Journal of Animal Research: v.12 n.06, p. 993-999. December 2022

DOI: 10.30954/2277-940X.06.2022.24

Detection of Drug Resistance in *Mycobacterium tuberculosis* Complex Isolates From Cattle and Buffaloes

Poloju Deepa[1], Gursimran Filia[2*], Deepti Narang[1], Leishangthem Geeta Devi[3] and Sikh Tejinder Singh[4]

[1]Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana Punjab, INDIA

[2]Animal Disease Research Centre, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA

[3]Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA

[4]Directorate of Livestock farm, Guru Angad Dev Veterinary and Animal Sciences University, INDIA

**Corresponding author: G Filia; E-mail: harpalfilia@rediffmail.com*

Received: 27 Oct., 2022 **Revised:** 28 Nov., 2022 **Accepted:** 30 Nov., 2022

ABSTRACT

Drug-resistant mycobacterial strains are increasingly being isolated from human cases. The identification of mycobacterial species in cattle and buffaloes is crucial for identifying the danger of drug-resistant strains being transmitted from animals to humans. In present study, a total of 200 animals (105 cattle and 95 buffaloes) from organized dairy farm were screened by CITT. Fifteen blood samples from animals tested by CITT, 57 tissue samples from dead animals and 6 trans-tracheal wash samples were collected from live animals and inoculated on Middlebrook 7H11 media and L-J media after proper decontamination. The isolates were identified by biochemical tests. DNA extracted from specimens and isolates was subjected to PCR for detection of MTC. The isolates positive for MTC were subjected to PCR for detection of drug resistance to rifampicin, isoniazid and streptomycin. Out of 57 tissue samples, 6 transtracheal washes and 15 blood samples, a total of 24 tissue samples, one transtracheal wash, and two blood samples were found to be positive by PCR respectively. Only 17 isolates were obtained from all of the samples isolated (16 from tissue and 1 from transtracheal wash), out of these, six isolates (5 tissues and 1 transtracheal washes) were confirmed as *Mycobacterium bovis* by biochemical tests and were found to be positive for MTC by PCR. Out of these 6 isolates, 4 isolates showed resistance to rifampicin. None of the isolates showed resistance to isoniazid and streptomycin.

HIGHLIGHTS

- Isolation and Identification of MTC from Cattle and buffaloes.
- Detection of drug resistance for rifampicin, isoniazid and streptomycin in MTC isolates.

Keywords: Drug resistance, isolation, identification, MTC, polymerase chain reaction

Mycobacterium tuberculosis complex includes *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium canettii, Mycobacterium africanum, Mycobacterium microti, Mycobacterium caprae* and *Mycobacterium pinnipedii* (Velayati *et al.* 2017). Bovine tuberculosis is an economically important and zoonotic disease caused by *Mycobacterium bovis* (*M.bovis*). Diagnosis of bovine tuberculosis by Intradermal tuberculin skin test is considered as primary method of choice by World organization of animal health (OIE) and the difference in skin thickness should be at least 4 mm after 72 hours to be identified as positive (Awah-Ndukum *et al.* 2016). The bacteria can be transmitted to humans particularly in areas

How to cite this article: Deepa, P., Filia, G., Narang, D., Devi, L.G. and Singh, S.T. (2022). Detection of Drug Resistance in *Mycobacterium tuberculosis* Complex Isolates From Cattle and Buffaloes. *J. Anim. Res.*, **12**(06): 993-999.

Source of Support: ICAR; **Conflict of Interest:** None

where there is close association between number of cattle and people due to the consumption of unpasteurised milk and milk products thus representing an important public health risk (Kock *et al.* 2021).

To identify the etiologic agent of bovine TB, bacterial culture and certain biochemical assays are being used. Although culture of bacteria is gold standard test it requires 90 days. Diagnosis using molecular testing, such as PCR, can deliver rapid and trustworthy findings while drastically reducing confirmation time (Araujo *et al*., 2014).

To aid in the recovery *of M. bovis*, a variety of pre-treatment methods (homogenization, decontamination, and concentration) as well as the use of an appropriate culture medium are used. The type of decontaminant and the media utilised are known to have an impact on the success of primary isolation.

