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Evaluation of various diagnostic methods for detection of *Mycoplasma bovis* from bovine mastitis milk in Egypt

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Bovine mastitis caused by *Mycoplasma bovis* represents a major problem for dairy industry all over the world. The present study was designed to determine the occurrence of *Mycoplasma bovis* in mastitis milk from different dairy herds in Egypt, and determination of the most appropriate diagnostic protocol for rapid, accurate diagnosis. That will allow a quicker segregation and culling of infected animals and lower morbidity rates as well, and leading to reduction in economic losses. Mastitis milk samples (n=249) were collected and examined for *Mycoplasma* isolation using conventional cultural method then identified by *Mycoplasma bovis* specific PCR using *mb-mp 81* gene primers. Among the 249 examined milk samples, 18 samples (7.23%) were positive for *Mycoplasma bovis*. For comparison of various diagnostic methods for detection of *Mycoplasma bovis*, twenty-two milk samples were selected randomly from the collected samples and analyzed by three methods, PCR directly from milk, Culture followed by PCR, and the antigenic ELISA. The finding of the comparative study between the three used techniques revealed that Culture followed by PCR 9/22 (40.9% Positive results) was the most sensitive methods for detection of *Mycoplasma bovis* in milk samples followed by AgELISA 7/22 (31.82% Positive results) and PCR directly from milk 2/22(9.09% positive results).

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

INTRODUCTION

Mycoplasma bovis is one of the major pathogens causing bovine mastitis that resulting in a major problem for dairy industry and animal welfare throughout the world. It causes harsh damage to the udder of cattle that mostly responds poorly to chemotherapy (Fox and Gay, 1993; Nicholas et al. 2016). *Mycoplasmas* are among the smallest free-living microorganisms with capability of auto-replication, it belongs to the class *Mollicutes;* order *Mycoplasmatales*, family *Mycoplasmacae* (Razin et al. 1998; McAuliffe et al. 2003). The first record of *Mycoplasma bovis* as a causative agent of sever mastitis outbreak was in 1961s in a commercial dairy herd in the USA and due to its close relatedness with the small ruminant *Mycoplasma agalactiae*, it was classified as a subspecies of it and named *Mycoplasma agalactiae* var. *bovis* (Hale et al. 1962). Then elevation to species level occurred and named *Mycoplasma bovis* in 1976s (Askaa and Erno, 1976).

Bovine mastitis caused by *Mycoplasma* species has destructive economic impact resulting from reduction in milk production, calf losses, culling of animals, veterinary and treatment costs

with all dairy animals including calves, heifers, dry and lactating cows being susceptible to *Mycoplasma bovis* infection (Currin et al. 2005). In the USA, it was estimated that economic losses of bovine mastitis caused by *Mycoplasma bovis* was about $108 million per year with morbidity rate reaching to 70% of a herd (Rosengarten and Citti, 1999). *Mycoplasma* can be transmitted by infected milk, milk clusters or milkers' hands (Calcutt et al., 2018), also large dairy herd size and introduction of new animals from outside of the herd act as great risk factors for occurrence of *Mycoplasma* mastitis outbreaks (Punyapornwithaya et al., 2010). *Mycoplasma* mastitis characterized by milk changes with the milk in the earlier stage appearing as watery secretion containing small sandy flakes with the rapid development of agalactia. By the 3rd to 6th day, the milk secretion appears as yellowish brown fluid with high fibrin and inflammatory cells and finally by the 4th to 14th days after the first clinical signs, the milk become very purulent with cottage cheese appearance (Bushnell, 1984).

Mycoplasma bovis are pleomorphic in shape due to their lack of a rigid lack cell wall with low G+C content (27.8%–32.9%) and small size of genome of 1,080 kbp (Hermann, 1992) which limits their metabolic activity, rendering them highly dependent on outsources of nucleic acid precursors, lipids and amino acids (Calcutt et al., 2018).

