Journal of Current Veterinary Research

ISSN: 2636-4026

Journal home page: http://www.jcvr.journals.ekb.eg

Internal medicine & Infectious disease

Bacteriological and Molecular Identification of *Mycoplasma Bovis* and *Mycoplasma bovigenitalium* Isolated from Cattle and Buffaloes

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ABSTRACT

This study was intended to detect the most common Mycoplasma species involved in respiratory diseases in both cattle and buffaloes. In this study, examination of 300 cattle and 200 buffaloes was carried out and then the collected samples were subjected to isolation of Mycoplasmaspecies followed by PCR confirmation. The results showed that the percent of mollicutes isolation from pneumonic lung tissues of cattle& buffaloes was 22% and 17%, respectively. Moreover, the percent of mollicutes isolation from nasal swabs of diseased cattle & buffaloes was 18% and 20%, respectively. PCR was effective in the detection of both *M. bovis* and *M. bovigenitalium* through successful amplification of the *Mbo* and *Mbg* gene at 360 and 312 bp, respectively. The minimum inhibitory concentration was performed on two field strains of *M. bovis* against seven different antimicrobial agents. The first strain was sensitive to Draxxin® 10 %, Marbocyle® 10%, Lincospectin®, Duocycline ® 20%, and Tilmicosin® 30%. The second strain was sensitive to Duocycline® 20% and Tilmicosin® 30% and resistant to other antimicrobial agents. Regarding the associated risk factors with Mycoplasmosis; the season was significantly correlated and the infection was 6.5 times more prevalent in the winter season than summer season. As well as the sex revealed a significant effect and 5.08 times in males more than females. Additionally, cattle were more susceptible than buffaloes for the disease prevalence. Meanwhile, the age was not a significant risk for the disease prevalence.

Keywords: PCR, MIC, *M. bovis*, *M. bovigenitalium*, Cattle, Buffaloes, Risk factors.

INTRODUCTION

Bovine respiratory disease complex (BRDC) caused by a variety of variables, including environmental factors, pathogen exposure, and animal management (Horwood et al., 2014). Numerous substances, including chemical, physical, and biological substances can cause pneumonia. The biological agents consist of bacteria, fungus, viruses, Mycoplasma, protozoa, and parasites (Taylor et al., 2010). *M. bovis*, *M. mycoides subsp. mycoides*, *M. dispar*, *M. californicum*, *M. agalactiae*, *M. canis*, *M. bovirhinis*, *M. alkalescens*, *M. arginini*, *M. bovoculi*,*M. bovigenitalium*, etc. are among the Mycoplasma species that cause financial losses in animal livestock (Maunsell and Donovan, 2009). *Mycoplasma* species including *M. bovis*, *M .bovigenitalium*, *M. dispar*, and *M. bovirhinis* cause severe

respiratory problems. These microorganisms are typically found in the upper respiratory tract as natural flora. Furthermore, *M. bovis* is typically correlated with concurrent viral infections. It is also one of the main pathogens responsible for arthritis, mastitis, otitis media, and respiratory illnesses. *M. bovis* is the main cause of pneumonia in calves and is responsible for 25% to 33% of calf pneumonia outbreaks (Nicholas et al., 2002; Gagea et al., 2006). Cattle, buffaloes, and small ruminants are susceptible to *M. bovis* (Pfützner and Sachse, 1996). Additionally, *M. bovis* affects all cattle age classes and also all cattle sectors (beef, milk, and rearing) (Nicholas & McAuliffe, 2008). Overcrowding, bad feed, frequent animal movements, and increased milk production were linked to a clinical *M. bovis* disease outbreak (Aebi et al., 2015). *M. bovis* is responsible for nearly 25-33% of economic losses to the respiratory diseases in the cattle industry (Nicholas and Ayling, 2003) through reduction in animal production, carcass quality, treatment costs, mortality, culling rate, and diagnosis and control measures (Horwood et al., 2014). *Mycoplasma* and, in particular, *M. bovis* from pneumonic lungs can be directly diagnosed using the PCR, which reduces the need for microorganism cultivation. This is an accurate method that also takes less time and effort for diagnosis of Mycoplasma, and especially *M. bovis*. Numerous international studies have endorsed the use of PCR in place of or in addition to culture (Hamad et al., 2019). The widespread of unchecked use of antibiotics in the treatment of bovine pneumonia has contributed to the fast development of antimicrobial resistance to *M. bovis* strains worldwide. Due to absenceofcell wall, β-lactam antibiotics are ineffective against *Mycoplasma* (Francoz et al., 2005). Consequently, *M. bovis* develops increased resistance to a number of antibiotic classes, such as tetracycline, fluoroquinolones and macrolides (Lysnyansky and Ayling, 2016; Sulyok et al., 2018). Tulathromycin® and florfenicol are officially accepted in the United States for the treatment of BRD caused by *M. bovis* (Godinho et al., 2005). In Egypt, several studies have reported that wide range of prevalence rates of *M. bovis* from cattle suffering from respiratory manifestation recorded at 8%, 13.8%, 18.9%, 40%, and 61% (Emran et al., 2013; Mahdy et al., 2015; Abdeen et al., 2017; Ammar et al., 2022; Hashem et al., 2022). The present work was designed to throw spotlights on the isolation of *Mycoplasma* species by traditional methods and PCR besides the determination of the MIC for some field isolates and estimation of some risk factors in cattle and buffaloes.

