JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 1579-1584

Detection and Identification of Bovine Tuberculosis in Indian Cattle (*Bos indicus*)

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(Received: 25 November 2015; accepted: 18 February 2016)

Bovine tuberculosis (bTB) in cattle is a serious zoonotic disease of worldwide distribution. Bovine tuberculosis is increasingly prevalent among farmed cattle in India, exerting a heavy economic burden on the farming industry and Government. This study was aimed to determine the prevalence of bovine tuberculosis in Indian cattle (*Bos indicus*), based on Post-mortem, Ziehl-Neelsen, Mycobacterial culture and ELISA. In post-mortem were found eight positive samples, twelve negative samples and three doubtful samples. With the help of Ziehl-Neelsen technique fifteen cattle sample were screened, seven positive samples, six negative samples and two doubtful samples. Thirty two cattle sample were identified based on mycobacterial culture, eleven positive samples, fourteen negative samples and seven doubtful samples. Sixteen positive samples, thirteen negative samples and thirty one doubtful samples were detected in ELISA test with *M. bovis* standard positive and control serum sample. The study found a high infection rate of bTB among cattle and majority of the lesions were found on lungs. We conclude that different laboratory testing requires the combination of a set of available diagnostic tools for better sensitivity and specificity of results.

Keywords: Post-mortem, Z-N Technique, Mycobacterial culture, ELISA.

M. bovis is the causative agent of bovine tuberculosis (bTB) and has a wide host range including cattle and various wildlife species[1]. Bovine tuberculosis is a chronic and progressive disease where animals may be infected for months or years before developing lesions and early diagnosis relies primarily on the detection of cell mediated immunity (CMI) to *M. bovis* antigens [2,3]. In cattle, bTB not only cause reduced productivity and death, but also considered as a main zoonotic risk, therefore intensively controlled in several countries. As a result, the detection of *M. bovis* infected cattle is important in preventing the transmission of bTB to other species, including domesticated livestock, as well as the geographic spread of this pathogen through movement of infected animals[4,5,6]. Infection of dairy cattle can lead to reduced animal health, welfare and economic losses through decreased milk yield and premature culling[7]. It is generally believed that cattle are primarily infected as calves, either while still in the uterus or as neonates by ingestion of faecal matter, milk or colostrum from infected cows in the herd[8]. As the TB infection progresses, a shift from the predominant cell-mediated immune response towards humoral response occurs[9]. The antibodies (Abs) produced are generally targeted at immunodominant antigens that elicit a humoral response, notably MPB70 and MPB83 released in large amounts by *M. bovis* in the later stages of the disease. In general, the profile of the humoral response varied individually and among animal species.

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M. bovis belongs to the *M. tuberculosis* complex, a group of mycobacteria which also includes *M. tuberculosis, M. africanum* and *M. microti.* A presumptive postmortem diagnosis of bovine tuberculosis can be made following the finding of lesions suspected to be tuberculous during necropsy, but a definitive diagnosis is dependent on the isolation and identification of *M. bovis*[10,11]. The identification of this pathogen has traditionally relied upon colony morphology, staining characteristics and biochemical tests, although recently rapid techniques using monoclonal antibodies and DNA technology have been developed. These methodologies allow for the rapid identification of mycobacterial organisms in less than one day[12].

MATERIALS AND METHODS

Experimental animals

Cattle owners were interviewed according to their readiness to participate and given verbal consent, on the same day their cattle were tested for bTB.

Post mortem examination

In cold season at dairy farm of Barsana, Mathura, Uttar Pradesh, Post mortem examination was done by the experts of *M. bovis* infected animal. The tonsils, retropharyngeal, parotid and sub-mandibular lymph nodes, the left-and right bronchial lymph nodes and lungs were excised and multiple parallel incisions were made into these tissues. Samples collected for mycobacterial culture included lesions suggestive of bTB, or if no such lesion was present, pooled samples taken from the bronchial and retropharyngeal lymph nodes. Suspected lesions of lymph nodes and lungs were fixed in 10% Formal saline embedded in paraffin and 5 gm thick sections cut from each block were stained with Harris's haematoxylin and eosin.