Experimental vaccines against *Mycoplasma* mastitis were ineffective; even it may worsen the condition (Ross, 1993). *Mycoplasma* mastitis is largely incurable by intramammary or systemic antimicrobial drugs so that treatment of infected cow is not recommended and cows that repeatedly suffers from clinical mastitis or become negative upon culturing usually remain subclinical carriers with intermittent shedding of *Mycoplasma* microorganisms, and should be regarded as permanently infected (Maunsell et al., 2011). Therefore, *Mycoplasma* mastitis control strategy depends mainly on detection of infected animals in an early stage with segregation or culling of infected animals to prevent dissemination of infection from infected to non-infected animals (Pfützner, 1990).

Mycoplasmas are highly fastidious microorganisms, difficult to culture, slowly growing with special growth requirements needed for their growth (Hogan et al. 1999). *Mycoplasma* grows on solid media giving the characteristic "fried egg" microscopic colonies. *Mycoplasma bovis* produces film and spots on the surface of solid media indicating the presence of lipolytic activity (Thorns and Boughton, 1978). For accurate diagnosis of *Mycoplasma* by culturing, differentiation between *Mycoplasma* spp. and *Acholeplasma* spp. is needed, as *Acholeplasma* spp. are capable of growing on Hayflick medium with formation of *Mycoplasma*-like-colonies. Several discriminative tools are available as Digitonin sensitivity test, Nisin sensitivity test and molecular assays (Tang et al., 2000; Boonyayatra, 2012). Therefore, the aim of our study was to determine the occurrence of *Mycoplasma bovis* in mastitis milk from different dairy herds, and determination of the most appropriate diagnostic protocol for rapid, accurate diagnosis between culture followed by PCR, PCR directly from milk and AgELISA. That will allow quicker segregation and culling of infected animals and lower morbidity rates as well, and finally leading to reduction in economic losses.

MATERIALS AND METHODS

Samples

Two hundred and forty nine mastitis milk samples were collected under aseptic condition from four dairy farms located in different governorates in Egypt and submitted for isolation of *Mycoplasma* using conventional cultural method followed by identification, using *Mycoplasma bovis* specific PCR. Twenty-two samples from the aforementioned mastitis milk samples were randomly selected and subjected for the three diagnostic methods, conventional culture followed by PCR, antigenic ELISA and PCR directly from milk.

Isolation of *Mycoplasma* from mastitis milk using conventional cultural method

It was performed according to Nicholas *et al*., (2008), Hazelton et al., (2018). *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) were used for isolation of *Mycoplasma* from milk samples. 0.1 ml of milk was inoculated into 5 ml *Mycoplasma* broth then incubated at 37ºc in candle jar with elevated Co_2 levels for 7 days and examined for growth daily with final reading on the 7th day 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 a negative sample. For subculturing from agar to broth, agar block with colonies was cut using sterile

spatula and dropped into broth. Isolates confirmed as *Mycoplasma* by Digitonin sensitivity test (Freundt et al., 1973), Glucose fermentation test and Arginine hydrolysis test (Erno and Stepkovits 1973).

Molecular identification of *Mycoplasma bovis* isolates

Extraction of DNA from *Mycoplasma* isolates

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

Extraction of bacterial DNA from milk:

Milk samples were submitted for extraction of bacterial DNA using milk bacterial DNA isolation kit (Norgen Biotek Corp.): Catalogue no. 21550.

Mycoplasma bovis specific PCR

Positive culture isolates were submitted to *Mycoplasma bovis* specific PCR using forward primer *mb-mp 81* F 5'-TATTGGATCAACTGCTGGAT-3' and reverse primer *mb-mp 81* R 5'-AGATGCTCCACTTATCTTAG -3' targeting *mb-mp 81* gene with amplicon size 447 bp (Foddai et al. 2005). 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. Amplicons were detected by electrophoresis on 2% agarose gel stained by ethidium bromide and examined by gel documentation system (Bio-Rad). Reference strain *Mycoplasma bovis* NCTC 10131 was used as control positive and reference strain *Mycoplasma bovigenitalium* NCTC 10122 was used as control negative.