MATERIAL AND METHODS

1. Sampling and animal examination

In this study (553) cattle and (342) buffaloes were subjected to clinical examination. A total of 500 samples (200 nasal swabs & 300 lung tissues) were collected from diseased cattle and buffaloes in this study. Nasal swabs were collected from live diseased animals from Menofia governorate and lung tissue was collected from El Basateen slaughterhouse, Cairo. Nasal swabs were collected from 100 cattle and 100 buffaloes showing fever, coughing, and rales by auscultation, nasal discharge, lacrimation, and conjunctivitis. As well as lung tissue from 200 slaughtered cattle &100 buffaloes showed signs of pneumonia at postmortem examination. All samples were collected aseptically and transferred cooled in an ice box to the laboratory.

2. Phenotypic Isolation and Identification of Mycoplasma

The samples cultivated for three days at 37 ^{o}C in PPLO broth, followed by three more days at 37 ^{o}C in PPLO agar medium. Every two or three days, the

samples were inspected under a stereo microscope. If the distinctive "fried egg" colonies showed up, the agar blocks were moved into broth medium, where they were cultured for two to three days at 37 ^{o}C before being purified. Identification of mycoplasma species was done through digitonin sensitivity disc (Freundt, 1973). Biochemical Characterization for the suspected mycoplasma isolates through arginine deamination test and glucose fermentation test were performed according to (Sabry, 1996).

3. Minimum Inhibitory Concentration (MIC) (Hannan, 2000)

Using seven different antibiotics, the minimum inhibitory concentration was tested on two field strains of M. bovis.Tilmicosin (Tilmovet 30%), Draxxin® 10 %, Zuprevo®18 %, Lincospectin® 100/50 (Zoetis), (duocycline®) Oxytetracycline®20%, Marbocyl® 10% and Tylosin (Tylovet® 20%).

4. Polymerase Chain Reaction for molecular identification of mycoplasma strains:

DNA Extraction by using (GF-1 Tissue DNA Extraction Kit, vivantis). The PCR reaction was done in 50 µl volume, including 25 µl My Taq Red Mix, 2x, 1 µl from each primer (20 µM of each), and DNA Template 200 ng and complete with sterile water up to 50 µl. Primers used for molecular diagnosis of *M. bovis* and *M. bovigenitalium* were listed in table (1).

Table 1. primers used for diagnosis of different Mycoplasma spp., *M.bovis,*and *M. bovigenitalium.*

Species	Designation	Sequence	Reference	Fragment
M. bovis	MboF MboR	5′- CCT TTT AGA TTGGGATAGCGGATG-3′ 5′- CCGTCAAGGTAGCGTCAT TTCCTAC-3′	**(Yleana et al., 1995)**	**360 bp.**
M. bovigenitalium	Mbg F Mbg R	5′- CGT AGA TGC CGC ATG GCA TTT ACG G-3′ 5′- CAT TCA ATA TAG TGG CAT TTC CTA C-3′	(**Kobayashi et al., 1998**)	**312 bp**

5. Statistical analysis

Data on species, age, sex, treatment, and season were collected. Using a univariate logistic regression analysis model, the relationship between positive samples and various animal traits was determined on an individual basis using IBM SPSS Statistics for Windows version 21.0. (IBM SPSS Inc., Armonk, NY). A multivariate logistic regression model was applied to investigate the relationship between mycoplasma infection and animal attributes (species, season, age, sex, and treatment).

