

DETECTION OF SPECIFIC CAUSES OF TUBERCULOSIS IN THE DAIRY CATTLE OF BANGLADESH AGRICULTURAL UNIVERSITY BY SEQUENCING AND SEQUENCE ANALYSIS

A. Yasmin, M. Z. Hossain, U. K. Rima, T. Ruba and M. A. H. N. A. Khan*

Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

ABSTRACT

Tuberculosis (TB) is a chronic disease of man and animal. In this study intradermal tuberculin test, necropsy, histopathology, polymerase chain reaction (PCR) and sequencing techniques were used to diagnose specific cause of TB in dairy cattle of Bangladesh Agricultural University, Mymensingh. Intradermal tuberculin tests were carried out on randomly selected 100 dairy cattle. Tuberculin test positive cattle (N=05) were examined at necropsy and granulomas in lungs was seen in three cattle. Caseous necrosis and swelling of lymphnodes was seen in prescapular (N=01) and mesenteric (N=02) lymphnodes. In a case nodular lesion was seen in lungs and mesenteric lymphnodes. Portion of infected lungs and lymphnodes were snap frozen, extracted genomic DNA and PCR protocols was adapted targeting MPB83 gene. Result of PCR showed amplification of 600bp fragments in five cases. The MPB83 gene although specific for *M. Bovis*, the gene is less abundantly expressed by *M. tuberculosis*. To differentiate infectivity due to *M. Bovis* and *M. Tuberculosis*, two more PCR were adapted targeting *pncA* and *oxyR* genes. Out of five cattle tested in PCR all samples generated *pncA* specific 185bp and *oxyR* gene specific 270bp amplicons. The sequencing of MPB83, *pncA* and *oxyR* genes were carried out. Results of sequencing did not show mutation in MPB83 gene. Sequencing of *pncA* gene showed replacement of nucleic acid base (guanine to cytosin) in position 169 in cattle no. 5. Similarly, sequence analysis of *oxyR* gene (n=05) showed replacement of nucleic acid base (adenine to guanine) in position 285 in cattle no. 5. The cattle no. 5 was confirmedly infected with *M. Tuberculosis* and rest of the cattle were infected with *M. Bovis*. The tuberculin tests, necropsy, histopathology and PCR amplification of MPB83 gene may not contribute species specific detection of Mycobacterial infectivity in cattle. Sequencing and sequence analysis of *pncA* and *oxyR* genes found to differentiate infectivity in cattle due to *M. Bovis* and *M. Tuberculosis*. Both of the bacterial species are extremely zoonotic and dairy cattle of Bangladesh Agricultural University were infected with both *M. Bovis* and *M. Tuberculosis*. It needs to tests all the dairy cattle twice in a year with tuberculin tests and dispose test reactors in order to minimize zoonotic risk.

Keywords: Dairy cattle, BTB, tuberculin test, Ziehl Neelsen staining, PCR, *pncA*, *oxyR* genes, MPB83 gene.

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic debilitating disease of dairy animals, caused by intracellular acid-fast bacterium *M. bovis* (Ameni *et al.*, 2007) and *M. tuberculosis* (Hossain *et al.*, 2016). Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide (Liebana *et al.*, 1995). Bovine TB caused by *M. bovis* is member of *M. tuberculosis* complex (MTBC) and refers to a genetically related group of Mycobacterium species that can cause TB in men and animals. *M. tuberculosis* complex includes: *M. tuberculosis*, *M. africanum*, *M. bovis* and the *Bacillus Calmette–Guérin strain*, *M. microti*, *M. canettii*, *M. caprae*, *M. pinnipedii* and *M. mung* (Hamidou *et al.*, 2006). Among them *M. bovis* and *M. tuberculosis* are common in men and dairy cattle (Ayele *et al.*, 2004; Thoen *et al.*, 2006; Yumi and Tooru, 2007). TB caused by *M. tuberculosis* (MTB) is a specific infectious cause of illness and death of human. In 2013, approximately 9 million people in the world developed active TB and 1.5 million die (WHO, 2014). These numbers are increasing globally, especially in Africa, China, India, Eastern Europe and the former Soviet Union (WHO, 2012). The human burden of TB caused by *M. bovis* is largely unknown (Garnier *et al.*, 2003). In 2014, TB killed 1.5 million people (1.1 million HIV-negative and 0.4 million HIV-positive). Death toll comprised 890000 for men, 480000 for women and 140000 for children (WHO, 2015). In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) women and 1.0 million (10%) children. People living with HIV accounted for 1.2 million (11%) of all new TB cases. Six countries accounted for 60% of the new cases including India, Indonesia, China, Nigeria, Pakistan and South Africa and Bangladesh got 7th (WHO, 2016).

