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RESEARCH ARTICLE

Molecular Detection and Phylogenetic Analysis of *Mycoplasma Bovis* in Bovine Calves

Ali Raza[1], Riaz Ahmed Gul[1], Khalid Mehmood[1*], Riaz Hussain[2], Kashif Iqbal[3], Abdul Sameed Saher[1], Muhammad Taslim Ghori[1], Sadaqat Ali[1], Muhammad Altaf[1], Amir Iftikhar Malik[1], Muhammad Zahid[1], Dalia Fouad[4] and Kun Li[5*]

[1]Department of Clinical Medicine and Surgery, Faculty of Veterinary and Animal Sciences, The Islamia University Bahawalpur 63100, Pakistan; [2]Department of Pathology, Faculty of Veterinary and Animal Sciences, The Islamia University Bahawalpur 63100, Pakistan; [3]Department of Microbiology, Faculty of Veterinary and Animal Sciences, The Islamia University Bahawalpur 63100, Pakistan; [4]Department of Zoology, College of Science, King Saud University, PO Box 22452, Riyadh 11495, Saudi Arabia; [5]Institute of Traditional Chinese Veterinary Medicine & MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China
*Corresponding author: khalid.mehmood@iub.edu.pk; lk3005@njau.edu.cn

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A B S T R A C T

Mycoplasma bovis (*M. bovis*) is one of the important pathogens of cattle, which have been detected from pneumonic lungs of dairy cattle especially calves. *M. bovis* is main reason for morbidity and mortality in dairy calves because it is overlooked due to its late detection. It is considered that some typical clinical ailments, postmortem and microscopic lesions are useful for the diagnosis of disease but molecular confirmation through PCR is more reliable technique. The present study was conducted for detection and phylogenetic analysis of *M. bovis* induced mortality in bovine calves. A total of 193 lung and pleural fluid samples (bovine calves) were collected from slaughterhouses and private farms in Bahawalpur region. The DNA was extracted from samples by using DNA extraction kit. The samples were analyzed by PCR using *M. bovis*-specific primers having a length of 270bp. The PCR results showed that *M. bovis* was detected in 16(8.29%) isolates, which included 14(7.90%) lung tissues and 2(12.50%) pleural fluid samples. The season-wise positive samples were 12.12% in winter, 10.34% in spring and 2.89% in summer. The sequences were submitted to NCBI under accession number allotted as PP738878-PP738885. The BLAST was performed for sequence alignment by windows 7.1 software MEGA Muscle 37. The phylogenetic analysis showed the maximum and minimum identity of 97-100% among these geographical obtained strains from local isolates. The reference strain PP738878 had 100% identity with Swiss strain AF003959.1 of *M. bovis* and other strains showed a minimum identity of 99% with Poland, Switzerland and Egypt. This is the first study of the molecular detection and phylogenetic analysis of *M. bovis* in bovine calves in Pakistan.

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INTRODUCTION

Mycoplasma bovis (*M. bovis*) belongs to Mycoplasmataceae, which targets dairy animals and major threat to large-scale rearing calves in tropical and subtropical countries like Pakistan (Ali *et al.,* 2022; Saher *et al.,* 2024). *M. bovis* is recognized as the causative agent of arthritis, otitis, pneumonia and mastitis while in late 80s mycoplasma was marked as the root of respiratory infection in calves (Dudek *et al.,* 2020). The progress of dairy and beef industry is affected by *M. bovis* infection considerably in the world (Gelgie *et al.,* 2024). *M. bovis* was isolated locally from joint exudates, diaphragmatic fluid and lung tissues (Mahmood *et al.,* 2017). The antibiotic response upon treatment is low and diagnosing asymptomatic carrier animals is difficult (Schibrowski *et al.* 2018). Previous studies reported that newly bought calves and unauthorized semen used in artificial insemination can introduce *M. bovis* infection in calves (Murai and Higuchi, 2019; Haapala *et al.,* 2021). Therefore, its infection can be transmitted from older cattle to youngers in head-to-head enclosures (Vähänikkilä *et al.,* 2019).