Ziehl-Neelsen Technique

Z–N staining procedure was used for microscopic examination. Sections of each sample were cut and stained by the Ziehl-Neelsen method. Stained sections were examined for the presence of typical tuberculous lesions and acid-fast bacilli. Staining protocol and microscopic reporting were done according to WHO laboratory guidelines[13]. Heat fixed sputum smears were stained using the Z–N method with carbolfuchsin. After rinsing with water and decolorizing using 3% acid-alcohol, the smear were stained with methylene blue. When the smear was examined under light microscope in·100 oil immersion, tubercle bacilli appeared as fine red rods, slightly curved, more or less granular, isolated, in pairs or in groups against the blue background fig.2.

Mycobacterial Culture and Identification

For bacteriological culture to detect *M. bovis*, samples of lymph nodes tissue & infected lungs part were decontaminated according to standard methods and inoculated on two slopes of agar-based medium (Middle brook 7H11) and an egg-based medium (Lowenstein-Jensen with 0.5% pyruvate) for primary isolation Becton Dickinson (Diffco) Private Limited, Gurgaon, India). It is required to decontaminate samples before culture because contaminating microflora may obscure the isolation of *M. bovis*. Samples requiring decontamination were processed using the following methods. Homogenate samples were centrifuged at 4000 × g at 4°C for 30 min and pellets resuspended in 1 ml of 2% HCl (Method A) and 4% NaOH (Method B) both containing 10 µl phenolphthalein as a pH indicator. After 15 min exposure to acid or base, suspensions were neutralized by addition of 1% NaOH or HCl. Then suspension were centrifuged as above and pellets were resuspended in 0.5 ml of 0.85% NSS. We used here two types of decontaminants to see the effects of decontaminants on recovery of *M. bovis*.

Decontaminated homogenates were inoculated on to two media (one egg media-Lowenstein-Jensen (LJ) slants, one agar media-Middlebrook 7H11 agar (MDA) containing 0.5% glycerol and oleic acid, albumin, dextrose and catalase (OADC) as a supplement. Inoculated media were incubated at 37 ^{0}C and observed once weekly for 12 weeks. Several factors were identified that significantly influenced the sensitivity of the primary isolation of *M. bovis* from tissue samples. The isolation of mycobacteria was also affected by culture medium employed.

All lesions found during this study were cultured for mycobacteria. To obtain the greatest sensitivity on primary isolation using solid media, the use of multiple culture medium types: agar based medium to exploit the faster growth rate or another egg based medium such as Lowenstein–Jensen with pyruvate for greater sensitivity and

the inhibition of contaminants[14]; the use of two or more slopes of each medium; and an incubation time of e” 12 weeks for all media was suggested. This part of our study done is ICGEB, New Delhi.

Enzyme-Linked Immunosorbent Assay (ELISA)

High adsorption capacity 96-well polystyrene microtiter plates (Thermo Fisher Scientific, Mumbai, India) were coated with 50 µl/well of 0.02 mg/ml solution of antigen in carbonate bicarbonate buffer (Sigma-Aldrich, Mumbai, India) and left at 4–8 ^{0}C overnight to remove nonspecific anti-*Mycobacterium* sp. Antibodies[17,16]. The plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). After three washes, 200 µl/well (2% BSA in PBS containing 0.05% Tween 20) and 1 h incubation period at room temperature. The plates were washed three times with a washing solution. After three washes, the serum samples were diluted (1:10, v/v) and 100 µl/well was added into duplicate wells of the antigen-coated plate. After a 1 h incubation period at room temperature, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). Protein G horseradish peroxidase conjugate (Sigma-Aldrich) was added (0.002 mg/ml in blocking solution) and incubated at room temperature for 1h. After three washes, 200 µl/well of substrate solution (Fast OPD, Sigma-Aldrich) was added. The reaction was stopped with 50 µl/well of $H_2SO_4$3N and optical density (OD) was measured in a spectrophotometer (Bio-Rad Laboratories, **Gurgaon**, India) at 450 nm.