Traditionally the Petroff method , which uses 4% sodium hydroxide (NaOH) solution, has been used to extract *M. bovis* from bovine tissues, however earlier studies has revealed that *M. bovis* vitality is decreased in samples treated with 4% NaOH. Hexadecyl pyridinium chloride (HPC) and sulphuric acid are two alternatives to the Petroff procedure (H_2SO_4). The culture medium, in addition to the decontaminants, affect the sensitivity of *M. bovis* isolation. (Issa *et al*., 2017).

The culture medium, in addition to the decontaminants, affect the sensitivity of *M. bovis* isolation. Due to high demand of nutrients Mycobacterium develops in about five weeks on a Stone brink's medium and isolation of *M. bovis* using the Middlebrook 7H11 medium, enhanced with OADC supplement (oleic acid, albumin, dextrose, and catalase), reduces the incubation period to three weeks or less. However, Middlebrook 7H11 is more prone to the growth of pollutants than Stone brink's medium due to the higher quantity of nutrients (De Azevedo Issa *et al*., 2017).

In humans, the global spread of drug resistance tuberculosis is a major issue, with documented cases of multi-drug resistant tuberculosis (MDR-TB) reaching 26.8%. Attempts to treat with anti-tuberculosis drugs have had inconsistent results. The increasing incidence of *M. tuberculosis* in endemic areas such as India may be due to human infection from the mixing of human and animal habitats. Furthermore, cattle may contract drug-resistant mycobacterium from the endemic environment. (Sweeline *et al*., 2019)

MTC have shown the ability to develop antimicrobial resistance, resulting in multi-drug resistant (MDR) and extensively drug resistant (XDR) human tuberculosis. MDR-TB is defined as tuberculosis that is resistant to both isoniazid (INH) and rifampicin. XDR (Extensively Drug Resistant) is defined by the World Health Organization Global Task Force as TB that resistant to rifampicin and isoniazid, as well as any member of the fluroquinolone family and one or more of the second-line antituberculosis drugs including kanamycin, amikacin, capreomycin (WHO, 2013). Failure to convert prodrugs to active forms, poor drug permeability are direct consequences of acquired resistance (Koch *et al*., 2018)

In 2020, 71% of people diagnosed with bacteriologically confirmed pulmonary TB were tested for rifampicin resistance (WHO, 2021). Antitubercular drug susceptibility testing of mycobacteria isolated from animals has only been done in a few case (Franco *et al*., 2017). Therefore, monitoring of antitubercular drug resistance of mycobacteria isolated from animals may contribute to reducing the risk of transmission of drug-resistant mycobacteria from animals to humans and among human beings. As there is lack of data on drug resistance patterns of MTC in dairy cattle and buffalo, this study is aimed to assess the drug resistance of mycobacterial isolates from cattle and buffaloes.

MATERIALS AND METHODS

Selection of animals

A total of 200 animals (105 cattle and 95 buffaloes) of different age groups from organized dairy farms were screened for the cell mediated immune response by Comparative Intradermal Tuberculin Test (CITT).

Collection of samples

Fifteen blood samples of cattle and buffaloes tested positive by CITT were collected in EDTA vial from organized dairy farm in Ludhiana. Blood samples were gently mixed and transported to the laboratory at ambient temperature

Tissue samples (lymph nodes, lungs with tuberculous

lesions) were taken in a sterile container from 57 suspected cases of bovine tuberculosis used for the study, samples were collected from post-mortem hall, Department of Veterinary Pathology, GADVASU, Ludhiana and post mortem of the animals carried out by field veterinarians.

Transtracheal washes (n= 6) were collected from cattle and buffaloes with a history or incidence of cases of respiratory affections from Teaching Veterinary Clinical Complex (TVCC), GADVASU, Ludhiana. All the samples were kept at -20 °C till further use.

Decontamination and isolation of mycobacteria from samples

Blood samples were decontaminated by modified Petroff method (Srivastava *et al.,* 2008). Tissue samples were decontaminated with 0.75% HPC as per (Nasr *et al*., 2016). Decontamination of transtracheal washes was done as per (Slathia *et al*., 2020). Samples were inoculated on LJ media and Middlebrook *7H11* media and the slants were incubated at 37°C in a BOD incubator for 6 weeks.