Neonatal calves may get infection from older animals and thus infection is sustained within a herd (McAloon *et al.,* 2022). Calves below 4 months are more susceptible to this infection and the positive cases are less between 8-12 months age (Serrano *et al.,* 2018). *M. bovis* infection arises due to direct and indirect contact of calves within a flock (Timonen *et al.,* 2020). In chronic cases, the prominent lesion upon slaughtering led to a putrefaction of lungs with multifocal to coalescing pulmonary nodes comprising white-yellowish caseous exudation bordered by fibrotic tissues and fibrin necrotic rheumatism and tendosynovitis were noticed (Saher *et al.,* 2024). These clinical observations verify that principal reason for respiratory disease in healthy calves is *M. bovis* (Maunsell *et al.,* 2011). Animals with no apparent symptoms may act as infection reservoir, increasing the disease's occurrence and spreading within the flock without clinical intervention (Dudek *et al.,* 2020).

The remedy to overcome the infection is slaughtering and solicitation of infected individuals (Cantón *et al.,* 2022), sterilization of milk for feeding and grouping the calves of different ages. The present study aimed for molecular confirmation and phylogenetic analysis of *M. bovis* strains in Pakistan to provide the data on the position of circulating *M. bovis* for disease prevention.

MATERIALS AND METHODS

Study Area: Cattle calves on public slaughterhouses and private farms of large herd size were included in the present study in different areas of Bahawalpur district, Punjab, Pakistan (Fig. 1). The history of calf and other health parameters were collected during sampling.

Clinical signs observation: Before slaughtering of animals, the calves having runny eyes and nasal secretion with white creamy discharge, in advanced disease condition: acute to chronic respiratory illness, cough, fever, arthritis, lameness, swelling of the knee, elbow, carpal or tarsal joints were selected (Fig. 2). After slaughtering, the lungs were collected from animals having severe multifocal to coalescent nodules containing whitish-yellow caseous fluid (Ahmad *et al.,* 2024). The pleural fluid was collected at necropsy examination from suspected animals containing white creamy froth (putrefactive smell) in the pleura (Gaurivaud and Tardy, 2022).

Sample collection: A total of 193 calves were included for sample collection from May-2023 to April-2024. The samples of lungs from morbid calves were collected in sterile plastic bags from slaughterhouses located at Bahawalpur, Yazman, AhmedPur East and Hasilpur and then stored at -20°C. The inflammatory exudates and fluid (pulmonary) were collected and stored at -20°C till further processing.

Genomic DNA Extraction: The DNA was extracted from the tissue sample by using a DNA extraction kit (Thermo Fischer 182001, China). The suspected lung samples (0.5g) were cut into small fragments with sterilized surgical blades and homogenate (80µl) was prepared (Hashem *et al.,* 2022). Then Proteinase K (20µl) was added and incubated for 3 hours at 56°C. After that, 20µl of RNase was added and incubated for 10 minutes at room temperature. The lysis solution (200µl) was mixed in it and vortexed for 15 s until a homogeneous blend was obtained. Further process was followed according to DNA extraction kit (Thermo Fischer, China) as per manufacturer's instructions (Okella *et al.,* 2023).

Molecular detection of *M. bovis*: The samples were analyzed by PCR using *M. bovis*-specific primers as mentioned in Table 1 having a length of 270 bp (Salina *et al.,* 2020). The amplification of specific genes of *M. bovis* was performed according to Abdeen *et al.* (2017). The reaction mixture of 20µl, having 10µl of master mix, 1ul of forward and reverse (10pmol concentration) primer, 5µl of template DNA, and 3µl of PCR water was added and then vortexed for 15 s. The reaction steps were followed at 95°C for 7.5 min as initial denaturation and final denaturation for 30sec, annealing at 56°C for 30sec, initial extension at 72°C for 1min and 5min final extension for 30 cycles, trailed through final extension at 72°C for 5 minutes (Bernitz *et al.,* 2021; Rehman *et al.,* 2022).

Table 1: The primers used for detection of *M. bovis.*

Primers	Oligonucleotide sequence	Amplicon	Gene
GPO-3-F	5'GGGAGCAAACAGGATTAG ATACCCT 3'	270bp	16S rRNA
MGSO-R	5'TGCACCATCTGTCACTCTG TTAACCT3'		

Gel electrophoresis: The amplified products were stained with ethidium bromide stain through electrophoresis in 2% (w/v) agarose gel using 50x TBE buffer. After 45-minutes, the gel was visualized on a UV transilluminator to spot bands. A Bio-Rad Gel EZ-imager device (λ=302nm) visualized and snapped DNA fragments.

