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Isolation and Identification of *M. tuberculosis* from Sheep Tissue Samples and Sero-Diagnosis Study in an Organized Sheep Farm

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A B S T R A C T

A 2 years old female Madras Red sheep with the medical history of reduction in feed intake, poor weight gain and emaciation was found dead in an organised farm. The sheep did not have any obvious respiratory symptoms. Edematous and caseous lesions were observed in mesenteric, bronchial, mediastinal and prescapular lymph nodes of the sheep during post mortem examination. Other internal organs were free of any specific lesions. The lymph node samples were decontaminated and cultured by inoculating into BACTEC Mycobacteria Growth Indicator Tube (MGIT) system and Lowenstein Jensen slants. The cultures turned positive and acid fast staining of the bacterial culturerevealed the presence of Mycobacteria. The bacteria was further confirmed as *Mycobacterium tuberculosis* by multiplex PCR and nucleotide sequencing. A Tuberculosis sero-diagnostic study was conducted for all the animals in the farm using commercially available ELISA kit to know the incidence of tuberculosis in the farm. Three sheep out of the total 205 sheep were positive for tuberculosis by ELISA with the estimated 1.5% positivity. This shows the active circulation of tuberculosis in sheep farm and there may be possibility of human to animal transmission and vice versa. The role of sheep in the epidemiology and transmission of tuberculosis needs further study.

Introduction

Tuberculosis (TB) is a chronic bacterial disease caused by *Mycobacterium tuberculosis* complex (MTC) leading to decreased productivity, economic losses and poses a significant threat to human health. Among MTC organisms, the major agents are *M. tuberculosis* and *M. bovis*. The primary host for *M. bovis* is cattle and *M. tuberculosis* is human. However, occurrence of *M. tuberculosis* in animals and *M. bovis* infection in humans has been reported previously (Ocepek *et al.,* 2005).

In ovine, the occurrence of tuberculosis is very rare although there are few reports indicating the presence of *M. bovis* in sheep and goat (Kassa *et al.,* 2012; Marianelli *et al.,* 2010). This primarily occurs in areas with high intensity sheep population and when there exists close contact between infected cattle and sheep facilitating transmission between these species. India accounts for one fourth of the global TB burden (Central TB division, GOI, 2017).

Tuberculosis in animals is not well studied in India; the lack of nation-wide epidemiological studies makes the disease burden largely unknown (Neeraja *et al.,* 2014a). Few studies have documented the prevalence of TB in animals in India (Parmer *et al.,* 2014; Srivastava, 2008). Tuberculosis causes huge economic loss in farm animals and the production loss in infected animals will be 10 to 20 percent (Verma *et al.,* 2004).

Tuberculosis is often unnoticed in animals and the infected animals continue to spread the disease to other susceptible animals and human by excreting the organisms through milk, faeces and respiratory droplets. Hence to control tuberculosis both animals and human has to be monitored for disease prevalence. In this study Mycobateria was isolated from a TB infected sheep and M. tuberculosis was identified by multiplex PCR and gene sequencing. Then all the sheep in the farm were screened for TB sero-positivity.

Materials and Methods

Sample collection

Post mortem examination was carried out on one Madras red sheep that had died in an organized farm. The mesenteric, pre-scapular, bronchial and mediastinal lymphnodes were edematous and caseous. Samples from these lymphnodes were collected in sterile PBS and transported to laboratory on ice for mycobacterial culture.

Isolation *Mycobacterium* Sp. from tissue samples

The samples were decontaminated and processed following the modified Petroff's method (Kent and Kubica, 1985). A portion of the decontaminated sediments were inoculated into Mycobacterial Growth Indicator Tubes (MGIT)^{TM} from Becton Dickinson (BD) and incubated in BACTEC MGIT 960 instrument for 49 days at 37 °C.

The remaining sediments were inoculated into one tube each of OADC-supplemented Middlebrook 7H10 agar and Lowenstein-Jensen (LJ) medium with sodium pyruvate and glycerol and each tube was incubated for 8 weeks at 37 °C.

Acid fast staining

Heat fixed smears prepared from the sediment and MGIT cultures declared as positive by the BACTEC 960 and typical growths on Middlebrook 7H10 and LJ media were screened for presence of acid fast bacilli. The heat-fixed smears were stained for acid fast bacilli as per the standard protocol.

Polymerase chain reaction confirmation and sequencing

The DNA extraction from MGIT liquid culture and colonies on 7H10 agar/LJ media was performed according to the CTAB –NaCl method. These DNA samples were subjected to conventional polymerase chain reaction (PCR) with specific primers reported by Zumarrga *et al.,* (1999) and Bakshi *et al.,* (2005). Then amplified PCR products were sequenced to confirm the mycobacterium species.

