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Original article

Prevalence, molecular characterization and antibiogram of *Mycoplasma bovis* isolated from milk in Pakistan

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Abstract

Mycoplasma bovis is a highly contagious pathogen that causes clinical or subclinical mastitis. The present study was aimed for the isolation, molecular characterization and antibiogram determination of *M. bovis* from raw milk samples. Milk samples were collected randomly from lactating cows and buffaloes from different tehsils of district Faisalabad, Pakistan. Samples were inoculated on modified Hayflick medium and biochemical tests were performed for further confirmation of isolated *M. bovis*. Out of total 400 milk samples, 184 (46%) samples were found positive for culture method. The 16S-rRNA gene polymerase chain reaction was performed for molecular characterization of isolated *M. bovis* strains. Out of total 400 milk samples, 240 (60%) positive for *M. bovis* through PCR method were examined. The 16S-rRNA gene PCR positive isolated *M. bovis* strains were sequenced and results were compared using Maximum-likelihood method and sequenced strains of *M. bovis* were aligned and analyzed by Clustal W software. Antibiogram of isolated *M. bovis* strains was analyzed by disc diffusion assay against eight commonly used antibiotics. Tylosin (30µg) and Tilmicosin (15ug) showed inhibition zones of 32.34 ± 1.10 mm and 17.12 ± 0.93 mm respectively against isolated *M. bovis* which were found sensitive. Isolated *M. bovis* was found resistant to other commonly used antibiotics. Statistical analysis revealed that p-value was < 0.05 and the odds ratio was > 1.0 at 95% CI. This study complemented the lack of epidemiological knowledge of molecular characterization, comparative effectiveness and resistance trends of isolated *M. bovis* strains against commonly used antibiotics.

Keywords: milk, *Mycoplasma bovis*, modified hayflick medium, PCR, DNA sequencing, disc diffusion, antibiotic susceptibility

Introduction

Livestock is considered a more consistent source of income for small farmers. Livestock contributes approximately 58.3% to agriculture and 11.4% to the country's (GDP) Gross domestic production (Ilyas et al. 2022). Most of the local population in Pakistan is involved in the breeding market and about 8 million people are doing business in dairy farming (Khan 2022). Agriculture and animal breeding are quite popular around the world countries, especially among the poor and non-agricultural communities (Buller et al. 2018). According to WHO (World Health Organization), a good drug resistance investigation program, data on the occurrence, and an understanding of the bacteria that cause infection are required to create prevention and control programs, as well as to conduct successful therapy procedures (Talebi et al. 2019).

Mastitis has been considered the most common and infectious disease plaguing the whole dairy industry and responsible for the damage of over two-thirds of the total world economy (Pal and Chakravarty 2020). Globally, mastitis is responsible to a great financial loss of US Dollar 35 billion (Imran et al. 2021). Mastitis losses are due to reduced milk production, cost of treatments, and culling, accounting for 78, 8 and 14%, respectively (Romero et al. 2018). Bovine mastitis is considered the most common disease leading to economic loss in dairy industries due to reduced yield and poor quality of milk (Cheng and Han 2020). Pakistan is the third-largest milk-producing country in the world having 87.8 million cows and buffaloes. Mastitis infections are rising around the world and are a hot issue in Pakistan owing to the absence of proper disease monitoring and evaluation systems. In Pakistan, the annual losses due to dairy animal diseases are reported to be US\$ 200 million (Ghafar et al. 2020). In Punjab, Pakistan, the total annual loss caused by mastitis is about 240 million rupees (Imran et al. 2021).

Mycoplasma bovis, along with other viral and bacterial agents, has been one of the primary causes of bovine respiratory disease (Deeney et al. 2021). By the way of the umbilical cord, direct contact with their mothers during birth, respiratory contact with other infected animals and consumption of tainted milk, calves can contact *M. bovis* vertically. The infection causes a range of clinical symptoms, including polyarthritis, enzootic pneumonia and death. Arthritis, pleuro-pneumonia, conjunctivitis and mucopurulent discharge are all common lesions in young calves, especially in those animals between the ages of 2 and 3 weeks. Furthermore, *M. bovis* produces caseous necrotic pneumonia, keratoconjunctivitis and polyarthritis (Mahmood et al. 2017).

M. bovis grows well in a variety of mediums (like Eaton's, Hayflick's, and Frey's) and produces orange color "centered" colonies with fried egg appearance after 3-5 days and generates films and spots. Mycoplasma can be identified using hyper immune rabbit serum, methods for metabolic inhibition, growth inhibition, film inhibition, and fluorescent antibodies (Klein et al. 2017). *M. bovis* can be biochemically characterized by glucose fermentation, tetrazolium reduction, casein digestion, arginine hydrolysis, phosphatase activity and film spot formation (Nicholas et al. 2016).

Milk samples from clinically mastitis suspicious cows or bulk tank milk samples can be used to identify farms with *M. bovis* problems and the infected cases within a herd. PCR technique has become the most common method for the detection of Mycoplasma spp. *M. bovis* is an infectious pathogen causing mastitis both in buffaloes and dairy cattle detected through the PCR method (Behera et al 2018). Advance approach to study molecular epidemiology is the use of sequencing methods. Gene sequencing has been used for wide range of Mycoplasma species including *M. bovis*. Sequence differences in the 16S-rRNA genes of homologous operons are of interest when attempting to elucidate the changes between and within species (Konigsson et al. 2020).

Disc diffusion was used to check the inhibition activity of many types of antibiotics against the growth in *M. bovis*. The inhibition zones were measured by using zone reader.