Identification

All the tissue samples and transtracheal washes were subjected to acid fast staining and the colonies obtained after inoculation of samples were also subjected to acid fast (Ziehl- Neelson) staining and biochemical tests such as niacin test, nitrate reduction test, semi quantitative catalase test, heat stable catalase test, thiophen-2-carboxylic acid hydrazide (TCH) susceptibility test were done as per manufacturer protocol (Hi media). The organisms were identified by on the basis of results of biochemical tests.

Molecular diagnosis

DNA was extracted from specimens using DN easy blood and tissue kit and from isolate as per instructions of HiPure DNA Purification kit (Hi media**)** followed by amplification by PCR using specific primers for detection of MTC.

Amplification of insertion sequence *IS6110*

Identification of MTC was done using a specific pair of primers, INS1 (forward) 5' CGTGAGGGCA TCGAGGTGGC 3'/INS2(reverse) 5'GCGTAGGCGTCGG TGACAAA 3'to amplify an insertion sequence *IS6110* for 245 base pair fragments in MTC. PCR was performed as per (Filia *et al*., 2016). For amplification reaction volume of 25 µl was made containing 12.5 µl of Taq PCR Master Mix, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 5.5 µl nuclease free water and 5 µl of DNA template of the sample. Positive control was obtained from ADRC, GADVASU, Ludhiana and for negative control 5 µl of Nuclease Free Water (NFW) was used as template. Thermal cycling was performed in T. Gradient Thermo cycler (Biometra, Germany) and cycling conditions were as follows: initial denaturation at 94˚C for 5 mins, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at 62˚C for 1 min, extension at 72˚C for 1 mins and final extension at 72˚C for 7 mins. Polymerase chain reaction products were analysed by gel electrophoresis.

Polymerase Chain reaction for detection of drug resistance from mycobacterial isolates

All the isolates were subjected to amplification by PCR targeting *rpoB* gene for detecting rifampicin resistance, *katG* gene for detecting isoniazid resistance and *rspL* gene for detecting streptomycin resistance using specific primers and conditions (Table 1).

RESULTS AND DISCUSSION

In the present study, out of 105 cattle, seven animals were found to be positive reactors and three were inconclusive and out of 95 buffaloes, eight animals were found to be positive reactors and six animals were inconclusive, rest of the animals were negative.

This study was nearer to (Kaur, 2020), studied cell mediated immune response of 230 animals (74 cattle and 156 buffaloes) from an organised dairy farm and one unorganised dairy farm. The author reported that out of 230 animals, 17 animals were positive reactors to CITT, 34 animals were inconclusive reactors and 179 were negative.

Bovine tuberculosis (BTB) is generally characterized by formation of granulomatous nodules called tubercles. The locations of these tubercles depend mainly on the route of infection (Terefe, 2014).

Table 1: Primers and PCR conditions

Primer	Primer Sequence (5'-3')	Initial Denaturation	Denaturation	Annealing	Extension	Final extension	Size (bp)	Reference
katG -F	GCA GAT GGG GCT GAT CTA CG	96 (3 min)	95 (50s)	68 (40s)	72 (1 min)	72 (10 min)	435bp	(Gupta *et al.,* 2013).
katG- R	AAC GGG TCC GGG ATG GTG							
*rpo*B-F	TGC ACG TCG CGG ACC TCC A	95 (15 min)	95 (30s)	60 (30s)	72 (20s)	72 (5 min)	157 bp	(Feuerriegel *et al*., 2014.)
rpoB-R	TCG CCG CGA TCA AGG AGT							
rspL-F	ATG AGA CGA ATC GAG TTT GAG	95 (15 min)	95 (30s)	55 (30s)	72 (1 min)	72 (15 min	632bp	(Djemal *et al.*, 2018)
rspL-R	GCT CAA GCG CAC CAT AAA CAA							

The lesions observed in the affected lungs or lymph nodes of the suspected carcass were the presence of circumscribed yellowish white lesions and in some miliary lesions were observed. Majority of lesions were found to be calcified. (Fig. 1).