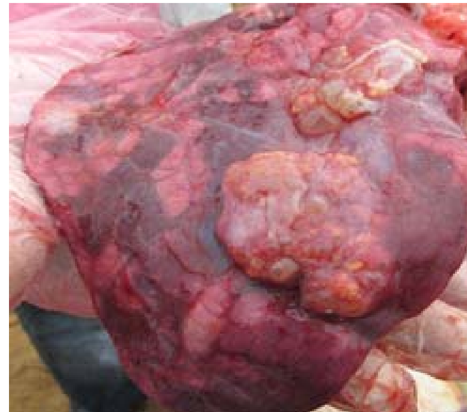
Animal negative control sera were included in every plate in duplicate. OD values of animals between different plates were normalized according to the values of the negative controls included in each plate. Sera from culture confirmed positive animals were included as positive controls.

RESULTS

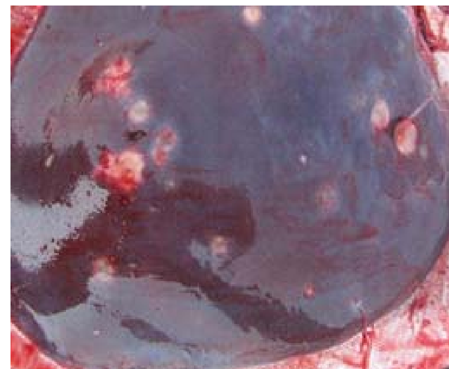
Post Mortem Examination

In Post-mortem examination, eight positive samples, twelve negative samples and three doubtful were found. Only positive animal samples were collected based on the results of histologic examination and microscopic examination, evidence of tuberculosis was found by bacteriologic culturing, histologic examination or microscopic examination. Eight positive samples of the infected cattle had evidence of tuberculosis in the Liver, Lung and medial retropharyngeal lymph nodes (Fig.1).

Ziehl-Neelsen Technique



(a) Lung



(b) Liver

(c) Lymph node

Fig. 1. Photograph of the affected Lungs showing massive granulomatous (tuberculous) lesions (A), Liver (B) Lymph node (C) from slaughtered cattle, during Post mortem

In this methods fifteen cattle sample were screened. We found seven positive samples, six negative samples and two doubtful samples. Among the study participants, cattle were finally classified clinically and microscopically (smear positive) as active bTB cases. Macroscopically, the most common change seen in affected lymph nodes was the presence of circumscribed yellowish white lesions of various sizes and numbers. The involved tissue in these lesions was dry and gritty. Histologically, granulomatous changes were observed only in the lymph nodes that had gross lesions.

Mycobacterial Culture and Identification

Thirty two cattle sample were identified based on mycobacterial culture, eleven positive samples, fourteen negative samples and seven doubtful samples. Our findings corroborate out of the eleven positive clinical samples considered standard to diagnose *M. bovis* infection. After 8 weeks post inoculation on L-J slants small, moist, shiny, slightly rough and and fragile colonies appeared. The growth of *M. bovis* was promoted with the addition of 0.5% sodium pyruvate to L-J medium. Isolation of Mycobacterium was significantly affected by the method of decontamination and culture medium employed for culturing. Recovery of Mycobacteria on Lowenstein Jensen media and Middle brook 7H11 media following HCl treatment was greater than NaOH treatment. Isolation of Mycobacteria from decontaminated samples was significantly high on LJ medium compared to MDA 7H11.