Sero prevalence study using ELISA

Sheep sera samples from study farm were screened for tuberculosis antibodies using the commercial ELISA kit (IDEXX), USA as per manufacturer's instruction.

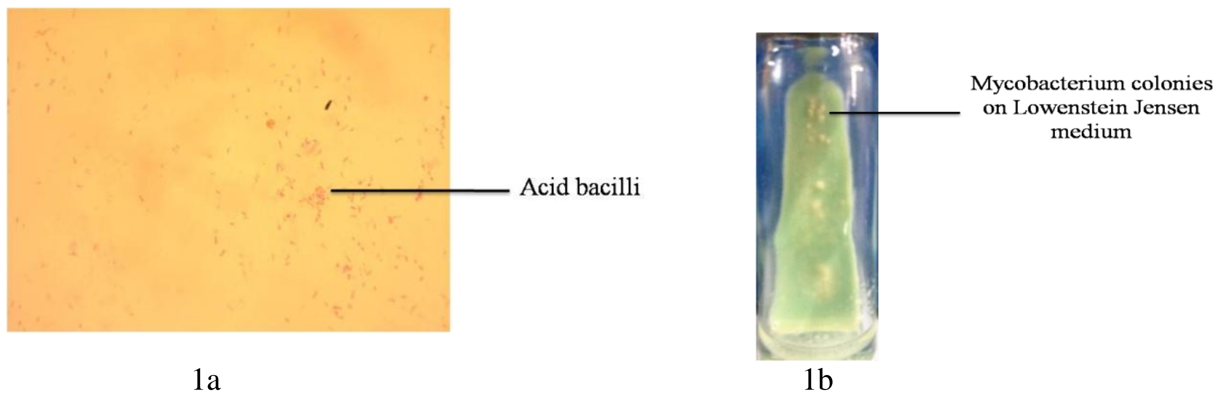
Result and Discussion

The post mortem caseous, edematous lymphnode tissue samples collected from the tuberculosis-suspected sheep were subjected to acid fast staining, bacterial culture and PCR. Staining of tissue smear from sheep lymph node revealed that presence of rod shaped, acid fast bacilli indicating the presence of mycobacterium infection (Figure 1a).

Bacterial culture study is the gold standard for laboratory confirmation of TB. Hence the tissue samples were cultured in LJ medium resulting in colonies that were rough, granular and whitish initially and later on the colonies turned yellowish (Figure 1b).

DNA amplification by PCR provides a rapid and sensitive method for the detection of *M. tuberculosis* complex (MTC) from post-mortem samples and cultures (Clarridge *et al.,* 1993). DNA extracted from LJ medium culture were subjected to multiplex PCR method. PCR product was further analyzed by agarose gel electrophoresis. There was no band around 168 bp which is *M. bovis* specific whereas *M. tuberculosis* specific band around 337 bp was visualized (Figure 2).

Fig.1a Acid Fast bacilli in Ziehl-Neelsen staining; **Fig.1b** Characteristic Mycobacterium colonies on Lowenstein Jensen medium



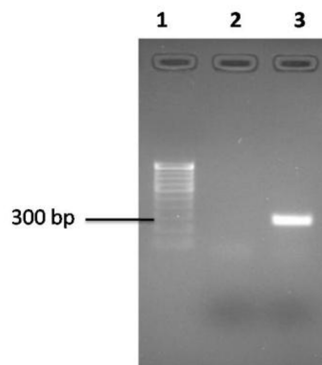


Figure 2 – Multiplex PCR; Lane 1 – 100 bp marker
Lane 2 – Negative control; Lane 3 – Sample

The PCR product was subjected to gene sequencing and confirmed as *M. Tuberculosis*.

Further, the circulation TB in the sheep farm was identified by using ELISA to estimate sero-prevalence. Generally humans are the maintenance hosts for *M. tuberculsois*. The sheep is considered to be the spill-over hosts for *M. bovis*, can maintain the organism only when its population density is high and is generally considered very rare in small ruminants (Tschopp *et al.,* 2011).

However, presence of MTB in sheep indicates a possible transmission of infection from human to animal. In this study out of 205 sheep 3 were sero-positives and indicates 1.5% sero-prevalence of TB was observed in study population. Lack of a robust animal TB surveillance system and vaccine use in animals aids in the transmission of TB between animals and from animals to human or *vice versa*. Thus there is an urgent and unmet need for implementation of animal TB control programs in developing countries through extensive surveillance. The license for the use of BCG vaccine in animals also warrants further studies.

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