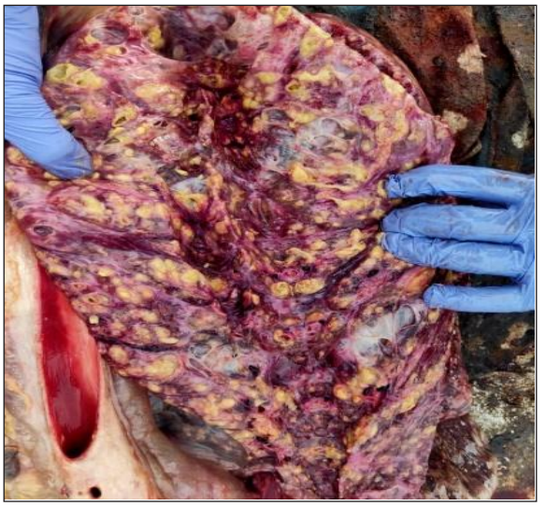

Fig. 1: Gross lesions of Lung

Culture is the most sensitive approach for diagnosing bovine tuberculosis, but it is also the most time-consuming. As a result of the decontamination methods utilised during sample isolation, the quantity of live mycobacteria reduces. An invasion of contaminating bacteria and fungi creates a challenge during sample culture. As a result of these circumstances, fewer isolates and fewer culture-positive animals are identified. A total of 17 isolates (tissue isolates = 16 and transtracheal wash isolates = 1) were obtained from 57 tissue samples (lungs and lymph nodes), 6 transtracheal washes and 15 blood samples subjected to isolation. (Fig. 2 & 3).

Fig. 2: Growth of mycobacteria on LJ media

Fig. 3: Growth of mycobacteria on Middlebrook media

No isolates were obtained from blood samples. Acid fast bacilli found in ZN staining were shown in (Figs. 4 and 5). A total of 24 tissue samples out of 57 and all the isolates were found to be positive for ZN staining. Out of 17 isolates obtained, six isolates (five from tissue and one from transtracheal wash) were identified as *M. bovis* by biochemical tests and remaining other isolates were other mycobacteria.

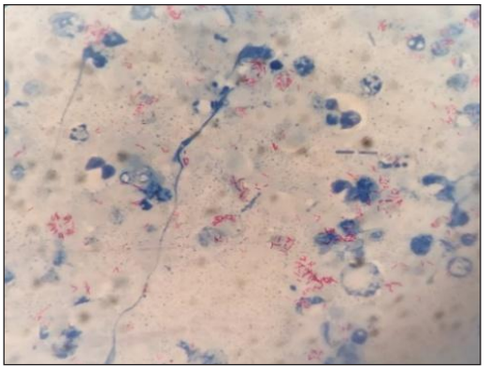

Fig. 4: Clumps of acid fast bacilli from the tissue

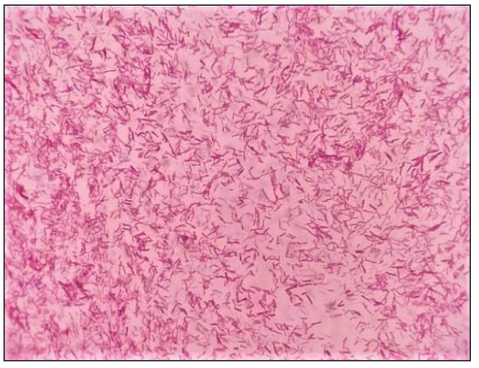

Fig. 5: Clumps of acid fast bacilli from isolate

Similar studies on tissue samples were conducted by (Javed, 2019) who subjected the twenty-five suspected tissue samples (lung and lymph nodes tissues) for isolation of *M. bovis* after preliminary decontamination. The author reported that 4 out of 25 samples were found positive by gold standard isolation. Similar studies on isolation of mycobacteria from transtracheal washes were carried out by (Slathia *et al*., 2020), collected 50 trans-tracheal washes from cattle (n=41) and buffaloes (n=9) with respiratory distress and inoculated the sample on Middlebrook *7H10* media after proper decontamination with 4% NaOH. Out of 50 trans-tracheal washes only one isolate of *Mycobacterium kansasii* (n=1) was obtained.