M. bovis is identified and treated with antimicrobials such as tetracyclines, some fluoroquinolones, and macrolides. Prophylactic antibiotic use is normally the next level target. It may be proved helpful when calves are delivered to a site that is heavily contaminated with *M. bovis* infection and has a high mortality rate (Maunsell and Donovan 2009, Adorno et al. 2021). Antibody responses to Mycoplasma infections are not very protective. *M. bovis*-related diseases are said to be resistant to all sorts of treatment (Maunsell et al. 2011). Antibiotics are commonly misused to cure infections, sometimes effectively to reduce subsequent bacterial infections but widely ineffective to heal Mycoplasma infections (Shao et al. 2021).

Antibiotic therapy and sanitary measures are used to control *M. bovis* infections until widely available vaccinations are developed. *M. bovis* is immune to all antimicrobials that attack the cell wall including β -lactams. *M. bovis* is resistant to commonly used antibiotics like polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin. The disc diffusion assay is mostly used for the selection of more suitable antibacterial against specific Mycoplasma infections. *M. bovis* infection must be identified early and treated

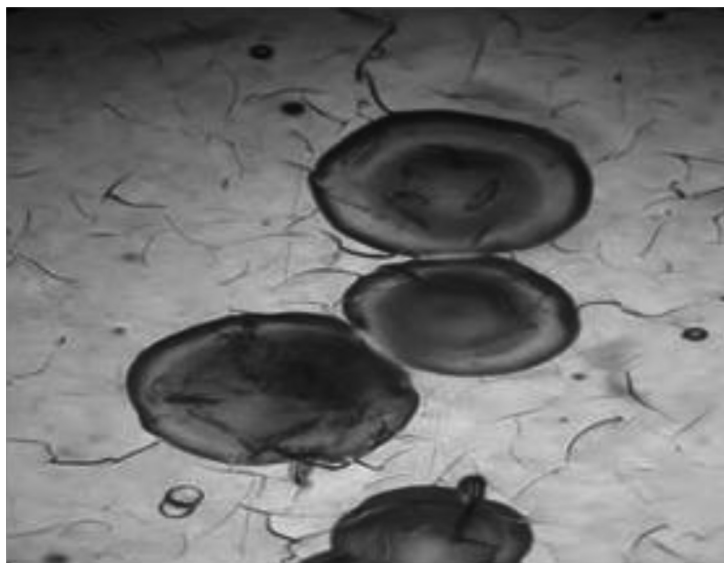


Fig. 1. Isolated *Mycoplasma bovis* colonies with typical “fried-egg” appearance on modified Hayflick agar at x40 under light microscopy.

with antimicrobials agents such as tetracyclines, fluoroquinolones, and certain macrolides (Hata et al. 2019). Antibiotics have been used to treat *M. bovis* but it is difficult because the bacteria penetrate completely into the host's body (Alhussen et al. 2021). Injectable antibiotics such as enrofloxacin, tulathromycin, gamithromycin, and florfenicol are also used for treatment and are effective against *Mycoplasma*, but it is more difficult to treat calves in the early stages of development.

Controlling *M. bovis* infections remains difficult due to the absence of an effective commercial vaccine. Most have been commercialized, primarily in the US, however there is not much information to assess their immunogenicity and protective qualities. They must be incorporated into multivalent vaccines that comprise the pathogenic bacteria and viruses now available for bovine respiratory illness for them to be effective (Perez-Casal et al. 2017) *M. bovis*-associated mastitis is a severe problem in large dairy herds frequently incurable and there is no vaccination currently available.

Keeping in view the above-mentioned information, bovine mastitis is highly prevalent disease in Pakistan. There is no data present regarding prevalence of *M. bovis* associated mastitis in Punjab, Pakistan. Study was planned to check the prevalence of *M. bovis* in selected region of district Faisalabad, Pakistan. The bacterium has not been characterized either through culture or using molecular diagnostic techniques like PCR from milk samples. Moreover, the antibiogram study and the status of antibiotic resistance have not been established so far in the selected study area. Therefore, research is designed to investigate the prevalence of *M. bovis* through conventional culture technique along with molecular characterization (Polymerase

Chain Reaction) and determination of antibiogram study of *M. bovis*. The findings help in updating knowledge regarding the prevalence, and conventional and molecular characterization of *M. bovis*. Moreover, an antibiogram study will help to develop appropriate treatment regimens against *M. bovis*-associated mastitis in Pakistan.

Materials and Methods

Study area

This study was conducted to establish the prevalence of *M. bovis* present in milk samples of selected study areas of district Faisalabad, Pakistan, and antibiogram of *M. bovis*.

Collection of samples

According to available livestock census of Punjab, Pakistan in 2014, out of the total livestock population, Faisalabad comprises 0.46 million (34%), Jaranwala 0.36 million (26%), Tandlianwala 0.33 million (24%), and Samundari 0.22 million (16%) animals (cows and buffaloes), respectively. Among this population of Faisalabad district, (Pakistan) a total of n=400 raw milk samples were collected randomly based on 10% proportional allocation of animals of dairy herds, from lactating buffaloes (n=265) and lactating cows (n=135). Out of total n=400, 136 from Faisalabad, (90 buffaloes, 46 cows), 104 from Jaranwala (70 buffaloes, 34 cows), 96 from Tandlianwala (65 buffaloes, 31 cows), and from Sumandri 64 (40 buffaloes, 24 cows) were collected from October 2020 to March 2021 as shown in Fig. 1.

Table 1. PCR primers sequence for 16S-rRNA gene of *Mycoplasma bovis* species.

Specie	Primers	Oligonucleotide sequence	Basepair	Reference
<i>Mycoplasma bovis</i>	MBOF2	5'GAAGCGAAAGTAGCATAGGAAATGAT3'	734 bp	Francis et al. 2020
	MBOR2	5'-CGTCGTCCCCACCTTCCTCCCG-3'		

Preparation of milk samples for culturing

For the preparation of milk samples, the 10 ml volume of each collected sample was taken into sterilized Falcon tubes and mixed with an equal volume of chloroform and centrifuged at 1957 x g for 5 minutes (Rossetti et al. 2010).

