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

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Abstract

Bovine Tuberculosis (BTB) is a chronic devastating 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, necropsied 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 pncA and oxyR at 185bp and 280bp respectively, while no products in *M. tuberculosis* specific pncA and oxyR gene.

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

1. Introduction

Bovine Tuberculosis (BTB) is a chronic devastating 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, 11]. 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, necropsied animals and slaughtered animal tissue samples. The smears were stained with Ziehl-Neelsen (ZN) staining kit (Hi-media) as per manufacturer’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 tissue samples. 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°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 manufacturer’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 pncA and oxyR genes of Mycobacterium spp. species using oligonucleotide primers. The same forward primer for pncA gene pncAMTB-1,2F (5’-ATGCGGGCGTTGATCATCCTGTC-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 pncAMTB1R (5’-CGGTGTGCCGGAGAAGCGG-3’), specific for M. tuberculosis pncA gene was used while in another reaction, primer pncAMB2R (5’-CGGTGTTGCGGAAGACCGG-3’), specific for M. bovis pncA gene was employed. Similarly, the same protocol was applied for oxyR gene. Here, the same forward primer for oxyR gene oxyRMTB-1,2F (5’-TGCCGGGCTTGCAGCGT-3’) was designed for both the species; however the reverse primers were different. In one reaction, reverse primer oxyRMT1R (5’-GCACGACGGTGCAGCCAGGA-3’), specific for M. tuberculosis oxyR gene was used while, in another reaction primer oxyRMB2R (5’-TGCGGCCCCTTGCAGGTA-3’), specific for M. bovis oxyR gene was used (Monteros et al., 1998) [7]. The amplification of bacterial DNA for pncA and oxyR 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 nucleic 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 oxyR 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 al. (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 (L-J) 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 Siddigi, 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 pncA and oxyR 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 pncA and oxyR at 185bp and 280bp respectively (Fig.5 & Fig.6), while no products in M. tuberculosis specific pncA and oxyR 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.
Mycobacterium tuberculosis strains had specific polymorphism (C instead of G at position 169) in pncA 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 pncA8 and pncA11 specific for Mycobacterium bovis pncA gene. Specification of organism has been necessary for proper curative treatment. Thus, the present study confirms the utility of pncA and oxyR 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 Targeting with Reverse Primer For *pncA* 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>
<thead>
<tr>
<th>Sample</th>
<th>Nos. of culture</th>
<th>Amplification for <em>pncA</em> gene</th>
<th>Amplification for <em>oxyR</em> gene</th>
<th>Species identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swabs</td>
<td>12</td>
<td>185bp</td>
<td>280bp</td>
<td><em>Mycobacterium bovis</em></td>
</tr>
<tr>
<td>Slaughtered animals tissue</td>
<td>12</td>
<td>185bp</td>
<td>280bp</td>
<td><em>Mycobacterium bovis</em></td>
</tr>
<tr>
<td>Necropsied animals tissue</td>
<td>3</td>
<td>280bp</td>
<td>280bp</td>
<td><em>Mycobacterium bovis</em></td>
</tr>
</tbody>
</table>

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 *pncA* and *oxyR* 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.

5. Reference