By PCR a total of 24 tissue samples out of 57 and one transtracheal wash out of 6 was found to be positive (Fig. 6). Out of 17 isolates obtained, 6 isolates which are identified as *M. bovis* by biochemical tests and were identified as MTC by PCR (Fig. 7). Out of 17 isolates, four of MTC the isolates showed resistance to rifampicin. None of the isolates showed resistance to isoniazid and streptomycin. Similar study was conducted by (Kaur, 2020) employing IS*6110* PCR. DNA was extracted from a total of 25 tissue samples (20 TB suspected samples and 5 healthy tissue samples), a total of 11 TB suspected tissue samples were detected positive by PCR. Similar study also carried by (Basit *et al*., 2015), who collected a total of 200 tissue samples of lungs, lymph nodes and liver from sheep and goats, cattle and buffalo. All the tissue samples were screened by acid fast staining and all the samples subjected to PCR for the detection of *M. bovis* and *M. tuberculosis*. The author concluded that PCR is more reliable diagnostic tool for diagnosis of bovine tuberculosis.

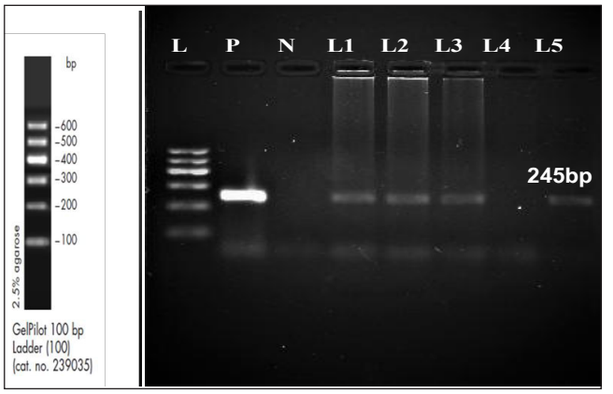

Fig. 6: Agarose gel electrophoresis showing an amplicon of 245 bp L: Marker (100 bp DNA ladder), P: Positive control, N: Negative control and L1-5: samples

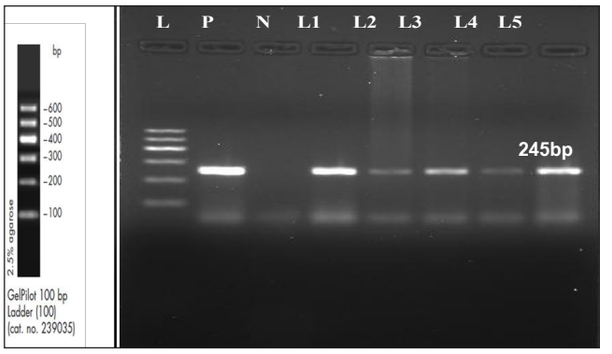

Fig. 7: Agarose gel electrophoresis showing an amplicon of 245 bp L: Marker (100 bp DNA ladder), P: Positive control, N: Negative control and L1-5: isolates

Unlike many bacteria that have evolved to propagate drug resistance in populations by horizontal gene transfer Drug resistance in mycobacteria is due to mutations in chromosomal genes (Portelli *et al*., 2018).

In the present study, rifampicin resistance was found in four of the six MTC-positive isolates (Fig. 8). None of the isolates tested positive for isoniazid or streptomycin resistance. The present study is in contrast to that of (Garfein *et al*., 2019), who obtained a total of 94 isolates from lymph nodes, raw milk and from human sputum samples and the authors reported, 29 isolates were found to be resistant to pyrazinamide, 55 isolates to isoniazid, 80 isolates to rifampicin, and 92 isolates to ethambutol, demonstrating multidrug resistance.