Isolation of *Mycoplasma bovis*

For the isolation of *M. bovis*, processed milk samples were inoculated simultaneously in modified Hayflick broth tubes and on modified Hayflick agar plates and were incubated in an anaerobic condition with 5% CO₂ at 37°C for 3-7 days. Incubated test tubes were inspected regularly for the appearance of turbidity and change in color and incubated plates were examined for “fried egg” appearance growth (Ahmad et al. 2014).

Cultural examination of *Mycoplasma bovis*

Preliminary identification of bacterial isolates was based on the typical *Mycoplasma bovis* colony grown on solid media. A typical characteristic colony of a fried egg appearance, tinny, flat, 0.1-1mm in diameter, with thick elevated centers embedded in the media, indicate the presence of *Mycoplasma bovis* in the collected milk samples (Ahmad et al. 2011).

Biochemical identification of isolated *Mycoplasma bovis*

Differentiation of *Mycoplasma bovis* isolates from other *Mycoplasma* species was done by using different biochemical tests. An amount of 0.5 µL of each isolate was diluted in 5 ml of modified Hayflick broth and was characterized by various biochemical tests, including pyruvate utilization, glucose fermentation, casein digestion, film and spot formation, phosphatase activity, tetrazolium reduction, urease activity, arginine hydrolysis and tween hydrolysis (Nicholas et al. 2016).

Molecular characterization of *Mycoplasma bovis*

After identification of *Mycoplasma bovis* colonies along with biochemical testing the confirmatory identification was made through 16S-rRNA Polymerase Chain Reaction. For PCR, DNA was extracted using Thermo Fisher Scientific USA Kit. After extraction

of DNA, its quality was confirmed on gel electrophoresis. PCR was performed using 16S-rRNA primers. Further PCR was confirmed on gel electrophoresis and purified PCR products using RNAase (Wisselink et al. 2019).

DNA extraction of *Mycoplasma bovis*

The positive samples were subjected to DNA extraction using the commercially available kit (Thermo Fisher, scientific USA) according to instructions of manufacturer.

Quantification of DNA

The extracted DNAs were further quantified for concentrations and purity (Spectra 260/280) with the NanoDrop-2000 (Thermo Fisher Scientific, USA) spectrophotometer (BioTek).

Polymerase chain reaction

The Polymerase Chain Reaction (PCR) was conducted for the identification of *Mycoplasma bovis* using *Mycoplasma bovis* specie-specific primers given in Table 1.

PCR conditions

Afterwards, the samples were subjected to amplification processes that included a 5- minute initial denaturation cycle at 94°C, followed by 40 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 59°C, 30 seconds of extension at 72°C, and 5 minutes hold at 4°C (Salina et al. 2020).

Gel electrophoresis

The amplified DNA was examined in gel electrophoresis, which was characterized by dissolving agarose at a concentration of 1% in Erlenmeyer flask containing 1X TBE buffer (Thermo Fisher, scientific USA).

Gel purification for DNA extraction of amplified PCR product

PCR amplified product was purified from gel using the manufacturer's protocol. Purified DNA was stored at 4°C.

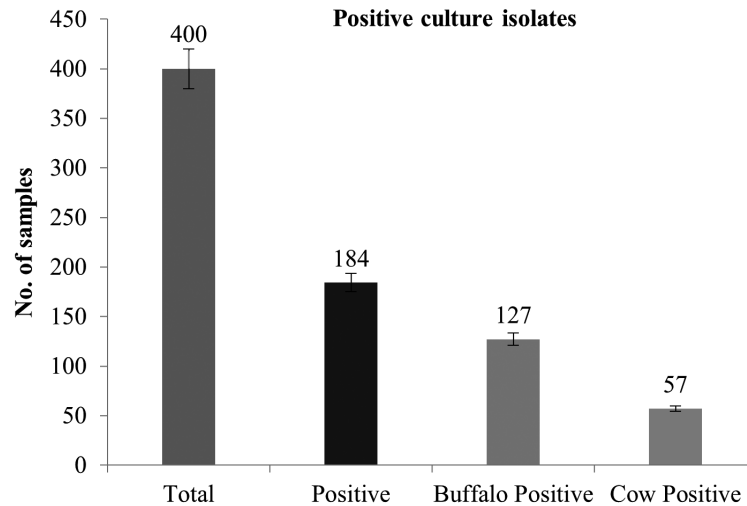


Fig. 2. Culture positive samples of isolated *M. bovis*.

DNA sequence analysis

The 16S-rRNA gene amplified *Mycoplasma bovis* products were subjected to DNA sequencing at sequencing laboratory, Advance Bioscience Internationals, Lahore, Pakistan, to determine PCR products nucleotide sequence. All the sequences were aligned using EditSeq and MegAlign software by the Clustal W method (Vereecke et al. 2020).

Phylogenetic analysis of *Mycoplasma bovis*

Strains of *M. bovis* isolated from different geographical areas of the district Faisalabad, Pakistan were compared using the maximum likelihood method with Bootstrap 1000 in Mega 7.0 software (MEGA4.exe). The four selected strains were further analyzed for the phylogenetic tree. The phylogenetic tree that was based on the molecular analysis and BLAST comparison results was observed and confirmed (Bokma et al. 2020). All four geographically isolated strains from different regions of the district Faisalabad, Pakistan, were submitted to Gene Bank to make the Phylogenetic tree.

Antibiogram of *M. bovis* by disc diffusion assay against commonly used antibiotics

Antibiogram of *M. bovis* by disc diffusion assay against commonly used antibiotics like tylosin (30 µg), telmicosin (95 µg), oxytetracycline (30 µg), spiramycin (100 µg), florfenicol (30 µg), gentamycin (10 µg), lincomycin (10 µg) and enrofloxacin (10 µg). Kirby-Bauer method was used to test the antimicrobial susceptibility. This method used disc diffusion assay to determine the sensitivity or resistance (Hudzicki 2009).

