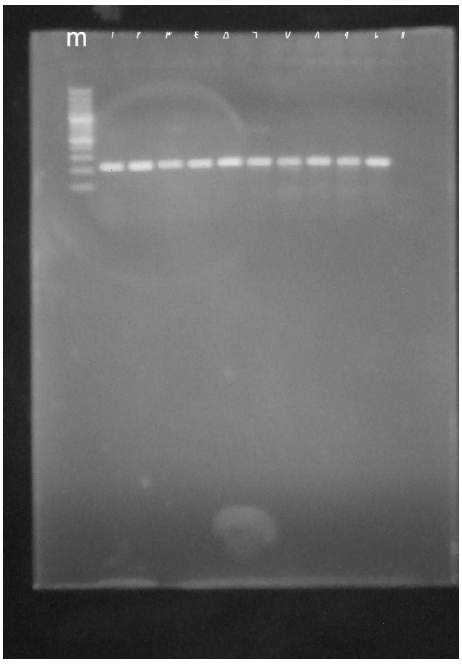

Photo (1): Agarose gel electrophoresis showing amplification of the 227 bp fragments of *M. bovis* from the extracted DNA of *M. bovis* isolates.

Lane M shows the 100 bp- 1.5 Kb DNA ladder.
Lane 1 shows amplification of the 227 bp fragment of *M. bovis* from the extracted DNA of *M. bovis* reference strain
Lane 2-10 showing amplification of the 227bp of *M. bovis*
Lane 11 showing no amplification of the 227bp of *M. bovis* (negative control).

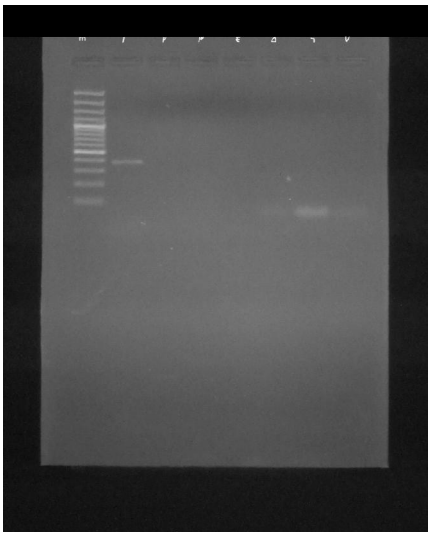

Photo (2): Agarose gel electrophoresis showing amplification of the 375 bp fragment of *M. agalactia* from the extracted DNA of *M. agalactia* reference strain.

Lane M: showing the 100 bp- 1.5 Kb DNA ladder.
Lane 1: *M. agalactia* reference strain
Lanes 2-6 showing no amplification of the 375 bp fragment of *M. agalactia* from the extracted DNA of other *Mycoplasma* isolates.
Lane 7 shows no amplification of the 375 bp fragment of *M. agalactia* (negative control).

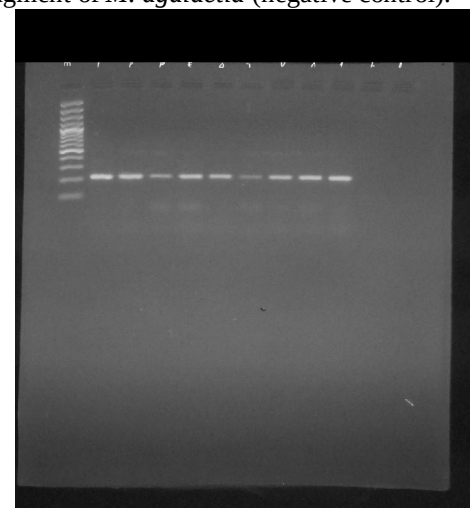

Photo. (3). Agarose gel electrophoresis showing amplification of the 227 bp fragment of of *M. bovis* from the extracted DNA of *M. bovis* reference strain.

Lane M showing the 100 bp- 1.5 Kb DNA ladder.
Lane 1: *M. bovis* reference strain.
Lanes 2-9 showing amplification of the 227 bp fragment of *M. bovis* from the extracted DNA of other *Mycoplasma* agalactia (which gives positive culture and negative PCR agalactia).
Lane 10 showing no amplification.

4. Conclusion:

In conclusion, *Mycoplasma*s isolates were grown slowly and were difficult to culture. Traditionally, very complex media had been used for culture, based on rich growth media have recently been found to be inhibitory in some cases. Incubation and observation should continue for 7-10 days before the plates were recorded as negative but false-negative results were common due to low numbers of organisms in the sample, or the fragility of *Mycoplasma* itself. Although serological methods are easier to perform and less costly, however, they are also generally non-specific, insensitive, and retrospective. PCR-based technology for *Mycoplasma* yields the highest level of sensitivity and specificity. The detection of *Mycoplasma* spp in cattle, buffaloaes, sheep and goats by polymerase chain reaction (PCR) was based on the *in vitro* amplification of the highly-conserved 16S rRNA gene, so using PCR technique to differentiate between *M. bovis* and *M. agalactia* because of the close relation between each other and this technique is rapid, sensitive and specific. Recommended future work to apply PCR technique directly on milk samples and udder tissues to make a comparison between results of culture and PCR.

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