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Isolation and species specification *Mycobacterium* spp. from bovine tuberculosis

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Abstract
Bovine Tuberculosis (BTB) is a chronic devasting disease which occurs in diverse group of animal (domestic, certain free and captive wild species) may allow the host to survive for many months, even years, without any clinical symptoms. Smears/impression smears were prepared from nasal swabs of single intra dermal (TST) positive and doubtful animals, necropcised animals and slaughtered animal tissue samples. Isolation of *Mycobacterium* spp. was attempted from nasal swabs of TST positive, doubtful and negative animals; necropsied and slaughtered animals tissues. DNA was extracted from the isolated *Mycobacterium* spp. with the help of DNA Sure Tissue Mini Kit (Genetics Brand) following manufacture's instruction. In Impression smears from nasal swabs, Acid fast bacilli were demonstrated in the nasal smear of tuberculin positive animals. The tested animals in the present investigation might be in active form of the disease leading to shedding of organism in nasal discharges. Impression smears from tissue samples, Acid-fast bacilli were observed in the impression smear of lungs, liver and lymph nodes obtained from slaughtered as well as necropsied animal. The organism could be demonstrated more in lung tissues in comparison to liver and lymph nodes. The isolated colonies of *Mycobacterium* spp. were characterized by small moist and granular, yellowish in colour with whitish orange shine. In Isolation from slaughtered animal samples, *Mycobacterium* spp. was isolated from the lungs and liver samples of slaughtered animals. All the *Mycobacterium bovis* isolates generated products of *Mycobacterium bovis* specific *pnc*A and *oxy*R at 185bp and 280bp respectively, while no products in *M. tuberculosis* specific *pnc*A and *oxy*R gene.

Keywords: Isolation, pathology, species, specification, *Mycobacterium* spp., bovine, tuberculosi

1. Introduction
Bovine Tuberculosis (BTB) is a chronic devasting disease which occurs in diverse group of animal (domestic, certain free and captive wild species) may allow the host to survive for many months, even years, without any clinical symptoms (De Lisle *et al.,* 2002). It is also a major zoonotic disease mainly occurring amongst farm workers of dairy farms, veterinarians, slaughter house workers and consumers who consume contaminated raw milk and other products. Clinically the disease is characterized by debilitated condition, cough, decreasing milk production, labored breathing etc. (Srivastava *et al*., 2008) [12]. Tuberculin skin test (TST) has traditionally been used to determine the prevalence of infection in human and animals by using the purified protein derivatives (PPD) of *Mycobacterium bovis*. Prescapular lymph gland is the most appropriate site for isolation of *Mycobacterium bovis* from infected cattle (Srivastava *et al*., 2008) [12] in comparison to other organs like lung and liver (Niaz and Siddiqi, 1979; Sulieman and Hamid, 2002) [10, 13]. In Lowenstein-Jensen Media (LJ Media), *Mycobacterium bovis* produces granular, yellowish colonies with whitish orange sheen in 4-8 weeks of incubation.
In the field, diagnosis of Bovine Tuberculosis is based on clinical and postmortem alterations observed by the Veterinarians, which may be confused with some other diseases like Aspergillosis, Aspiration Pneumonia, Pleurisy and Contagious Bovine Pleuropneumonia. Now a day, molecular techniques like Polymerase Chain reaction (PCR) can detect *Mycobacterium* spp. DNA in the isolates obtained from clinical/tissue samples and represents a valid additional tool for the postmortem diagnosis of Bovine Tuberculosis. PCR amplification of gene can differentiate *M. bovis* from *M. tuberculosis* (Monteros *et al*., 1998) [7].

2. Material and Methods

2.1 Impression smears

Smears/impression smears were prepared from nasal swabs of single intra dermal (TST) positive and doubtful animals, necropcised animals and slaughtered animal tissue samples. The smears were stained with Ziehl-Neelsen (ZN) staining kit (Hi-media) as per manufacture's guideline (Chauhan, 2006).