Statistical analysis

The prevalence of isolated *Mycoplasma bovis* within the herds of district Faisalabad was determined by using the Chi-square test and Odds ratio at 95% CI. Antibiogram of *M. bovis* was analyzed by independent t-test for commonly used antibiotics. Then the data were subjected to the One-way Analysis of Variance to compare the eight different antibiotics. All results were statistically analyzed using SPSS (version 16.0) software (Mojsoska et al. 2021).

Results

Isolation and identification of *Mycoplasma bovis*

The presence of *Mycoplasma bovis* in milk samples was examined by change of color in modified Hayflick broth. *M. bovis* colonies were characterized by a dense central core that grew down in modified Hayflick agar and a lighter peripheral zone of surface growth surrounding this area having a “fried-egg” appearance on modified Hayflick agar as shown in Fig. 1.

Total milk samples with culture method

Out of total n=400 raw milk samples which were collected from lactating buffaloes and cows, 184 (46%) samples were positive for *M. bovis* by culture method. Out of 265 buffalo milk samples, 127 (47.9%) positive isolates were identified and out of cow samples 57 (42.2%) positive isolates were identified as given in Figs. 2 and 3.

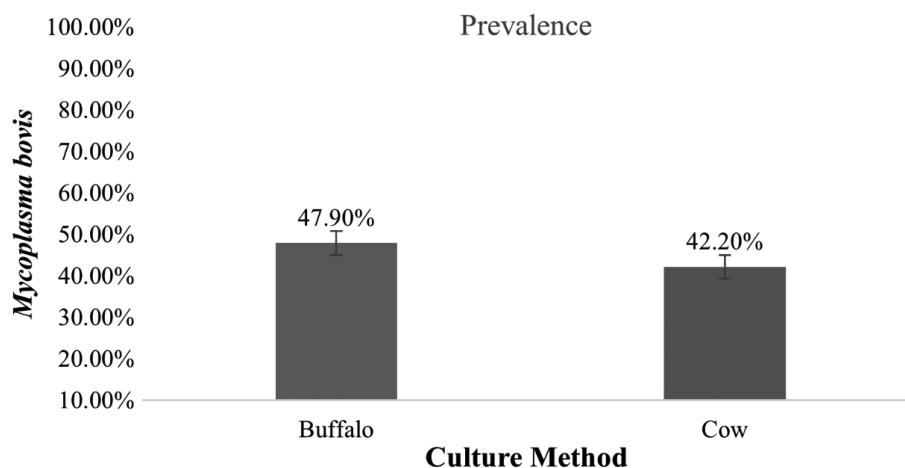


Fig. 3. Prevalence of isolated *M. bovis* in buffaloes and cows by culture method.

Table 2. Biochemical profiling of isolated *M. bovis*.

Biochemical Test	Results
Pyruvate	Positive
Phosphatase	Positive
Tetrazolium reduction	Positive
Film & spot formation	Positive
Glucose	Negative
Tween hydrolysis	Negative
Arginine hydrolysis	Negative
Urease activity	Negative
Casein digestion	Negative

Biochemical identification of isolated *Mycoplasma bovis*

Differentiation of *Mycoplasma bovis* isolates from other *Mycoplasma* species was done by using different biochemical tests. Results of *M. bovis* response to these biochemical tests were given in Table 2.

Molecular characterization of isolated *Mycoplasma bovis*

Polymerase Chain Reaction

Extraction of genomic DNA of *Mycoplasma bovis* isolates was done using a DNA extraction kit (Thermo Scientific) by following the guidelines of the manufacturer. PCR results showed a 734 bp PCR product as expected and the amplified product as shown in Fig 4.

Total milk sample with PCR

Out of total 400 samples, 240 (60%) were positive and 160 samples (40%) were negative according to the PCR of the 16S-rRNA gene. Out of 265 buffalo

samples, 169 (63.7%) samples were positive by PCR method. Out of 135 cow samples, 71 (52.6%) samples were positive for PCR as given in Figs. 5 and 6 and Table 3.

DNA sequence analysis

PCR products of amplified isolated *Mycoplasma bovis* multiple strains were sent to sequencing lab, Advance Bioscience Internationals, Lahore, Pakistan, to determine the nucleotide sequences. Multiple strains from this study from different geographical regions of district Faisalabad, Pakistan, were aligned and analyzed by Clustal W software and were compared to *Mycoplasma bovis* complete genome recorded at Gen Bank.

Phylogenetic analysis of *Mycoplasma bovis*

Four selected strains were compared using the Maximum likelihood method with Bootstrap 1000 in Mega 7.0 software (MEGA4.exe). Accession number assigned by NCBI and other details are given



Fig. 4. 16S-rRNA PCR positive 734 bp product of *M. bovis* in Lane 1, 7, 10, and 12 while Lane-M shows the molecular marker of 100 bp.

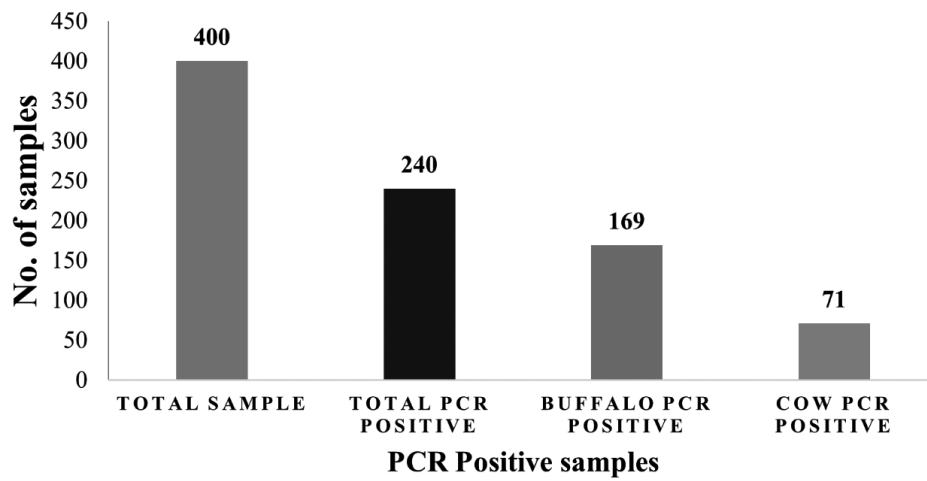


Fig. 5. PCR positive samples of isolated *M. bovis*.