RESULTS

1. Mycoplasma species prevalence and bacteriological identificationfrom lung tissues and nasal swabs collected from cattle and buffaloes

The percentage of mollicutes isolated from 200 lung tissue samples collected from cattle suffering from pneumonia and 100 buffaloes were 44 (22%) and 17 (17 %) respectively as shown in Table 2. The percent of mollicutes isolation from 100 nasal swabs from cattle and 100 nasal swabs from buffaloes were 18 (18 %) and 20 (20 %) respectively as shown in Table 2. Mycoplasma on PPLO media exhibited the typical fried-egg appearance, where a

dark central zone is usually surrounded by a lighter peripheral zone or finely granular berry-like colonies that penetrate the agar surface. as showed in figure 1 (A & B). Regarding the isolated Mycoplasma species, it was found that M. bovis and M. bovigenatlium were the most detected serotypes in our study confirmed through biochemical tests as in Table 3.

Table 2. The percentage of Mollicutes isolation from pneumonic lung tissues of cattle and buffaloes.

Animal species	**Lung tissues (200 cattle; 100 buffaloes)**		**Nasal swabs (100 for each)**	
	No	%	No	%
Cattle	44	22	18	18
buffaloes	17	14	20	20

Table 3. Biochemical tests of mycoplasma *M.bovis* and *M.bovigentalium*from pneumonic lung tissues of cattle and buffaloes.

Test	**Digitonin sensitivity test.**	**Arginine**	**Film & spot**	**Glucose fermentation**
M.bovis	+ve	-ve	+ve	-ve
M.bovigentalium	+ve	-ve	-ve	-ve

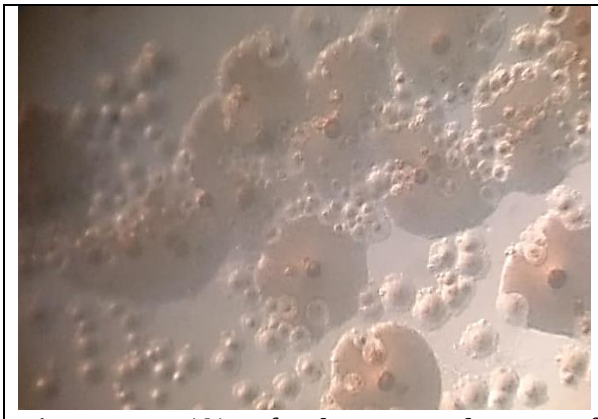

Figure 1 (A). fried egg colonies of mycoplasma species.

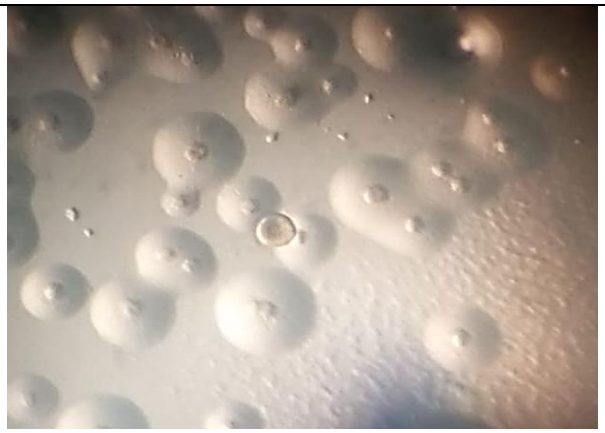

Figure 1 (B). fried egg colonies of mycoplasma species.

3.4. Evaluation of the Minimum inhibitoryconcentration against field M. bovis strains

The MIC was applied for 2 isolates of *Mycoplasma bovis* as shown in table 4. The first isolate was sensitive to (Draxxin®, Lincospectin®, Duocycline® (oxytetracycline), Tilmicosin®, and Marbocyle®) and resistant to (Tylosin and Zuprevo®). Moreover, the MIC of the second isolate of *M.bovis* was sensitive to (Duocycline® (oxytetracycline), Tilmicosin® and resistant to (Draxxin®, Lincospectin®, Tylosin, Marbocyle® and Zuprevo®).