2.2 Isolation of *Mycobacterium* spp

Isolation of *Mycobacterium* spp. was attempted from nasal swabs of TST positive, doubtful and negative animals; necropsied and slaughtered animals tissues. Isolation of *Mycobacterium* spp. was done on Lowenstein-Jensen (L-J) media slants with or without pyruvate (Hi-media Laboratories Pvt. Ltd., Mumbai) and incubated at 37^{0}C for 8 weeks.

2.3 Molecular identification of *Mycobacterium* spp.

DNA was extracted from the isolated *Mycobacterium* spp. with the help of DNA Sure Tissue Mini Kit (Genetics Brand) following manufacture's instruction. The isolated DNA was subjected to 2% agarose gel electrophoresis to confirm the presence of isolated genomic DNA. PCR was performed for each isolated culture of *Mycobacterium* spp. following the method described by Monteros *et al*. (1998) [7] to detect *pnc*A and *oxy*R genes of *Mycobacterium* species using oligonucleotide primers. The same forward primer for *pnc*A gene *pnc*AMTB-1,2F (5'-ATGCGGGCGTTGATCATCGTC-3') was designed for both *M. bovis* and *M. tuberculosis.* On the contrary, the reverse primers used were different for both the species. In one reaction, primer *pnc*AMT1R (5'-CGGTGTGCCGGAGAAGCCG-3'), specific for *M. tuberculosis pnc*A gene was used while in another reaction, primer *pnc*AMB2R (5'-CGGTGTGCCGGAGAAGCGG-3'), specific for *M. bovis pnc*A gene was employed. Similarly, the same protocol was applied for *oxy*R gene. Here, the same forward primer for *oxy*R gene *oxy*RMTB-1,2F (5'-TGGCCGGGCTTCGCGCGT-3') was designed for both the species; however the reverse primers were different. In one reaction, reverse primer *oxy*RMT1R (5'-GCACGACGGTGGCCAGGCA-3'), specific for *M. tuberculosis oxy*R gene was used while, in another reaction primer *oxy*RMB2R (5'-TGCACGACGGTGGCCAGGTA-3'), specific for *M. bovis oxy*R gene was used (Monteros *et al*., 1998) [7]. The amplification of bacterial DNA for *pnc*A and *oxy*R gene was performed in thermal cycler in 50µl volume containing 25µl of Taq polymerase master mix (Qiagen), 1µl of each forward and reverse primer, 6 µl of DNA template and 17µl of nuclease free water. The conditions of PCR for *pncA* 95 °C for 12 mins(initial denaturation), followed by 30 cycle including denaturation at 94 °C for 45 seconds, annealing at 67 °C for 1 min and extension at 72 °C for 1 min. For *oxy*R method 95 °C for 12 mins(initial denaturation), followed by 30 cycle including denaturation at 94 °C for 45 seconds, annealing plus extension at 72 °C for 1 min 30 seconds. The amplified PCR products of desired size were visualized by submarine gel electrophoresis using 6µl of PCR product on 2% w/v of agarose gel in 1X Tris-acetate–EDTA buffer containing ethidium bromide 0.5µg/ml at 120V for 45 min. The amplified DNA fragments of specific sizes were located by Gel Doc System and the image was captured using Kodak Imager Ep software. Molecular size markers of 100bp were loaded in each gel.

3. Results and Discussion

In Impression smears from nasal swabs, Acid fast bacilli were demonstrated in the nasal smear of tuberculin positive animals (Fig.1). On the contrary, Nahar *et al*. (2011) [9] could not able to demonstrate acid-fast bacilli in impression smear of upper respiratory tract and explained that diseased cattle seldom shed the organism at detection levels in nasal discharge. The tested animals in the present investigation might be in active form of the disease leading to shedding of organism in nasal discharges. Influence of dose of organism and frequency of nasal shedding in tuberculosis has been explained earlier (McCorry *et al*., 2005) [6].