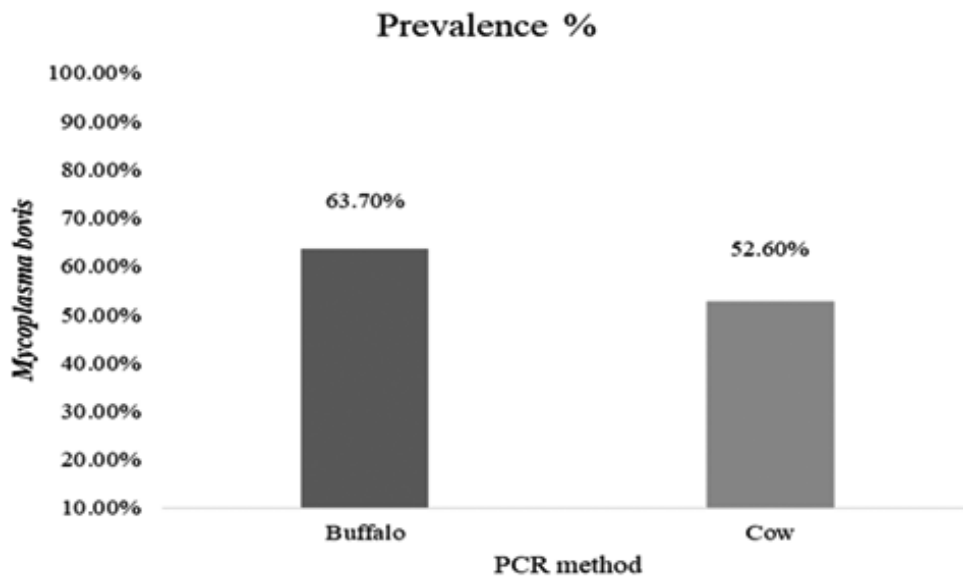


Fig. 6. Prevalence percentage of PCR positive samples of isolated *M. bovis*.

in Table 4. Finally, the four strains were further analyzed for the phylogenetic tree. Phylogenetic tree was generated and is given in Fig. 7.

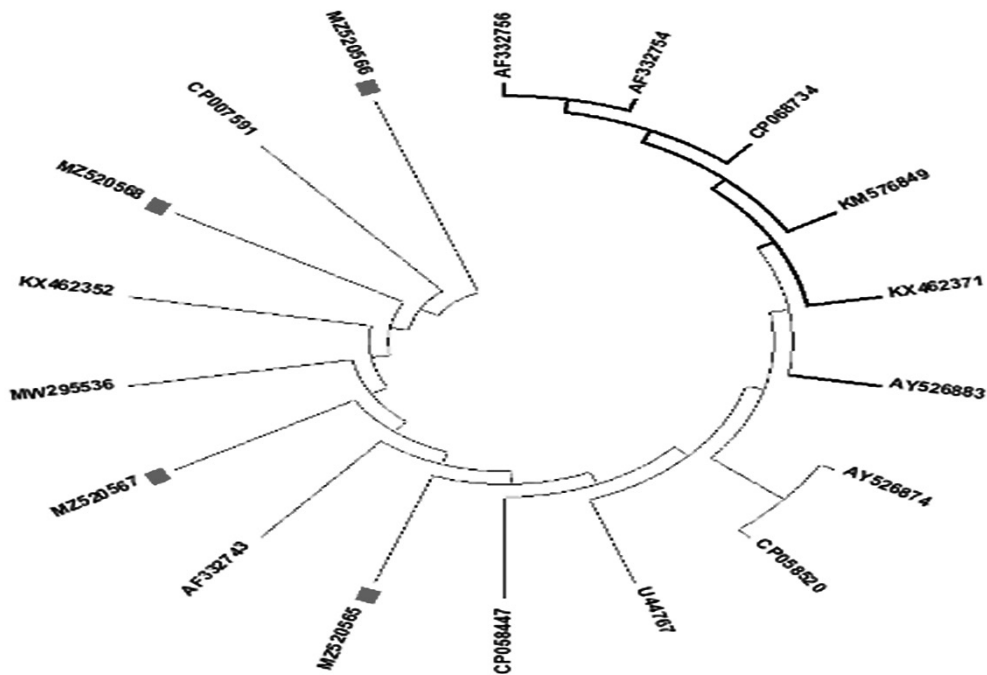


Fig. 7. Molecular phylogenetic analysis of isolated *M. bovis* by Maximum Likelihood method.