Table 4. Evaluation of the Minimum inhibitoryconcentration against field *M. bovis* strains.

Antibiotics	***M. bovis* 1**	***M.bovis* 2**
Draxxin®	Sensitive	Resistant
Lincospectin®	Sensitive	Resistant
Duocycline® (oxytetracycline)	Sensitive	**Sensitive**
Tilmicosin®	Sensitive	**Sensitive**
Tylosin	Resistant	Resistant
Marbocyle®	Sensitive	Resistant
Zuprevo®	Resistant	Resistant

5. Molecular Identification of Mycoplasma species in cattle using PCR

M. bovis isolates that were confirmed by biochemical tests were subjected to DNA extraction and molecular identification using a specific primer for *M. bovis* which amplified at 360 bp as in figure 2 and 3 from cattle and buffaloes respectively. While *M. bovigenitalium* isolates that were confirmed by biochemical tests was successfully amplified at 312 bp as shown in figure 4.

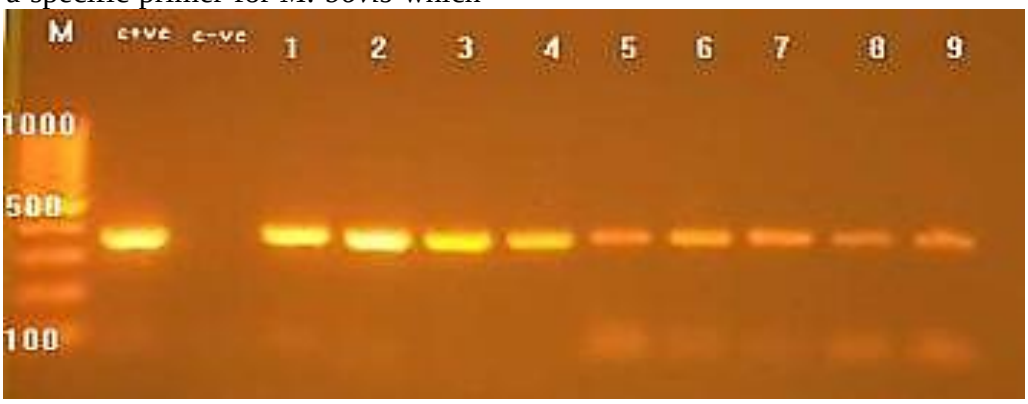

Figure 2. 1.5 % Agarose gel showing PCR product of *M. bovis using* specific primer at 360 bp. M: 100 bp 1 Kb DNA ladder, C+ve: control +ve,C-ve: control –ve, from 1: 9: positive M. *bovis*from cattle samples.

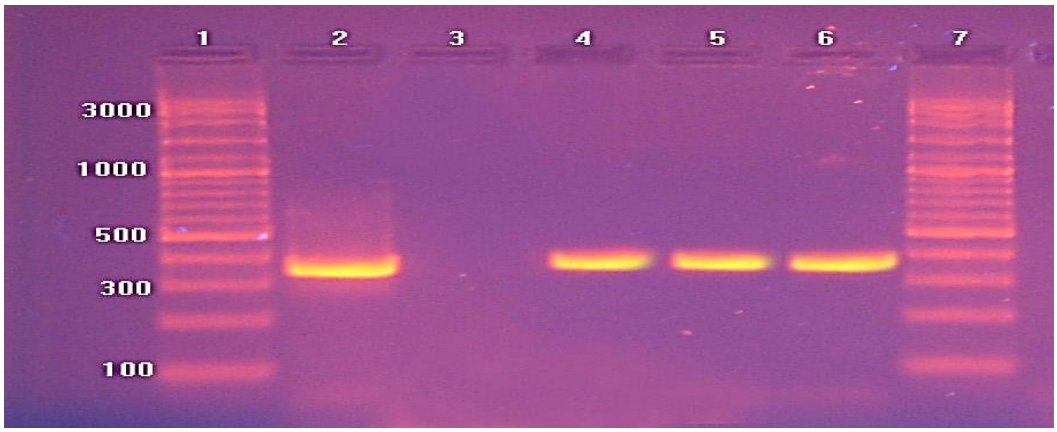

Figure 3. 1.5 % Agarose gel showing PCR product of *M.bovis* using specific primer at 360 bp Lane 1, lane 7: 100 bp DNA ladder. Lane 2: control +ve: Lane 3: control –ve, (Lane 4- 6): +ve *M. bovis* from buffaloes samples.