Impression smears from tissue samples, Acid-fast bacilli were observed in the impression smear of lungs, liver and lymph nodes obtained from slaughtered as well as necropsied animal. The organism could be demonstrated more in lung tissues in comparison to liver and lymph nodes. Goswami *et at*. (2014) [4] demonstrated acid fast bacilli in lung tissue, whereas Prakash *et al*. (2015) [11] demonstrated the organism in lung as well as mediastinal lymph nodes with Ziehl-Neelson's stain (Fig.2).

In Isolation from clinical samples, in the present study, *Mycobacterium* spp. was isolated in Lowenstein-Jensen (LJ) slants from nasal swab of clinically affected animals, both tuberculin positive and doubtful. Ashenafi *et al*. (2013) [1] also isolated *Mycobacterium* spp. from tuberculin reactor animals (cattle and small ruminant) (Fig.3). Organism was also isolated from tuberculin doubtful animals, it might be due to false negative result of tuberculin test. The isolated colonies of *Mycobacterium* spp. were characterized by small moist and granular, yellowish in colour with whitish orange shine. Thakur *et al*. (2010) [14] also observed similar colonies of *Mycobacterium* spp (Fig.4).

In Isolation from slaughtered animal samples, *Mycobacterium* spp. was isolated from the lungs and liver samples of slaughtered animals. Several workers (Naiz and Siddiqi, 1979; Gutierrez *et al*., 1995 and Muller *et al*., 2008) [10, 5, 8] isolated the *Mycobacterium* spp. from lungs, liver and lymph nodes, suspected for Tuberculosis from slaughtered animals of different abattoirs. Organisms isolated in the present study were mostly from lungs, since lung act as primary site of *Mycobacterium bovis* infection and association of liver was also found at weaker rate (Muller *et al.,* 2008) [8].

In Isolation from necropsied animal samples, In the present study *Mycobacterium* spp. was isolated from the tissue samples (lungs, liver, lymph nodes, pleura, peritoneum and spleen) of necropsied animals died of tuberculosis. Muller *et al.* (2008) [8] also isolated *Mycobacterium* spp. from lung, liver and peritoneum where as Gutierrez *et al*. (1995) [5] and Thakur *et al*. (2010) [14] isolated *Mycobacterium* spp. from lungs and lymph nodes. Isolation of *Mycobacterium* spp. from pleura and spleen in the present study might be due to generalized nature of the disease.

In the present investigation, the results of *pnc*A and *oxy*R PCR assay clearly revealed that all 27 cultures isolated from nasal swabs, tissue samples from both slaughtered and necropsied animals were *Mycobacterium bovis* species (Table.1). All the *Mycobacterium bovis* isolates generated products of *Mycobacterium bovis* specific *pnc*A and *oxy*R at 185bp and 280bp respectively (Fig.5 & Fig.6)), while no products in *M. tuberculosis* specific *pnc*A and *oxy*R gene. *Mycobacterium bovis* strains tested were found to carry an identical mutation in nucleotide sequence of the gene. This suggested that *Mycobacterium bovis* strains tested had the *Mycobacterium bovis* specific polymorphism (G instead of C

at position 169) while *Mycobacterium tuberculosis* strains had *Mycobacterium tuberculosis* specific polymorphism (C instead of G at position 169) in *pnc*A gene. In case of oxyR gene changes in the sequence occurs at 285 position (Monteros *et al*., 1998) [7]. Vathsala *et al*. (2007) [15] also diagnosed *Mycobacterium bovis* in wild animals with PCR by using primer sequence of *pnc*A8 and *pnc*A11 specific for *Mycobacterium bovis pnc*A gene. Specification of organism has been necessary for proper curative treatment. Thus, the present study confirms the utility of *pnc*A and *oxy*R gene based PCR to differentiate two closely related species i.e. *Mycobacterium bovis* and *Mycobacterium tuberculosis* with high specificity and sensitivity.