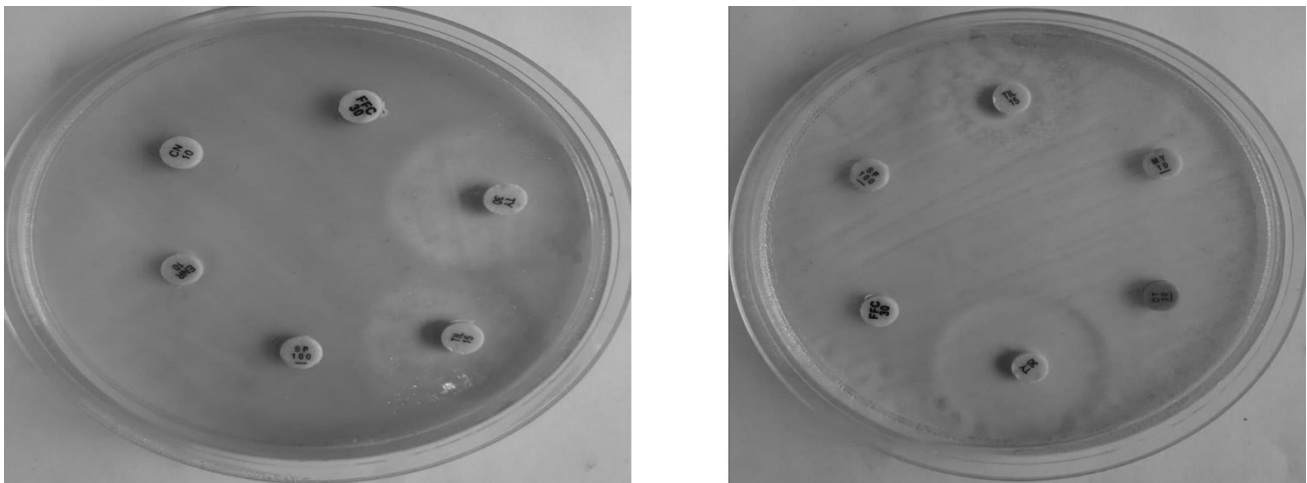


Fig. 8. Antibiogram assay of isolated *M. bovis* against florphenicol, gentamycin, enrofloxacin, spiramycin, tilmicosin, tylosin, oxytetracycline and lincomycin.

Antibiogram of isolated *Mycoplasma bovis* against commonly used antibiotics

Antibiogram studies of isolated *M. bovis* strains were performed by disc diffusion assay against commonly used antibiotics as given in Fig. 8 and Table 5.

Discussion

Salina et al. (2020) used Hayflick agar for *M. bovis* culture. They found 6% colony growth from culture of 100 samples (Passchyn et al. 2012) observed 1.5% *M. bovis* growth out of 200 dairy herds from bulk milk

tanks. They conclude that dairy herds of Flanders were containing more than one cow that is shedding *M. bovis* in milk samples. In this study, the prevalence of *M. bovis* was higher in buffaloes than in cow's raw milk; this may be due to the difference in the anatomy of teats (Verraes et al. 2014). The epithelium of the streak canal of teat is thicker and more compact in buffaloes than in cows. The sphincter muscle around the streak canal is thicker in buffaloes than in cows. Therefore, more force is required to open and close the streak canal (Caria et al. 2013).

Biochemical analysis of *M. bovis* revealed that film and spot formation, pyruvate reduction, phosphatase reduction, and tetrazolium reduction were positive while

Table 4. *Mycoplasma bovis* isolated strains submitted to NCBI with accession number.

Sr#	Strain	Origin	Collection year	Host	Accession No. from NCBI
1	IOM21	Faisalabad Pakistan	2021	Buffalo and cow	MZ520565.1
2	IOM21	Faisalabad Pakistan	2021	Buffalo and cow	MZ520566.1
3	IOM21	Faisalabad Pakistan	2021	Buffalo and cow	MZ520567.1
4	IOM21	Faisalabad Pakistan	2021	Buffalo and cow	MZ520568.1

Table 5. Antibiogram studies of isolated *M. bovis* strains against commonly used antibiotics.

Antibiotic Disc used	Disk code	Sensitive (S)	Intermediate (I)	Resistant (R)	Mean±SD	t-statistic	p-value
Tylosin (30µg)	TY30	≥24	-	-	32.34±1.10	3.53	0.047
					(S)		
Tilmicosin (15 µg)	TIL15	≥14	11-13	≤10	17.12±0.93	4.76	0.038
					(S)		
Spiramycin (100 µg)	SP100	≥14	11-13	≤10	0 (R)		
Enrofloxacin (10 µg)	ENR10	≥23	17-22	≤16	0 (R)		
Oxytetracyclin (30 µg)	OT30	≥19	15-18	≤14	0 (R)		
Lincomycin (10 µg)	MY10	≥15	13-14	≤12	0 (R)		
Gentamycin (10 µg)	CN10	≥15	13-14	≤12	0 (R)		
Florfenicol (30 µg)	FFC30	≥19	15-18	≤14	0 (R)		

* p-value of tyloin (30 µg) and tilmicosin (15 µg) is < 0.05 which indicates that data is significant Significance level 0.05

glucose utilization, tween hydrolysis, arginine hydrolysis, urease hydrolysis, and casein digestion were negative. These findings of biochemical analysis were also mentioned by (Nicholas et al. 2016) in their study.

Niu et al. (2021) analyzed biochemical identification of *M. bovis* from samples. Their results showed that none of the isolates fermented glucose, lactose, hydrolyzed gelatin and arginine, also could not decompose urea. The decomposition of urea could not be accomplished due to the lack of metabolic enzyme. These findings confirm that our results were similar.

The validation of culture method in the diagnosis of *M. bovis* was determined through PCR technique. This is considered highly sensitive and highly specific for the diagnosis of *M. bovis*. Out of total 400 raw milk samples from lactating cows and buffaloes, 60% samples showed positive result through PCR. Out of 265 buffalo milk samples, 63.7% were found positive and out of 135 cow samples, 52.6% were found positive with PCR. Imandar et al. (2018) explained that while performing PCR of 16S-rRNA gene of 328 samples, 31.97% amplification results were positive for *Mycoplasma* genus. Tiwari et al. (2022) described that samples were assessed for *M. bovis* using Polymerase

Chain Reaction (PCR). Prevalence of *M. bovis* was 42.5% in lactating cows, which agreed with current study results.