Monoscreen AgELISA for detection of *Mycoplasma bovis* in milk samples

The ELISA, BIO K 341/2 Monoscreen AgELISA *Mycoplasma bovis* 2X12 tests (Bio-X Diagnostics S.A. Belgium) was used according to manufacturer instructions. It is immunoenrichment technique; As *Mycoplasma bovis* is usually present in diseased specimens in very small amounts with presence of other contaminant microorganisms, which may hobble the growth of *Mycoplasma bovis*, so we made four sequential 9-fold dilutions per sample then enriched on Hayflick media on the kit's microplate for 3 days at 37°C with elevated CO_2 level. The kits provides positive control antigen consisting of a lyophilised inactivated *Mycoplasma bovis* culture and negative control for quality control and validation of test results. Sample Value was calculated as the following: Value = (OD of tested sample – OD of the corresponding negative control) / (OD of positive control – OD of the corresponding negative control) × 100%. A value > 7% is considered positive and a value < 7% is considered negative according to the manufacturer instructions.

Statistical analysis

By using the statistical software package IBM SPSS version 22.0, statistical analysis and kappa coefficient were done for each two techniques separately. The level of agreement between each two methods was calculated as the percentage of samples, which had the same result for both tests. Statistical significance was assigned as $P \leq 0.05$.

RESULTS

Incidence of *Mycoplasma bovis* recovered from mastitis milk using conventional culture method followed by PCR

The 18 *Mycoplasma bovis* isolates were recovered from 249 mastitis milk samples from four dairy herds in Egypt in an incidence of 7.23% (Table 1, Figure 1). In addition, from the 22 samples randomly selected from the 249 samples for validation of the different techniques, nine samples were found to be positive for *Mycoplasma bovis* in a percentage of 40.9% (Table 2, Figure 2).

Table 1. Incidence of *Mycoplasma bovis* recovered from mastitis milk samples

Farm	Number of samples	Number of positive samples	Percentage of positive samples*
1	27	1	3.70%
2	93	8	8.60%
3	126	8	6.35%
4	3	1	33.33%
Total	249	18	7.23%

*Percentage in relation to total number of samples collected from each farm

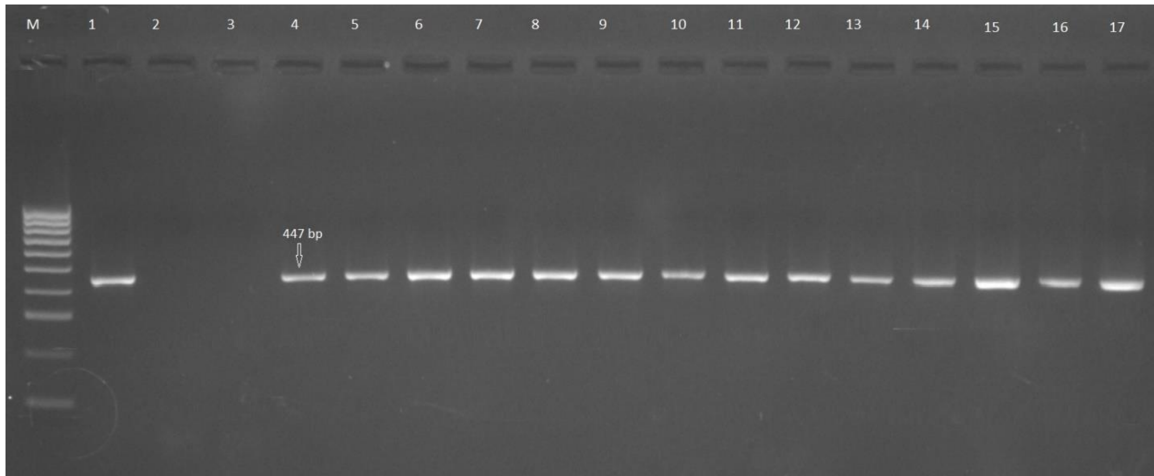

Figure. 1. Agarose gel electrophoresis of *Mycoplasma bovis* specific PCR amplified products from isolates obtained by conventional culture method. Lane M: Marker (GeneRuler100 bp DNA ladder, Thermo scientific™), Lane 1: Positive control (*Mycoplasma bovis* NCTC 10131), Lane 2: negative control (*Mycoplasma bovigenitalium* NCTC 10122), Lanes 4-17: Positive for *mb-mp 81* gene with 447 bp amplicon, lane 3: negative *mb-mp81*.