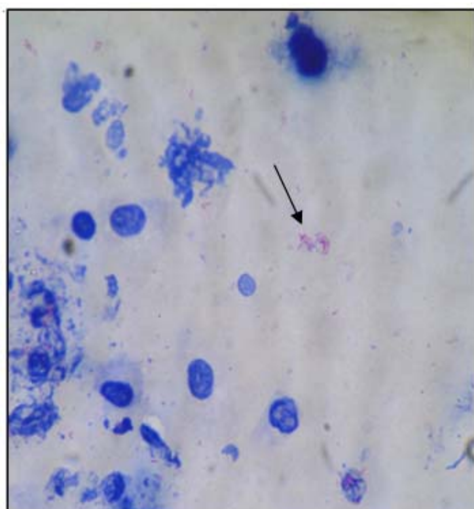

Fig 1: acid-fast bacilli in impression smear

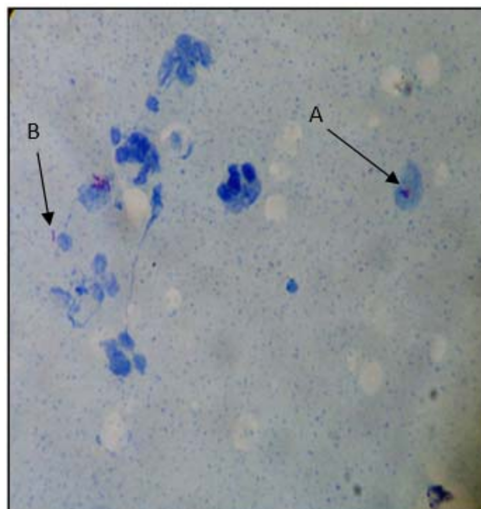

Fig 2: acid-fast bacilli in impression smear both (a) intra and (b) extra-cellularly