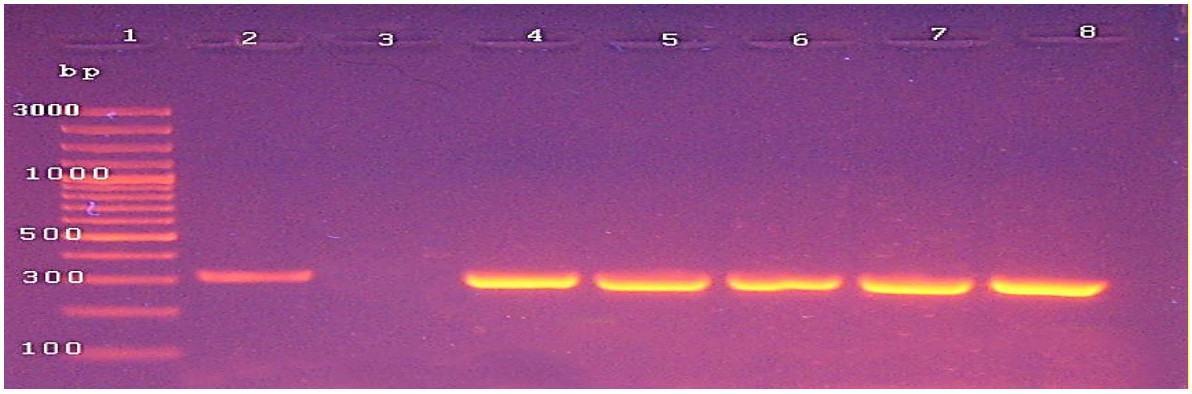

Figure 4. 1.5 %Agarose gel showing PCR product of *M. bovigenitalium* using specific primer at 312 bp.Lane 1: 100 bp DNA ladder, Lane 2: control +ve, Lane 3: control –ve: Lane 4, Lane 5, Lane 6, lane 7, Lane 8: +ve *M. bovigenitalium*from cattle and buffaloes.

6. Risk factors related to Mycoplasmosis in cattle and buffaloes

The examination of certain risk factors linked to *Mycoplasma* infection in cattle and buffaloes showed that, among the infected cattle and buffaloes under research, age was not a significant risk factor for mycoplasma infection. Mycoplasmosis was statistically significantly more common in the winter than in the summer, with a nearly 6.5-fold increase in prevalence during the winter. The findings regarding the impact of sex on the prevalence of mycoplasmosis showed that sex had a significant effect, with $p<0.047$ and 5.08 times more susceptibility in males than in females. The study findings indicate a statistically significant effect of treatment on the prevalence of mycoplasmosis, with a p-value of less than 0.001. Regarding the impact of species on the prevalence of Mycoplasmosis, the findings showed that the species had a significant effect ($p<0.032$) as shown in Table 5.

Table 5. Risk factors related to Mycoplasmosis in cattle and buffaloes.

Variables in the Equation							
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Exp(B)**
Step 1[a]	**Season**	1.910	.650	8.626	1	.003	6.751
	Species	-1.377	.633	4.740	1	.029	.252
	Age	-.269	.600	.201	1	.654	.764
	Sex	1.631	.820	3.956	1	.047	5.108
	Treatment	-2.827	.822	11.817	1	.001	.059
	Constant	-3.184	1.943	2.685	1	.101	.041
Step 2[a]	**Season**	1.881	.646	8.493	1	.004	6.563
	species	-1.349	.627	4.625	1	.032	.260
	sex	1.626	.820	3.935	1	.047	5.086

	treatment	-2.798	.818	11.712	1	.001	.061
	Constant	-3.584	1.738	4.253	1	.039	.028
a. Variable(s) entered on step 1: season, species, age, sex, treatment.							