Molecular analysis by phylogenetic tree and BLAST comparison confirmed 100% similarity of the isolated strains with previously reported reference strains of *M. bovis* in Gene Bank following the same phylogenetic roots (Imandar et al. 2018). Farid et al. (2018) stated that partial sequences of the 16S-rRNA genes of the *Mycoplasma* isolates were obtained and phylogenetic analysis of these sequences deposited in the Gene Bank database were studied, together with related sequences in Gen Bank. The 16S-rRNA sequences of the *M. bovis* strains deposited in Gen Bank were identical to those obtained in our study.

In the present study, isolated strains of *M. bovis* were tested against eight commonly used antibiotics. The antibiogram of *M. bovis* strains revealed that they were sensitive to Tylosin and Tilmicosin and resistant to enrofloxacin, spiramycin, oxytetracyclin, lincomycin, florfenicol, and gentamycin. Farid et al. (2018) described that *M. bovis* was sensitive to tylosin, tilmicosin and enrofloxacin and resistant to other antibiotics. Results agreed according to sensitivity of tylosin, tilmi-

cosin and resistance of florfenicol and disagreed according to Enrofloxacin. Bokma et al. (2021) stated that *M. bovis* resistance to enrofloxacin, spiramycin, oxytetracyclin, lincomycin, florfenicol, and gentamycin may be due to mutation in 23S-rRNA or change in alleles of 16S-rRNA. In the present study, it was concluded that Tylosin was more effective against the isolated *M. bovis* strains.

The results of our study are also related to findings of Amit et al. (2012) who described that tylosin and enrofloxacin showed greater zone of inhibitions against *M. bovis* isolated from nasal, tracheal, blood, serum, faecal and tissue samples. In this study, enrofloxacin was also found effective, which is not in accordance with our study.

Conclusions

Mycoplasma bovis associated mastitis is a perilous problem for dairy industry throughout world. This research findings disclosed that *Mycoplasma bovis* is prevalent in buffaloes and cows in the study area. Tylosin was the most effective for treatment of *Mycoplasma bovis* associated mastitis and Tilmicosin was also effective.

References

- Adorno BM, Salina A, Joaquim S, Guimarães FF, Lopes BC, Menozzi B, Langoni H (2021) Presence of Mollicutes and *Mycoplasma bovis* in nasal swabs from calves and in milk from cows with clinical mastitis. *Vet Zootec* 28: 001-009.
- Ahmad Z, Babar S, Abbas F, Awan MA, Abubakar M, Attique MA, Hassan Y, Rashid N, Ali M (2011) Identification and molecular characterization of *Mycoplasma* species from bovine lungs samples collected from slaughter house, Quetta, Balochistan, Pakistan. *Pak J Life Soc Sci* 9: 91-97.
- Ahmad Z, Babar S, Abbas F, Awan MA, Shafee M, Tariq MM, Mengal MA, Rashid N, Amin S, Taj K, Ali M (2014) Prevalence of *Mycoplasma bovis* in respiratory tract of cattle slaughtered in Balochistan, Pakistan. *Pak Vet J* 34: 46-49.
- Alhussen MA, Kirpichenko VV, Yatsentyuk SP, Nesterov AA, Byadovskaya OP, Zhanovaty TV, Sprygin AV (2021) *Mycoplasma bovis*, *M. bovis genitalium* and *M. dispar* as Bovine Pathogens: Brief characteristics of the pathogens (review). *Agric Biol* 56: 245-260.
- Kumar A, Verma AK, Gangwar NK, Rahal A (2012) Isolation, characterization and antibiogram of *Mycoplasma bovis* in sheep pneumonia. *Asian J Anim Vet Adv* 7: 149-157.
- Behera S, Rana R, Gupta PK, Kumar D, Sonal, Rekha V, Arun TR, Jena D (2018) Development of real-time PCR assay for the detection of *Mycoplasma bovis*. *Trop Anim Health Prod* 50: 875-882.
- Bokma J, Vereecke N, De Bleeker K, Callens J, Ribbens S, Nauwynck H, Haesebrouck F, Theuns S, Boyen F, Pardon B (2020) Phylogenomic analysis of *Mycoplasma bovis* from Belgian veal, dairy and beef herds. *Vet Res* 51: 121.
- Bokma J, Vereecke N, Nauwynck H, Haesebrouck F, Theuns S, Pardon B, Boyen F (2021) Genome-wide association study reveals genetic markers for antimicrobial resistance in *Mycoplasma bovis*. *Microbiol Spectr* 9: e0026221.
- Buller H, Blokhuis H, Jensen P, Keeling L (2018) Towards farm animal welfare and sustainability. *Animals* 2018, 8: 81.
- Caria M, Boselli C, Murgia L, Rosati R, Pazzona A (2013) Influence of low vacuum levels on milking characteristics of sheep, goat and buffalo. *J Agr Eng* 44: 217-220.
- Cheng WN, Han SG (2020) Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments – A review. *Asian-Australas J Anim Sci* 33: 1699-1713.
- Deeney A S, Collins R, Ridley AM (2021) Identification of *Mycoplasma* species and related organisms from ruminants in England and Wales during 2005-2019. *BMC Vet Res* 17: 325.
- Farid MA, Abo-Shosha AA, Belal ES, Hassan MM (2018) Genotyping of pathogenic *Mycoplasma bovis* isolated from cattle in Kafr El-Sheikh Province, Egypt. *J Pure Appl Microbiol* 12: 2103-2109.
- Ghafar A, McGill D, Stevenson MA, Badar M, Kumbher A, Warriach MH, Gasser RB, Jabbar A (2020) A participatory investigation of bovine health and production issues in Pakistan. *Front Vet Sci* 7: 248.
- Hata E, Harada T, Itoh M (2019) Relationship between antimicrobial susceptibility and multilocus sequence type of *Mycoplasma bovis* isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines, and spectinomycin. *Appl Environ Microbiol* 85: e0057519.
- Hudzicki J (2009) Kirby-Bauer disk diffusion susceptibility test protocol. *Am J Mol Biol* 8: 2009.
- Ilyas F, Gillani DQ, Yasin M, Iqbal MA, Javed I, Ahmad S, Nabi I (2022) Impact of Livestock and Fisheries on Economic Growth: An Empirical Analysis from Pakistan. *Sarhad J Agric* 38: 160-169.
- Imandar M, Pourbakhsh SA, Jamshidian M, Salehi TZ (2018) Isolation, identification and molecular characterization of *Mycoplasma bovis* in mastitic dairy cattle by PCR and culture methods. *J Hell Vet Med Soc* 69: 815-822.
- Imran M, Rehman I, Sulehria AQ, Butt YM, Khan AM, Ziauddin A (2021) Profile of Antimicrobial Susceptibility from Cattles's Milk Isolates Suffering from Mastitis in District Lahore. *J Biores Manag* 8: 6-14.
- Khan ZU (2022) Laws, Issues, Challenges, Analysis of Livestock Sector and International Best Practices. *J Dev Soc Sci* 3: 271-283.
- Klein U, de Jong A, Moyaert H, El Garch F, Leon R, Richard-Mazet A, Rose M, Maes D, Pridmore A, Thomson JR, Ayling RD (2017) Antimicrobial susceptibility monitoring of *Mycoplasma hyopneumoniae* and *Mycoplasma bovis* isolated in Europe. *Vet Microbiol* 204: 188-193.
- Konigsson MH, Bolske G, Johansson KE (2002) Intraspecific variation in the 16S-rRNA gene sequences of *Mycoplasma agalactiae* and *Mycoplasma bovis* strains. *Vet Microbiol* 85: 209-220.
- Mahmood F, Khan A, Hussain R, Khan IA, Abbas RZ, Ali HM, Younus M (2017) Patho-bacteriological investigation of an outbreak of *Mycoplasma bovis* infection in calves-