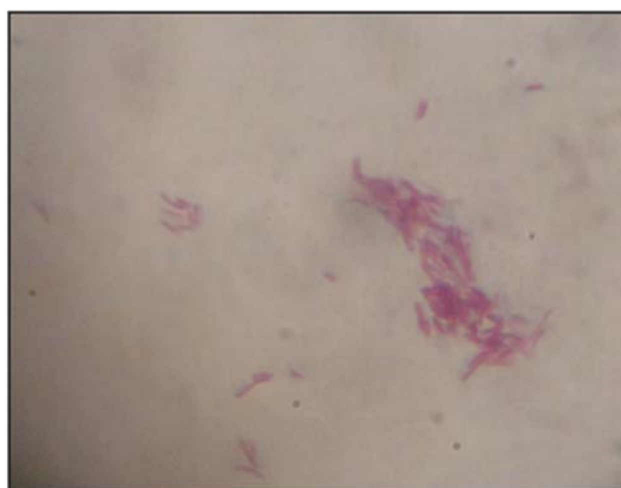

Fig 3: *Mycobacterium spp.* Stained with acid fast stain

1st week

2nd week

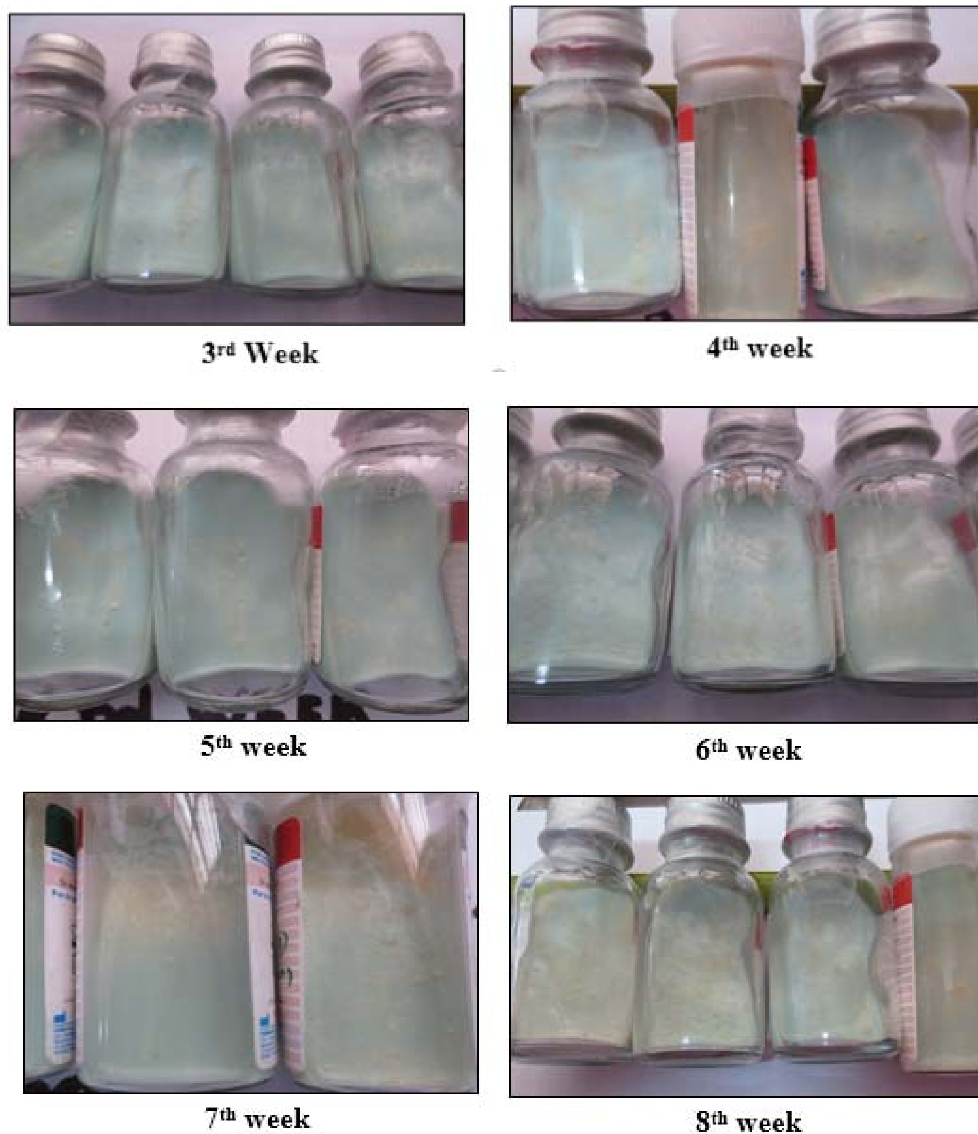

Fig 4: week-wise growth of *Mycobacterium* spp. In LJ slant

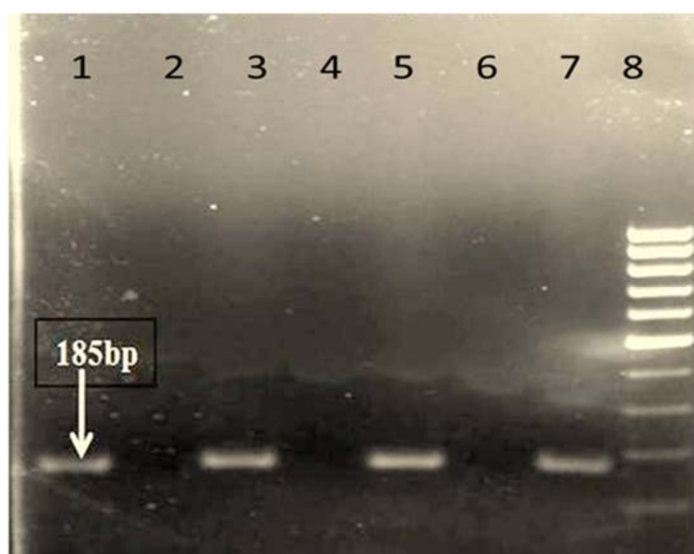

Fig 5: PCR For Identification of *M. Bovis* Showing Amplification of An 185bp Targating with Reverse Primer For *pnc*A AMB2 (Lanes: 3, 5 And 7) And Not with Reverse Primer For *pncA* MT1 *(Lanes: 4 And 6)*. Lane 1: Positive Control, Lane 2: Negative Control, Lane 8: 100 bp DNA Ladder