Sequencing and alignment: These isolates were numbered KM1- KM8 on gel electrophoresis. The amplified fragments were purified using Gene Jet PCR purification kit (Fermentas; Cat no. KO701). PCR product was purified to use in EZ-10 spin column DNA Gel Extraction Kit (Biobase, Canada) and sent for sequencing from private company (Macrogen, South Korea). The sequences results were blasted in NCBI to obtain accession number PP738878-PP738885. For similarity index and multiple alignment, we performed CLUSTAL W alignment tool and windows 7.1 official.

Phylogenetic analysis: The bioinformatics resource such as the NCBI's Basic Local Alignment Search Tool (BLAST) and BioEdit software's ClustalW technique (version 7.5.0.3) were used for comparison and align nucleotide sequences and analyses, respectively (Bokma *et al.,* 2020). The MEGA tool created a phylogenetic tree using the maximum likelihood technique with 1000 iterations of bootstrap analysis after multiple sequence alignment (Barry, 2013). The tree was created using neighbour-joining from a distance matrix corrected by the one-parameter nucleotide substitution model with already designated accession numbers on NCBI showing resemblance to *M. bovis* genes (Kumar *et al.,* 2018).

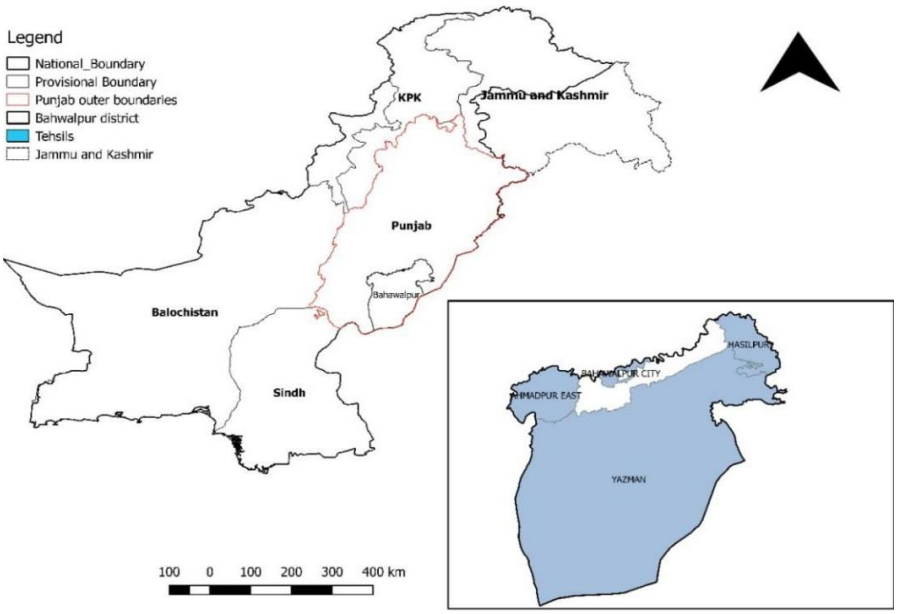

Fig. 1: Geographical orientation of the study area.

Fig. 2: Clinical signs and apparent pathological lesion in infected calves (a) Nasal discharge, arthritis in infected calves, (b) Caseous necrotic and coalescent nodules filled with off-white to yellow mass (c) Pleura effusions and fluid accumulation in the walls of the diaphragm (d) Lung consolidation, suppuration, white or blood-tinged froth in bronchi.

Table 2: Detection of *M. bovis* from lung tissue and pleural fluid samples of calves.

Sample	No +ve (Prevalence)	Total	95% CI	Odd Ratio/ P-value
Lung tissue	14 (7.90%)	177	4.57 to 12.62	Odds ratio= 1.66
Pleura fluid	2 (12.50%)	16	2.15 to 35.52	(reciprocal= 0.60)
Total	16 (8.29%)	193	4.98 to 12.84	
Seasons wise prevalence				
Summer (Jun-Aug)	2 (2.89%)	69	0.49 to 9.25	Mantel-Haenszel chi-sq
Winter (Dec-Feb)	8 (12.12%)	66	5.79 to 21.72	P = 0.081
Spring (Mar-May)	6 (10.34%)	58	4.30 to 20.27	
Total	16 (8.29%)	193	4.98 to 12.84	

Table 3: Nucleotide sequence similarity between Pakistani, Egyptian, Polish, and Swiss strains all over the Globe following reference paper.