- Emerging stealth assault. *Microb Pathog* 107: 404-408.
- Maunsell FP, Donovan GA, (2009) *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract* 25: 139-177.
- Maunsell FP, Woolums AR, Francoz D, Rosenbusch RF, Step DL, Wilson DJ, Janzen ED (2011) *Mycoplasma bovis* infections in cattle. *J Vet Inter Med* 25: 772-783.
- Mojsoska B, Ghoul M, Perron GG, Jenssen H, Alatraktchi FA (2021) Changes in toxin production of environmental *Pseudomonas aeruginosa* isolates exposed to sub-inhibitory concentrations of three common antibiotics. *PLoS One* 16: e0248014.
- Nicholas RA, Fox LK, Lysnyansky I (2016) *Mycoplasma* mastitis in cattle: To cull or not to cull. *Vet J* 216: 142-147.
- Niu J, Wang D, Yan M, Chang Z, Xu Y, Sizhu S, Li Z, Hu S, Bi D (2021) Isolation, identification and biological characteristics of *Mycoplasma bovis* in yaks. *Microb Pathog* 150: 104691.
- Pal A, Chakravarty AK (2020) Disease resistance for different livestock species. *Genet Breed Dis Resist Livest* 2020: 271-296.
- Passchyn P, Piepers S, De Meulemeester L, Boyen F, Haesebrouck F, De Vlieghe S (2012) Between-herd prevalence of *Mycoplasma bovis* in bulk milk in Flanders, Belgium. *Res Vet Sci* 92: 219-220.
- Perez-Casal J, Prysliak T, Maina T, Suleman M, Jimbo S (2017) Status of the development of a vaccine against *Mycoplasma bovis*. *Vaccine* 35: 2902-2907.
- Romero J, Benavides E, Meza C (2018) Assessing financial impacts of subclinical mastitis on Colombian dairy farms. *Front Vet Sci* 5: 273
- Rossetti BC, Frey J, Pilo P (2010) Direct detection of *Mycoplasma bovis* in milk and tissue samples by real-time PCR. *Molar Cell Pro.* 24: 321-323.
- Salina A, Timenetsky J, Barbosa MS, Azevedo CM, Langoni H (2020) Microbiological and molecular detection of *Mycoplasma bovis* in milk samples from bovine clinical mastitis. *Pesqui Vet Bras* 40: 82-87.
- Shao Y, Wang Y, Yuan Y, Xie Y (2021) A systematic review on antibiotics misuse in livestock and aquaculture and regulation implications in China. *Sci Total Environ* 798: 149205.
- Abadi AT, Rizvanov AA, Haertlé T, Blatt NL (2019) World Health Organization report: current crisis of antibiotic resistance. *BioNanoScience* 9: 778-788.
- Gogoi-Tiwari J, Tiwari HK, Wawegama NK, Premachandra C, Robertson ID, Fisher AD, Waichigio FK, Irons P, Aleri JW (2022) Prevalence of *Mycoplasma bovis* Infection in Calves and Dairy Cows in Western Australia. *Vet Sci* 9: 351-358.
- Vereecke N, Bokma J, Haesebrouck F, Nauwynck H, Boyen F, Pardon B, Theuns S (2020) High quality genome assemblies of *Mycoplasma bovis* using a taxon-specific Bonito base-caller for MinION and Flongle long-read nanopore sequencing. *BMC Bioinform* 21: 517.
- Verraes C, Claeys W, Cardoen S, Daube G, De Zutter L, Imberechts H, Dierick K, Herman L (2014) A review of the microbiological hazards of raw milk from animal species other than cows. *Inter Dairy J* 39: 121-130.
- Wisselink HJ, Smid B, Plater J, Ridley A, Andersson AM, Aspan A, Pohjanvirta T, Vahanikkila N, Larsen H, Hogberg J, Colin A, Tardy F (2019) A European interlaboratory trial to evaluate the performance of different PCR methods for *Mycoplasma bovis* diagnosis. *BMC Vet Res* 15: 86.