DISCUSSION

However, *Mycoplasma bovis* is the main cause of mastitis, arthritis, and bovine bronchopneumonia; additionally, infections can occur in the brain, ear, or eye. It is responsible for both significant financial losses and distress to animals even though it is not a zoonotic disease. Due to antibiotic resistance, *M. bovis* has also spread worldwide, making control difficult. The primary cause of this is a lack of effective vaccinations and therapies (Dudek et al., 2020). In this study, the main observed clinical findings on the examined animals were in contact with previous studies reported by (Stipkovits et al., 2000; Nicholas & McAuliffe, 2008). The common clinical signs of bovine mycoplasmosis were reported, including fever, dyspnea, and decreased appetite, tachypnea, nasal discharge and coughing. Animals with chronic illnesses find it difficult to gain weight. Mycoplasma pneumonia may also be related to otitis media, arthritis, lameness, and joint stiffness. Meanwhile, P.M. showed signs of pneumonia in lung tissues including reddening, localized necrosis, consolidation, sero fibrinous fluid in the thoracic cavity and fibrinopurulent membrane on the pleural surface. That is agreed with (Gagea et al., 2006; Hamad et al., 2019) as they reported reddening, consolidation, purulent focal and distinctive areas of coagulative necrosis in the lung with mycoplasmosis. In addition, affected lungs frequently have many necrotic foci filled with yellow caseous content, congestion, caseous nodules on the lung, a marbling appearance, hepatization, and in some slaughtered calves, the lungs had a putrid odor with surface ulceration. These observed signs in the postmortem examination were attributed to the severe infection and inflammatory response of the lung tissues against mycoplasma infection and its replication in the lung and pleura surface. In the present study the positive *Mycoplasma* species showed microscopically fried-egg appearance. This was in contact with (Dudek et al., 2020) who revealed the typical and characteristic shape of *Mycoplasma* colonies which appeared as fried egg colonies. Moreover, (Quinn et al., 2013) isolated the *Mycoplasma* on PPLO medium and produced micro-colonies that resembled "fried eggs" colonies by adding thallium acetate or antibiotics to the medium. Concerningthe percent of Mycoplasma isolation from pneumonic lung tissues was 13.5 % and 12% from pneumonic lung tissues of cattle and buffaloes. This result disagrees with those obtained by (Eissa et al., 2007) who found that the incidence of Mycoplasma from private cattle farm in Kalubia Governorate was 31.58%, and (Hashem, 2008) that isolated Mycoplasma from Egyptian cattle in an incidence of 31.63%. Also, the result of the present study disagrees with (Giovannini et al., 2013) who found that Mycoplasma species were isolated in an incidence of 37.05%. The observed variation may be due to the difference in a number of samples collected, breeds, season, locality, and the applied hygienic measures.

The results of Mycoplasma isolation recorded that the prevalence rate from nasal swabs of cattle and buffaloes

was 12% and 5% respectively. These results disagree with (Siugzdaite et al., 2012) who found that Mycoplasma was prevalent with 34.44% from nasal swabs collected from 90 calves in Lithuania. This variation may be attributed to the variation of age of the animals examined between studies, geographical distribution, and number and type of samples collected concerning the results of minimum inhibitory concentration that reported high sensitivity to Draxxin® 10 %, Marbocyle® 10%, Lincospectin®, Duocycline ® 20%, and Tilmicosin® 30% and resistant to other antimicrobial agents. The MIC results of this study is supported by (Godinho et al., 2005; Uemura et al., 2010) as well as (Maunsell et al., 2009) who said that Oxytetracycline, tilmicosin, spectinomycin, and tulathromycin are effective against *M. bovis.* On the other hand, disagree with (Nicholas et al., 2000) who reported that *M. bovis is* resistant to oxytetracyclines, tilmicosin. It is clear from the result the second strain was sensitive to Duocycline® 20%, and Tilmicosin ® 30% and resistant to other antimicrobial agents. This agrees with (Thomas et al., 2003) who reported that *M. bovis* was resistant to tylosin, spectinomycin and lincomycin. Also, agree with (Sato et al., 2017) who reported that tilmicosin is the main antibiotic used in cattle in Japan.