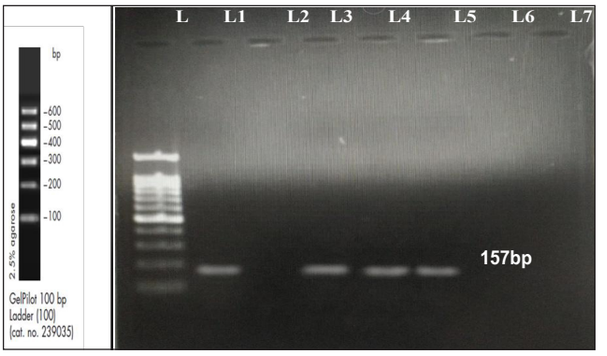

Fig. 8: Agarose gel electrophoresis showing an amplicon of 157 bp L: Marker (100 bp DNA ladder, L1-7: isolates

CONCLUSION

Early detection of drug resistance is critical for reducing and containing the spread of drug resistant strains. Drug resistant TB is a serious public health concern and it has hampered tuberculosis (TB) control . Because of the rising prevalence of MDR TB, it is critical to keep up-to-date on the mechanisms underlying drug resistance. One Health approach between medical and Veterinary departments should be encouraged to strengthen the health care and disease surveillance systems.

ACKNOWLEDGEMENTS

The authors acknowledged ICAR for providing funds to carry out the study.

REFERENCES

Awah-Ndukum, J., Temwa, J., Ngwa, V.N., Mouiche, M.M., Iyawa, D. and Zoli, P.A. 2016. Interpretation Criteria for Comparative Intradermal Tuberculin Test for Diagnosis of Bovine Tuberculosis in Cattle in Maroua Area of Cameroon. *Vet. Med. Int.*, *2016*: 4834851.

Araujo, C.P., Osorio, A.L., Jorge, K.S., Ramos, C.A., Souza Filho, A.F., Vidal, C.E., Vargas, A.P., Roxo, E., Rocha, A.S., Suffys, P.N., Fonseca, A.A., Jr, Silva, M.R., Barbosa Neto, J.D., Cerqueira, V.D. and Araújo, F.R. 2014. Direct detection of *Mycobacterium tuberculosis* complex in bovine and bubaline tissues through nested-PCR. *Braz. J. Microbiol.,* **45**(2): 633–640.

Basit, A., Hussain, M., Ayaz, S., Shahid, M., Rahim, K., Ahmad, I. and Gul, N. 2015. Isolation and identification of *Mycobacterium bovis* and *Mycobacterium tuberculosis* from animal tissues by conventional and molecular method. *Indian J. Anim. Res.*, **49**: 687-693.

De Azevedo Issa, M., Martins Soares Filho, P., Fonseca Júnior, A.A., Arrais Hodon, M., Cristian Dos Santos, L., Karlisson Pimenta Dos Reis, J. and Cerqueira Leite, R. 2017. Comparative study of *Mycobacterium bovis* primary isolation methods. *Braz. J. Microbiol* ., **48(**1): 139–144.

Djemal, S.E., Camperio, C., Armas, F., Siala, M., Smaoui, S., Messadi-Akrout, F., Gdoura, R. and Marianelli, C. 2018. Detection of a streptomycin-resistant *Mycobacterium bovis* strain through antitubercular drug susceptibility testing of Tunisian *Mycobacterium* tuberculosis complex isolates from cattle. *BMC Vet. Res*., **14**(1): 296.

Feuerriegel, S., Koser, C.U. and Niemann, S. 2014. Phylogenetic polymorphisms in antibiotic resistance genes of the *Mycobacterium tuberculosis* complex. *J. Antimicrob. Chemother*., **69**(5): 1205–1210.

Filia, G., Leishangthem, G.D., Mahajan, V. and Singh, A. 2016. Detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in Sahiwal cattle from an organized farm using ante-mortem techniques. *Vet. World.*, **9**(4): 383–387.

Franco, M.M.J., Ribeiro, M.G., Pavan, F.R., Miyata,M., Heinemann, M.B., de Souza Filho, A.F., Cardoso, R.F., de Almeida, A.L., Sakate, R.I. and Paes, A.C. 2017. Genotyping of rifampicin and isoniazid resistance in *Mycobacterium bovis* strains isolated from the lymph nodes of slaughtered cattle. *Tuberculosis,* **104**: 30–37.