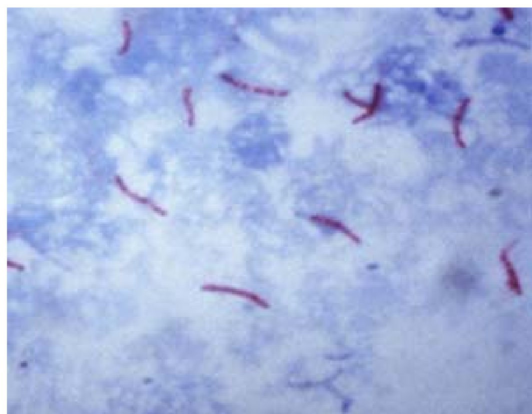


Fig. 2. Ziehl–Neelsen stain of tissue sample (Lung) of *M. bovis* infected animal

ELISA

Sixty cattle serum samples were selected for ELISA test, sixteen positive samples, thirteen negative samples and thirty one doubtful samples, with *M. bovis* standard positive and control serum sample. Although ELISA results showed higher mycobacterial infection rate than traditional culture or serological test. *M. bovis* ELISA provides an apparent test sensitivity of 63% and specificity of 98% with samples from cattle naturally infected with *M. bovis*.

DISCUSSION

However, the problems to complete the eradication of bovine TB in assured regions or nations should not be concentrated only to the boundaries of the current diagnostic assays or to the epidemiological situations presence of wildlife or infection with other non-tuberculous mycobacteria[17]. The late pattern of antibody production reported in the humoral response against TB by the irregular, in general mainly affected serological assays performance. The other factors related to the practices of all stakeholders involved (private and government veterinarians, managers and herd owners) should be addressed, evaluated and improved if necessary. The distribution and occurrence of suspected bTB lesions in different organs of postmortem cattle showed in the lungs and in the lymph nodes, while the heart, liver, spleen, intestines, and mammary glands made up the least number of bTB positives. On gross bTB lesions in different organs in postmortem cattle in Barsana with highly infection in lungs and lymph nodes, with the least number in the other affected visceral organs. This also agreed with the report Abubakar and his research team.[18]

The severity of infection ranged from latent infection to generalised disease. Conventional identification of fish mycobacteria are based on culture and biochemical characteristics. However, these methods have their own limitations. Of the four diagnostic procedures, bacteriological culture was the most sensitive and the most specific. Traditionally, culture followed by a panel of biochemical tests has been used for speciation of mycobacteria. This has inherent disadvantages, as most mycobacteria of clinical importance are slow growers and hence are difficult to isolate and

cultivate. The time required for primary isolation ranges from 4 to 6 weeks in the case of solid culture media and 10 to 15 days by radiometric and other automated systems. Moreover, the paucibacillary nature of clinical samples also accounts for the low efficiency of isolation of pathogenic mycobacteria[19]. In addition to difficulties in primary isolation of mycobacterial pathogens, particularly *M. bovis*, there have been reports of difficulty in differentiating closely related mycobacterial species[20]. Therefore, there is a need to develop molecular biological tools like PCR-based assays for reliable, early detection and speciation of mycobacteria in clinical samples and, as a consequence, for determination of the disease burden caused by diverse pathogenic mycobacteria.

In so many state of India one policy is there “no slaughter of cattle”. There is a trend to possess animals with a long production life without culling which increases their chance of involvement in spread of bovine TB. Although the numbers of positive animals were few, pooling milk from the farm does pose a great public health risk to milk consumers as it has been shown that one cow can excrete enough viable bacilli to contaminate the milk from up to 100 cows when their milk is pooled[21].

CONCLUSION

Identification of positive animal certain limitations associated to the immunological response against the infection and to the accuracy of the current diagnostic tools that are more evident at the final steps of the eradication process. However, this can only be achieved if bTB is declared an important disease for animal and human health, and the required financial support is provided to support inspections, testing and compensation for losses. The early detection and removal of positive animals reduces the risk from tuberculosis that they will become a source of infection for other cattle. Veterinary Officer and farmers should be made aware of the increased risk associated with disease animal.

ACKNOWLEDGMENTS

I thanks the study participants who contributed to this work and I appreciate the staff of IVRI, Izatnagar and dairy farm of Barsana, Mathura, Uttar Pradesh. This study was funded (SB/YS/LS-78/2013) by DST-SERB (Department of Science and Technology-Science and Engineering Research Board) New Delhi, and I am highly thankful to Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly for providing research place for this work.

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