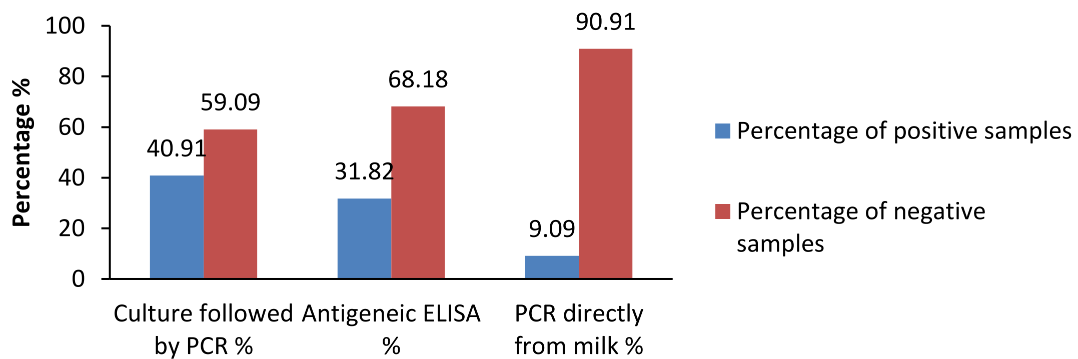

Figure. 2. Results for detection of *Mycoplasma bovis* by Culture followed by PCR, PCR directly from milk and AgELISA (n=22).

Table 2. Results for detection of *Mycoplasma bovis* by different diagnostic techniques

Milk samples (n=22)	Diagnostic methods		
	Culture followed by PCR	PCR directly from milk	AgELISA
1	-	-	-
2	-	-	-
3	+	-	-
4	-	-	-
5	-	-	-
6	-	-	+
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	+	-	+
13	+	+	+
14	+	+	+
15	+	-	+
16	-	-	-
17	+	-	+
18	+	-	+
19	+	-	-
20	+	-	-
21	-	-	-
22	-	-	-
Positive	9	2	7
Total	22	22	22
Percentage%*	40.9	9.09	31.82

* Percentage in relation to the total number of samples (n=22) subjected for the three diagnostic techniques.

Table 3. Results for detection of *Mycoplasma bovis* by the AgELISA four sequential 9-fold dilutions per sample.

Sample	First dilution	Second dilution	Third dilution	Fourth dilution
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	+	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	+	+	+	+
13	+	+	+	+
14	+	+	+	+
15	-	+	+	+
16	-	-	-	-
17	-	-	+	-
18	-	-	+	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-

Table 4. Comparison of culture followed by PCR, PCR directly from milk and AgELISA for detection of *Mycoplasma bovis* in mastitis milk samples

Statistical comparison			Milk samples	P value	Level of agreement	Kappa
Culture followed by PCR Vs PCR directly from milk	Culture followed by PCR positive samples	(n=9) 40.9%	All (n=22)	0.075	Fair (22%)	0.252
	PCR directly from milk positive samples	(n=2) 9.09%				
Culture followed by PCR Vs AgELISA	Culture followed by PCR positive samples	(n=9) 40.9%	All (n=22)	<0.004*	Good (67%)	0.611
	Antigenic ELISA positive samples	(n=7) 31.82%				
AgELISA vs PCR directly from milk	Antigenic ELISA positive samples	(n=7) 31.82 %	All (n=22)	0.030	Fair (29%)	0.353
	PCR directly from milk positive samples	**(n=2) 9.09%**				