*Corresponding e-mail address: hadi.khan@bau.edu.bd

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It has been estimated that more than 50 million cattle are infected with *M. bovis* worldwide and that the resulting economic losses are approximately \$3 billion per year (Garnier *et al.*, 2003). Literature available indicated that the occurrence of TB in cattle due to *M. bovis* and *M. tuberculosis* is common in many developing countries and due to lack of appropriate technology. Identification of the incidence of cattle TB is mostly ignored (Forrellad *et al.*, 2013).

In developed countries pasteurization of milk and control of bovine tuberculosis (BTB) by test and slaughter based control measures have dramatically reduced the transmission of *M. bovis* infection from cattle to humans. *M. bovis* is now predominantly an occupational zoonosis with potential risk for workers on farms, in abattoirs, and in zoos (Dalovisio *et al.*, 1992; Hossain *et al.*, 2016). Bovine TB has got a major impact on livestock productivity, and on the livelihoods of poor and marginalized communities. In 2015, there were an estimated 149 000 cases of zoonotic TB in human (WHO, 2016). The scenario of zoonotic TB in Bangladeshi cattle is less defined.

Bangladesh is one of the highest TB burden countries; total human case notification in 2013 was 1, 90,891 and ranked seventh in terms of global occurrence of TB (WHO, 2014). Tuberculosis in cattle is mostly caused by *M. bovis* and in human is due to *M. Tuberculosis*; both of the organisms are transmissible between man and animals and are highly zoonotic. Now a day, the incidence of *M. bovis* in cattle is increased in Bangladesh (Hossain *et al.*, 2016) and in human, the incidence of TB due to *M. bovis* is more than 35% (Hewinson *et al.*, 2006). Dairy industry in Bangladesh started rising but the incidence of TB due to *M. bovis* and *M. Tuberculosis* is largely unknown. Tuberculosis in elderly dairy cattle in farm condition is common and cross breed cattle are mostly susceptible (Hossain *et al.*, 2016). The dairy farm of Bangladesh Agricultural University has got local and cross breed cattle and there were sporadic infectivity of cattle due to *M. bovis* and *M. tuberculosis* (Al Mamun *et al.*, 2016). As tuberculosis is a slow growing infectious disease it is absolutely difficult to identify infected but symptomless cattle and thus infected cattle spreading diseases silently in farm cattle and human being working around. The Sustainable Development Goals (SDGs) for 2030 were adopted by the United Nations in 2015. One of the targets is to end the global TB epidemic. The WHO End TB Strategy, approved by the World Health Assembly in 2014, Detection of specific causes of tuberculosis in the dairy cattle calls for a 90% reduction in TB deaths and an 80% reduction in the TB incidence rate by 2030, compared with 2015 (WHO 2016). The incidence of TB in animals in developing country including Bangladesh is largely unknown and may warn expanding TB. This study was, aimed at specific identification of TB in dairy cattle using tuberculin test, necropsy and histopathology. The rapid and accurate detection of *Mycobacterium sp.* is of paramount importance in the effective control and eradication of TB from dairy animals and human.