GenBank Accession No.	Similarity (%)	GenBank Accession No.	Similarity (%)
AF003959.1(Switzwerland)	100	SUB14416526(KM1PP738878)	100
KP099618.1(Egy-10-FA-14)	100	SUB14416526(KM2PP738879)	99.57
KP099619.1(Egy-11-DK-14)	95.27	SUB14416526(KM3PP738880)	99.12
KP691394.1(Poland)	100	SUB14416526(KM4PP738881)	98.94
KP691397.1(Poland)	99.91	SUB14416526(KM5PP738882)	98.95
KP691398.1(Poland)	100	SUB14416526(KM6PP738883)	98.40
KP691399.1(Poland)	100	SUB14416526(KM7PP738884)	97.88
KP691400.1(Poland)	100	SUB14416526(KM8PP738885)	97.85

Statistical analysis: The PCR data was checked for positive and negative numbers together with 95% confidence intervals by WinPepi software. Multiple alignment was integrated by software MEGA 10 alignment and similarities were compared.

RESULTS

Molecular Detection of *M. bovis*: The phenotypic confirmed *M. bovis* isolates were identified by PCR genotypically, the isolates exhibiting a band of 270 bp were declared positive for the 16S rRNA gene of *M. bovis* as shown in Fig. 3. The positive isolates for *M. bovis* were sliced and sequenced to compare with already stated sequences of *M. bovis* isolates from all other countries. The strong positive samples 16S rDNA PCR amplicons confirmed that they were the same length as *Mycoplasma* sp. The *Mycoplasma* identity was verified by the 16S rDNA PCR products.

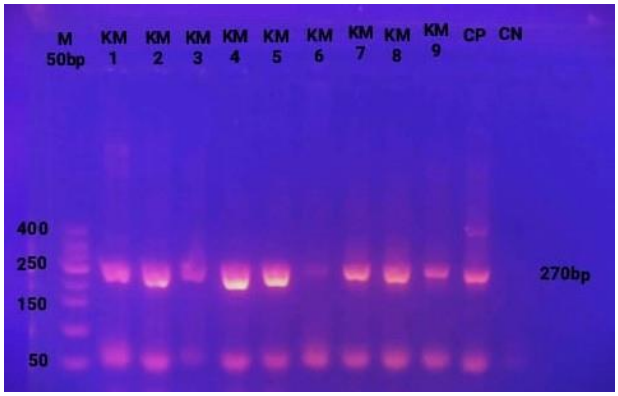

Fig. 3. GEL electrophoresis and PCR product analysis from 8 out of 16 *Mycoplasma* isolates attained by amplification with a universal primer of 16S rDNA. CP: Control positive; CN: Control negative. The size of the amplicons was 270 bp.

Seasonal Outcomes with sample types: The PCR results showed that *M. bovis* was detected in 16(8.29%) isolates (95% CI: 4.98-12.84), which including 14 lung tissues and 2 pleural fluid samples. On the basis of sample positiveness, the result showed 7.90% (95% CI: 4.57-12.62) in lung tissue while on pleural fluid sample positivity was 12.50% (95% CI: 2.15-35.52). The odd ratio indicted that both lung tissue and pleural fluid samples were associated with *M. bovis* detection. On the basis of season, the positive samples in Winter (Dec-Feb) 12.12% (95% CI: 5.79-21.72), Spring (March-May) 10.34% (95% CI: 4.30-20.27) and Summer (June-Aug) 2.89% (95% CI: 0.49-9.25) in Bahawalpur region (Table. 2). Although, the positive samples were more in winter and spring seasons compared with summer but the difference was not significant ($P \geq 0.081$).