* Statistically significant result P ≤ 0.05

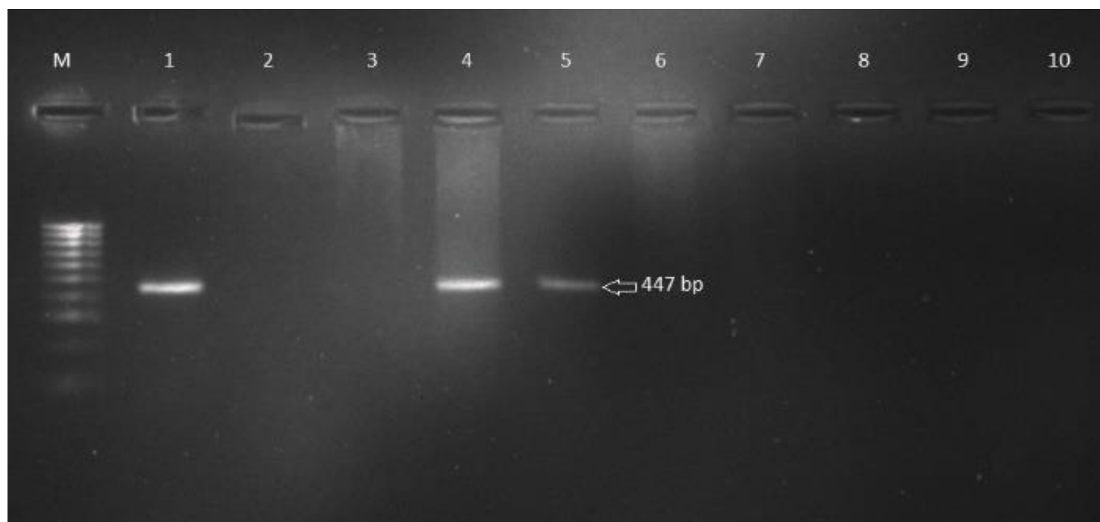

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Figure. 3. Agarose gel electrophoresis of *Mycoplasma bovis* specific PCR amplified products from bacterial DNA isolated directly from milk. Lane M: Marker (GeneRuler100 bp DNA ladder, Thermo scientific™), Lane 1: Positive control (*Mycoplasma bovis* NCTC 10131), Lane 2: negative control (*Mycoplasma bovigenitalium* NCTC 10122), Lanes 4, 5: Positive for mb-mp 81 gene with 447 bp amplicon, lane 3, 6, 7, 8, 9, 10: negative *mb-mp81*.

Biochemical identification of *Mycoplasma bovis* isolates recovered from examined samples

All examined *Mycoplasma bovis* isolates showed >5 mm zones of growth inhibition in digitonin disc diffusion assay. In addition, Isolates were negative for glucose fermentation test and arginine hydrolysis test.

The detection of Mycoplasma bovis from milk samples by PCR using mb-mp 81 gene primers.

Only two samples from the 22 examined milk samples were positive for *Mycoplasma bovis* where it gave an amplicon of 447 bp in a percentage of 9.09% (Table 2, Figures 2, 3). Statistical analysis of the culture followed by PCR and PCR directly from milk showed that of the 22 examined milk samples, 9 (40.9%) samples were culture followed by PCR positive, while only 2 (9.09%) were PCR directly from milk positive with a Kappa coefficient of 0.252 and fair level of agreement (22%) with no significant difference observed (P=0.075) (Table 4).

AgELISA for detection of Mycoplasma bovis directly from milk

By using AgELISA method, results showed that seven samples from the 22 examined samples were found positive for *Mycoplasma bovis* in a percentage of 31.82% (Table 2, Figure 2). Of these 7 positive samples, 3 samples gave positive results at all dilutions, 2 samples gave positive results only with the third dilution, one sample was negative for the first dilution and positive for the 2nd, 3rd and 4th dilutions and one sample gave positive result with the first dilution only with negative results for the other dilutions (Table 3). Analysis for AgELISA and Culture followed by PCR showed that of the 22 examined milk samples, 9 (40.9%) were culture followed by PCR positive, and 7 (31.82%) were ELISA positive with a Kappa coefficient of 0.611and a good level of agreement (67%) with a significant difference observed (P=0.004). (Table 4).