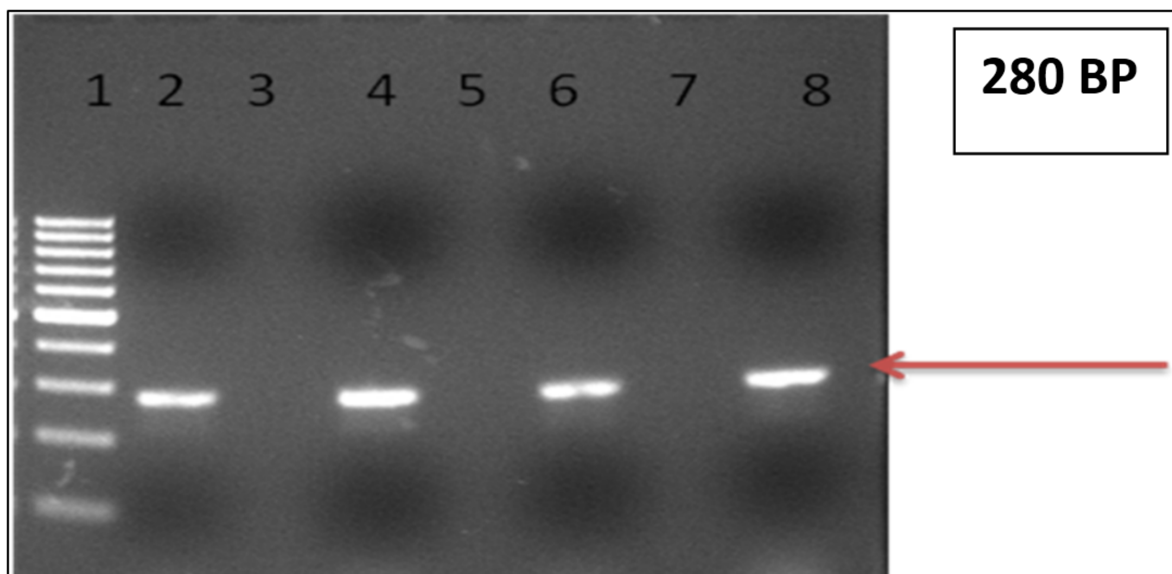

Fig. 6: PCR for Identification of *M. Bovis* Showing Amplification of A 280bp Targating with Reverse Primer for *oxyR*MB2 (Lanes: 4, 6 And 8) and Not with Reverse Primer for *oxyR*MT1 *(Lanes: 5 And 7)*. Lane 1: 100 bp DNA Ladder, Lane 2: Positive Control, Lane 3: Negative Control

Table 1: Species Specification of *Mycobacterium* Spp. From Different Categories of Samples

Sample	Nos. of culture	Amplification for *pnc* A gene	Amplification for *oxy* R gene	Species identified
Nasal swabs	12	185bp	280bp	*Mycobacterium bovis*
Slaughtered animals tissue	12	185bp	280bp	*Mycobacterium bovis*
Necropsied animals tissue	3	185bp	280bp	*Mycobacterium bovis*

4. Summary and Conclusion

In Impression smears from nasal swabs, Acid fast bacilli were demonstrated in the nasal smear of tuberculin positive animals. The organism could be demonstrated more in lung tissues in comparison to liver and lymph nodes. Organism was also isolated from tuberculin doubtful animals, it might be due to false negative result of tuberculin test. The isolated colonies of *Mycobacterium* spp. were characterized by small moist and granular, yellowish in colour with whitish orange shine. Thus, the present study confirms the utility of *pnc*A and *oxy*R gene based PCR to differentiate two closely related species i.e. *Mycobacterium bovis* and *Mycobacterium tuberculosis* with high specificity and sensitivity. This disease has zoonotic importance and it may be transmitted to the farm worker and veterinary practitioners frequently due their occupation.

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