Sequencing analysis: The sense and antisense sequences obtained as electropherograms were visualized in Chromas software version 2.6.5 (Technelysium Pty Ltd 1998-2018©) and manually checked for edge removal and identification of errors. *M. bovis* isolates succeeding Accession numbers from calf samples in the Bahawalpur region, Punjab, Pakistan is followed as SUB14416526 KM1 (PP738878), SUB14416526 KM2 (PP738879), SUB14416526 KM3 (PP738880), SUB14416526 KM4 (PP738881), SUB14416526 KM5 (PP738882), SUB14416526 KM6 (PP738883), SUB14416526 KM7 (PP738884), SUB14416526 KM8 (PP738885).

Phylogenetic analysis: These 8 reported strains from different geographical areas were compared on percentage similarity basis. The results showed the maximum and minimum identity of 97-100% among these geographical obtained strains as shown in Table 3. From the 8 selected local isolated reference strain (PP738878) had 100% identity with Swiss strain AF003959.1, which showed the maximum identity with the reference *M. bovis* sequence and the other strains showed a minimum identity of 99% with Poland, Switzerland and Egypt (Fig. 4a). Phylogenetic tree analysis describes that PP738878-PP738885 were clustered together with *M. bovis* reference strain (AF003959.1, KP099618.1, KP099619.1, KP691397.1, KP691398.1, KP691399.1, KP691400.1). The species wise relation to reference strains clustered and frequencies of different species with our local isolates is shown in Fig. 4b.

Sequence analysis and alignment: The obtained nucleotide sequences compared with reference *M. bovis* by Clustal OMEGA for Multiple sequences alignment using Phylip format revealed that the distance between current isolates and available *M. bovis* references was 0.011-0.008 as shown in Fig. 5. The selected 8 sequences were further analyzed by BLAST to compare the *M. bovis* amino acid and strain's nucleotide sequences against other strains that were published on GenBank. BLAST Alignment of local isolates of *M. bovis* with reference isolates after trimming ends and colored image to highlight similarities is given in Fig. 6.

DISCUSSION

M. bovis is a major bacterial pathogen, which have been detected from pneumonic lungs of dairy animals especially calves, which is an important threat for respiratory system in bovines to induce pneumonia (Oliveira *et al.*, 2020). Rapid and substantial propagation of infectious agents within the mucosae of respiratory tract causes damages to defensive walls and epithelium of respiratory systems leading to per acute, acute, sub-acute and chronic pneumonic changes (Torres *et al.*, 2021). Some typical clinical ailments, postmortem and microscopic lesions are useful for the diagnosis disease but molecular confirmation through PCR is more reliable technique. *M. bovis* is one of the main reasons for morbidity and mortality in dairy calves (Askar *et al.*, 2021). Previous study showed that it caused respiratory distress, lameness and swollen joints, ultimately death in young calves, which put significant economic burden on livestock farmers (Ashraf *et al.*, 2018). The present study isolated and detected *M. bovis* from calves through PCR. This is the first molecular confirmation study of *M. bovis* from calves in Pakistan. This study revealed overall positive rate of 8.29% (16/193) in Bahawalpur, Pakistan. Our results are in line with previous report in which prevalence of *M. bovis* from lungs samples of cattle collected from slaughterhouses was 9% in Balochistan. It