Garfein, R.S. and Doshi, R.P.2019. Synchronous and asynchronous video observed therapy (VOT) for tuberculosis treatment adherence monitoring and support. *J. Clin. Tuberc. Other Mycobact. Dis.,* **17**.

Gupta, A., Prakash, P., Singh, S.K. and Anupurba, S. 2013. Rapid genotypic detection of rpoB and katG gene mutations in

Mycobacterium tuberculosis clinical isolates from Northern India as determined by MAS-PCR. *J. Clin. Lab. Anal*., **27**(1): 31–37.

Issa, M.D.A., Soares, P.M., Fonseca, A.A., Hodon, M.A., Santos, L.C.D., Reis, J.K.P.D. and Cerqueira Leite, R. 2017. Comparative study of *Mycobacterium bovis* primary isolation methods. *Braz. J. Microbiol.*, **48**: 139-144.

Kock, R., Michel, A.L., Yeboah-Manu, D., Azhar, E.I., Torrelles, J.B., Cadmus, S.I., Brunton, L., Chakaya, J. M., Marais, B., Mboera, L., Rahim, Z., Haider and Zumla, A. 2021. Zoonotic Tuberculosis - The Changing Landscape. *Int. J. Infect. Dis.*, **113**: 68–72.

Javed, R. 2019. Studies on the diagnosis of bovine tuberculosis using fluorescence polarisation assay and in situ hybridisation. Ph.D. Thesis, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

Koch, A., Cox, H. and Mizrahi, V. 2018. Drug-resistant tuberculosis: challenges and opportunities for diagnosis and treatment. *Curr. Opin. Pharmacol.*, **42**: 7–15.

Kaur, G. 2020. Molecular detection of *Mycobacterium tuberculosis* complex and analysis of cytokine profiles associated with bovine tuberculosis M.V.Sc Thesis, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

Nasr, E.A, Marwah, M., Abdel Rahman, M. and Shafeek, H. 2016. Comparison of modified Decontamination methods with culture systems for primary isolation of *Mycobacterium bovis* from bovine tissues. *Benha. Med. J*., **30**: 59-67.

Portelli, S., Phelan, J.E., Ascher, D.B., Clark, T.G. and Furnham, N. 2018. Understanding molecular consequences of putative drug resistant mutations in *Mycobacterium tuberculosis*. *Scient. Reports*, **8**(1).

Slathia, P., Narang, D., Chandra, M., Sharma, A. and Narang, A. 2020. Detection of non-tuberculous mycobacterial species using PCR-RFLP analysis in trans-tracheal washes in cattle and buffaloes with respiratory distress. *Iran. J. Vet. Res*., **21**: 136–140.

Srivastava, K., Chauhan, DS., Gupta, P., Singh, H.B., Sharma, V.D., Yadav, V.S., Sreekumaran Thakral, S.S., Dharamdheeran, J.S., Nigam, P., Prasad, H.K. and Katoch, V.M. 2008. Isolation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* from cattle of some farms in north India-possible relevance in human health. Indian *J. Med. Res*., **128**: 26–31.

Sweetline Anne, N., Ronald, B.S.M., Senthil Kumar, T.M.A. and Thangavelu, A. 2019. Conventional and molecular determination of drug resistance in *Mycobacterium tuberculosis* and *Mycobacterium bovis* isolates in cattle. *Tuberculosis*, **114**: 113–118.

Terefe, D. 2014. Gross pathological lesions of bovine tuberculosis and efficiency of meat inspection procedure to detect-infected cattle in Adama municipal abattoir. *J. Vet. Med. Anim. Health.*, **6**(2): 48-53.

Velayati, A.A. and Farnia, P. 2017. The Species Concept. *Atlas of Mycobacterium Tuberculosis,* pp. 1–16.

World Health Organization (WHO). 2013. The use of bedaquiline in the treatment of multidrug-resistant tuberculosis: interim policy guidance (No. WHO/HTM/TB/2013.6).

World Health Organization. 2021. Global Tuberculosis Report , Geneva, Switzerland.