For AgELISA and PCR directly from milk, of the 22 examined milk samples, 7 (31.82%) were found AgELISA positive, while only 2 (9.09%) were PCR directly from milk positive with a kappa coefficient of 0.353 and a fair level of agreement (29%) with slight significant difference observed (P=0.030). (Table 4).

Discussion

Mycoplasma bovis is one of the major pathogens causing contagious mastitis (Fox and Gay, 1993), representing a major problem for milk production and animal welfare in large dairy herds resulting in severe economic losses in dairy industry all over the world (Nicholas et al., 2016). In the current study, we determined the occurrence of *Mycoplasma bovis* in mastitis milk from different dairy herds, and detected the most appropriate diagnostic methods for rapid, accurate diagnosis of *Mycoplasma bovis*. The results of *Mycoplasma bovis* isolation revealed that out of 249 Milk samples, 18 were positive for *Mycoplasma bovis* in a Percentage of 7.23%. Slightly lower percentage was obtained by Al-Farha et al. (2017) in South Australia with a percentage of 6.2%. While higher percentage was obtained by Karahan et al. (2010) with a percentage of 21.1% from eastern Turkey. This variation may be due to difference in herd sizes, control measures and biosecurity programs used.

Interestingly, we compared three different diagnostic methods for detection of *Mycoplasma bovis* in mastitis milk, conventional culture followed by *Mycoplasma bovis* specific PCR, *Mycoplasma bovis* specific PCR directly from Milk and AgELISA. Culture followed by PCR showed the highest level of detection, as nine samples were positive out of 22 samples. It also provides isolates that can be used for further investigations, but it is time consuming as *Mycoplasma* shows slow rate of growth with colonies being visible within 5 days (Nicholas and Baker, 1998) which represents a problem as delayed diagnosis without removal of infected animals from the herd, allows transmission of infection into new animals (Fox 2012).

Furthermore, the detection of *M. bovis* by PCR directly from milk showed the lowest level of detection of *Mycoplasma bovis*, as Hotzel et al., (1996) results. The previous publication used different methods for extraction of *Mycoplasma bovis* DNA from milk with Qiaamp DNA blood kit (Qiagen) which provides silica-membrane-based DNA purification, being one of those methods, which failed to detect *Mycoplasma bovis* in milk samples. In addition, PCR inhibitors present in milk may play a role in the false negative results obtained (Schrader et al., 2012).

AgELISA was the most expensive, less time-consuming technique with results showing statistical significance with culture followed by PCR. Seven samples were positive from the 22

examined samples. Moreover, from the seven positive samples, one sample gave negative results with the first dilution and two samples gave negative results with the first two dilutions, which could be attributed to the outgrown of *Mycoplasma bovis* by other pathogens present in the milk samples (Ayling et al., 1997). While in three from the seven positive samples, the latest dilutions did not give positive results which might be attributed to the over dilution of *Mycoplasma bovis* concentrations to below the detectable level. Negative ELISA, Positive Culture samples may be attributed to the *Mycoplasma bovis* antigenic variability with alteration of the antigenic characters of the surface components (Lysnyansky et al., 2001).

CONCLUSION

In conclusion, conventional cultural method followed by PCR is the most sensitive, accurate, and the cheapest technique in comparable with the other techniques, but it is time consuming due to the fastidious growth requirements of the *Mycoplasma bovis* and it may also give false negative results due to the outgrown of other contaminants present in milk sample. The AgELISA allows quicker diagnosis with results statistically significant to the culture followed by PCR results but it was the most expensive technique with the chance of false negative results due to the outgrown of *Mycoplasma bovis* by other contaminants or over dilution of milk sample. Further investigations are needed for PCR from milk concerning extraction methods and sensitivity of other molecular techniques.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AAA, FIA, NIH and MEE designed and performed the experiments, Analyzed data. All authors participated in writing and reviewing of the manuscript. All authors read and approved the final version.

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