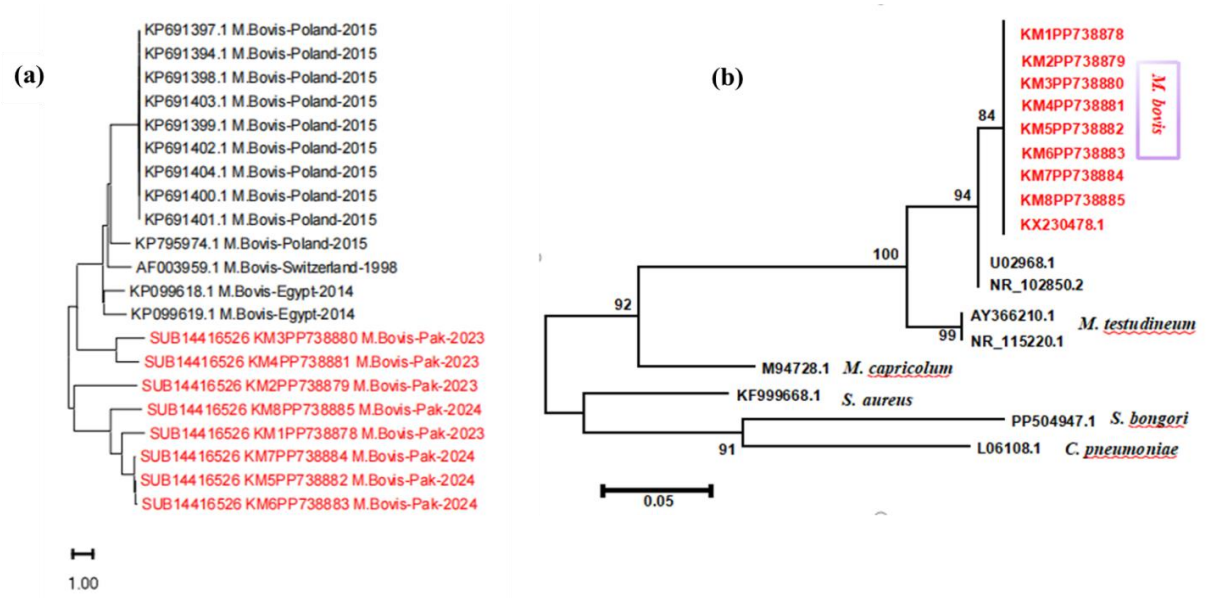

Fig. 4: Phylogenetic tree illustrating all groups and clusters based on *M. bovis* 16S rRNA sequences. The tree was constructed by using neighbour-joining from a distance matrix corrected by the one-parameter nucleotide substitution model. (a) Resemblance of genus *M. bovis* with other countries. (b) Phylogenetic tree illustrating specie wise resemblance with different species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. KM1		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
2. KM2	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
3. KM3	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
4. KM4	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
5. KM5	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
6. KM6	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
7. KM7	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
8. KM8	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
9. KX230478.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.008	0.008	1.368	0.716	1.597	0.601	0.021	0.021
10. U02968.1	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011		0.000	1.367	0.717	1.596	0.601	0.020	0.020
11. NR_102850.2	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.000		1.367	0.717	1.596	0.601	0.020	0.020
12. PP504947.1	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.321	0.321		1.088	0.967	1.186	1.367	1.367
13. KF999668.1	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.249	0.249	0.239		1.303	0.673	0.716	0.716
14. L06108.1	0.343	0.343	0.343	0.343	0.343	0.343	0.343	0.343	0.343	0.354	0.354	0.221	0.232		1.394	1.589	1.589
15. M94728.1	0.216	0.216	0.216	0.216	0.216	0.216	0.216	0.216	0.216	0.209	0.209	0.281	0.180	0.250		0.597	0.597
16. AY366210.1	0.065	0.065	0.065	0.065	0.065	0.065	0.065	0.065	0.065	0.059	0.059	0.320	0.266	0.336	0.196		0.000
17. NR_115220.1	0.065	0.065	0.065	0.065	0.065	0.065	0.065	0.065	0.065	0.059	0.059	0.320	0.266	0.336	0.196	0.000	

Fig. 5: Multiple sequence analysis of *M. bovis* isolates with reference sequences.