PCR methodology was found to be an effective direct method for diagnosing Mycoplasma, and specifically *M. bovis*, from pneumonic lungs due to difficulties related to traditional cultivation procedures. This approach will save effort as well as time and is accurate. Several international research suggested using PCR for diagnosis in addition to mycoplasma culturing (Hamad et al., 2019). In this study, PCR used aspecific gene Mbo, and Mbg genes for *M. bovis* and *M. bovigenitalium* respectively. This was previously described that PCR is a high-speed and specific successful approach for detection of mycoplasma infections and identifying this agent at the species level (Kilic et al., 2013). In a related study (Maya-Rodríguez et al., 2022) applied mPCR test as a highly sensitive method for the diagnosis and surveillance of *M. bovis*, *M. bovirhinis* and *M. dispar* from 335 nasal swabs from respiratory disease in Mexico. Additionally, (Mohamed et al., 2020) used 16S rRNA genes as universal primers for the detection of Mycoplasma species from nasal swabs from 93 camels. However, (Loens et al., 2003) identified *M. pneumoniae* by both the P1 adhesin gene and *16S rRNA* gene.Because it is present in all bacteria and has remained functional over time, (Janda & Abbott, 2007) employed the 16S rRNA gene as a common gene for bacterial identification. Also, (Miles et al., 2004; Marques et al., 2007) applied the 16S rRNA gene as a target species-specific PCRs for *M. dispar* and *M. bovirhinis*. In addition, (Hiroseet al., 2001) used many primers specific to *M. alkalescens*, *M. bovigenitalium*, *M. bovirhinis*, and *M. bovis*, with speciation determined by the product size on an agarose gel.

The study findings indicate a significant relationship between sex and the prevalence of mycoplasmosis, with $p<0.047$ and 5.08 times higher susceptibility in males than in females. This was agreed with (Cusack et al., 2007) reported a higher risk of bovine respiratory disease in male calves compared to the female calves. On the other hand, disagree with (Gogoi-Tiwari et al., 2022) who reported that female calves were found to become seropositive with *M. bovis* twice more than the male calves. The variation between this study and other studies may attributed to the difference in the number of examined animals and other factors related to the physiological status. Also, statistical analysis showed that age was not a significant risk factors for *Mycoplasma*

infection in the examined diseased cattle and buffaloes. These results come in agreement with (Nicholas & McAuliffe, 2008) who said that *M. bovis* affects all age groups of cattle and all cattle sectors such as milk, beef or rearing.In the present study, the prevalence of mycoplasmosis in relation to the season was statistically significant and the winter season was almost 6.5 times more than the summer season. These results agree with (Moroni et al., 2018) who said that cold, wet seasons also may increase the incidence of infection because the organisms may persist longer in the environment. Mycoplasmosis prevalence was examined in relation to treatment, and the findings indicated a highly significant effect on disease prevalence ($p<0.001$). This agrees with (Godinho et al., 2005) who reported that Tulathromycin and tildipirosin injections into cattle are crucial for both treating and preventing *M. bovis* infections. Meanwhile, species' impact on mycoplasmosis prevalence the study's findings showed a significant species effect with $p<0.032$. these results agree with (Caswell & Archambault, 2007) who reported that Buffaloes are less susceptible to Mycoplasma bovis pneumonia than feedlot and young dairy and veal calves.

CONCLUSION

According to the current investigation, *M. bovis* and *M. bovigenitalium* were found in cattle and buffaloes that had respiratory symptoms and were identified using traditional techniques. In addition, PCR was applied as a confirmatory technique, using certain primer sets, to detect *M. bovigenitalium* and *M. bovis*. The antimicrobial pattern of *M. bovis* isolates through MIC revealed higher sensitivity to Draxxin®, Lincospectin®, Duocycline®, Tilmicosin®, and Marbocyle® and resistance to Tylosin® and Zuprevo®. The season was found to be statistically significant in the study of the risk factors correlated with mycoplasmosis, and the infection was 6.5 times more common in the winter than in the summer.As well as sex revealed significant effect and 5.08 times in males more than females. Additionally, the effect of species on the prevalence of mycoplasmosis revealed a significant effect with more prevalence in cattle than buffaloes. Meanwhile, age was not a significant risk factor for mycoplasma infection in the cattle and buffaloes.

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