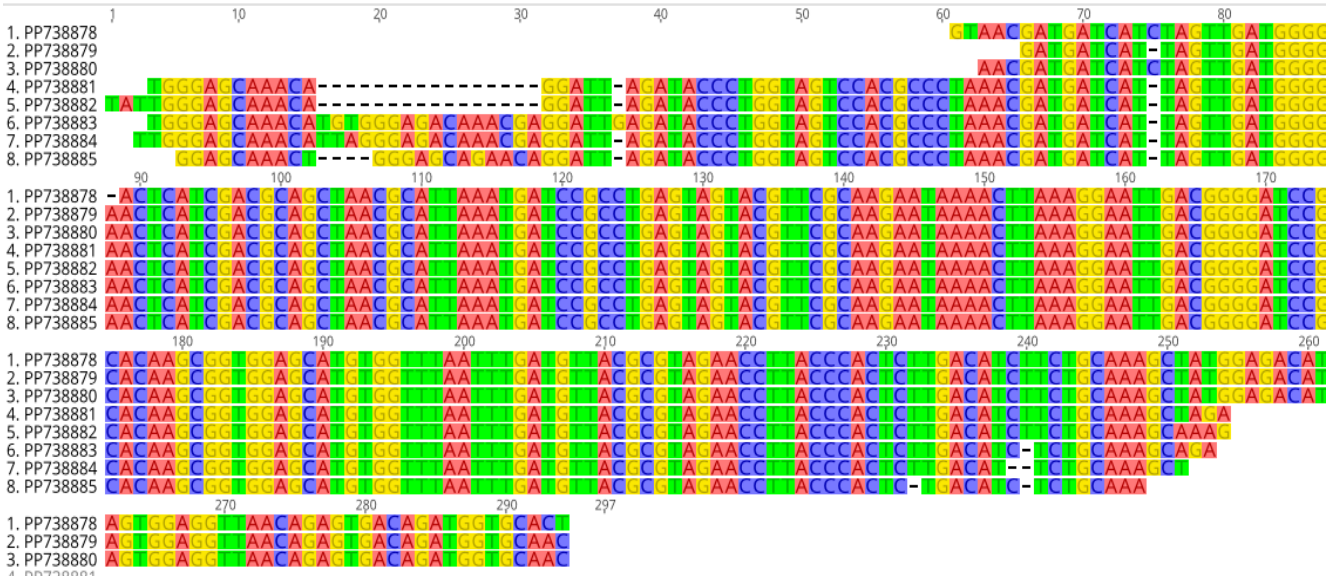

Fig. 6: Blast Alignment of local isolates with reference isolated of M. bovis after trimming ends and colored to highlight similarities followed and prepared by software MEGA X alignment with Muscle.

was highest in Quetta (16%) followed by Pishin (15%), Zhob (11%) and Kalat (10%), while lowest in Bolan (2%) followed by Jaffarabad (3%), Sibi (7%), and 4% each in Khuzdar, Mustung, and Killasaifullah district (Ahmad *et al.,* 2014) through microscopic confirmation. In Adamawa State and other two northwestern States of Nigeria have 8.9% isolation rate (Tambuwal and Egwu, 2017). A global prevalence of *M. bovis* during 2009-2019 was recorded 12.1%. The *M. bovis* accompanied by considerable morbidity (8-13%) and mortality rates (13-30%) (Taye *et al.,* 2021).

The detection and isolation rates of *M. bovis* varied in some studies when compared to our results. It is studied that *M. bovis* isolation rate was 70.5% in individual calves in Belgian, while 84.6% isolation rates were recorded when tested at herd level (Pardon *et al.,* 2011). In Egypt at molecular level 32% isolation rate was observed through PCR (Abdalhamed *et al.,* 2024). In Turkey, herds were positive with 87.6% (149/172) isolation rate from 2010-2015 (Sayin *et al.,* 2016). This variation in prevalence may be due to different geographical location and temperature fluctuation as *M. bovis* infection is more prevalent in winter season and these countries have prolonged winter season with low temperature than Pakistan. The main difference in prevalence might be due to the different environmental conditions, age, health score, feeding status and the strains of animals (Schibrowski *et al.,* 2018).

Our sample sequences presented 97-100% similarity when Sequence analysis of the gene nucleotide was performed according to previous study (Salina *et al.,* 2020). Swiss isolates showed 100% similarity, Polish strains showed 97-99% similarity and Egyptian strain showed 99% similarity to our isolates. In this research sequence analysis and alignment of strains were made possible by MEGA X software (Tamura *et al.,* 2021). Species wise phylogeny tree was compared the resemblance with other ones by Clustal OMEGA software and equated with sequences of *M. bovis* documented to Gene Bank. Results confirmed that protein patterns within *M. bovis* strains were supportive for the evaluation (Hala and Hotzel, 2013).

Our results showed that PCR is reliable and useful technique for the detection of *M. bovis* from infected lung tissues. Vähänikkilä *et al.* (2019) employed PCR in place of the microbial culture or together for the confirmatory diagnosis of *M. bovis*. The present results recommend that PCR technique should be preferred for the detection of *M. bovis* from pneumonic lungs and to differentiate with other Mycoplasma species.

Conflict of interest: None

Authors contributions: AR, RAG, KI, ASS: Writing - original draft, Resources, Methodology, Formal analysis; KM, RH, DF, KL: Conceptualization, Resources, Funding acquisition, Writing - original draft. MGT, SA, MA, AIM, MZ: Review and Editing. All the authors approved the publication.

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