MATERIALS AND METHODS

Selection of animals

The dairy cattle were tested in the Bangladesh Agricultural University (BAU) dairy farms; histopathology and polymerase chain reaction (PCR) was carried out in the Department of Pathology, Faculty of Veterinary Science, BAU, Mymensingh. A total of 25 Holstein Frisian X local cross breed, 25 red Chittagong and 50 local breed cattle were tested using intradermal tuberculin test during the period between July 2014 to June 2015.

Identification of TB test reactors

A short needled syringe (McLintock® preset syringe and tuberculin testing equipment, Glasgow, G81 1NH) was used to perform intra-dermal tuberculin test in dairy cattle. The tuberculin test was performed onto the caudal fold of the tail and the mid-neck region according to the standard protocol of OIE (OIE, 2009).

The caudal fold was pinched up with the tip of fingers and its thickness was measured by callipers. The tuberculin (Bovituber) was injected in the caudal fold; injection site was examined for swelling and thickness as measured using callipers at every 24 hrs of tuberculin injection until 72 hrs post injection (Monaghan *et al.*, 1994). A total of 100µl of Bovine PPD was injected within the dermis of the caudal tail fold using a short needled syringe. The test was read at 24, 48 and 72 hrs (+/- 6 hrs) after the tuberculin was administered. The injection site was inspected by both visual observation and palpation for indications that the animal has mounted an immune response to the PPD. The response was recognized by swelling or discoloration at the site where the PPD was injected. If any abnormalities such as discoloration or swelling are found at the injection site, the animal was classified as Caudal-fold Tuberculin Test (CFT) test responder (also known as suspect). If no response was noted, the animal was classified as CFT test-negative.

Differentiate *Mycobacterium* spp. using Comparative intradermal tuberculin test

The Caudal-fold Tuberculin Test (CFTT) responder cattle to bovine PPD may mount immune response against a number of *Mycobacterium* species including *M. Bovis*, *M. Tuberculosis*, *M. Avium sub-spp var paratuberculosis* and *M. avium*. The Comparative intradermal tuberculin tests (CITT) were used as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals were used to differentiate infectivity due to *M. bovis/ tuberculosis* and para TB. **This** investigation was carried out with the **selected** cattle test positive to CFTT. Two injection sites (neck region, up and down, 130±10mm apart) were clipped, shaved and cleansed with spirit soaked cotton. A fold of skin within each clipped area was measured using callipers before injection. A short needled syringe (McLintock® preset syringe and tuberculin testing equipment, Glasgow, G81 1NH) were loaded with either avian or bovine type tuberculin and 0.1ml of the content was injected 7 days after SITT. The bovine type tuberculin was injected, into the lower clipped area while the avian type was injected into the upper clipped area on the same side of the mid-neck of cattle. The skin fold thickness of each injection site was measured 24, 48 and 72 hours after the injections. A case was considered positive while the skin reaction with bovine type tuberculin was 4 mm or more than that with the avian type tuberculin. An inconclusive result was recorded when the bovine reaction recorded between 1 and 4 mm but greater than the avian reaction. A negative result was recorded while the bovine reaction appeared negative or if the positive or inconclusive bovine reaction equal to or less than the avian reaction site.

Pathological investigation of tuberculin test positive cattle

The routine necropsy was done on the tuberculin test responder cattle in the BAU Dairy Farm, Mymensingh. The tuberculin test positive cattle (N=05) were euthanized by intravenous injection of saturated MgSO₄ solution and a thorough post-mortem examination was carried out. At necropsy, gross tissue changes were observed and recorded carefully. Personal protection was taken not to get infected. The lungs, thoracic, prescapular, prefemoral and mesenteric lymphnodes were examined carefully. The organs were sectioned 1cm apart to visualised nodular lesions if any. Representative sections of lungs and lymphnodes were collected in 10% neutral buffered formalin for the histopathological study. The tissue sections of lungs and lymphnodes were stained with Hematoxylin and eosin (H&E) and Ziehl Neelsen staining (Luna, 1968). Portion of lungs and lymphnodes were snap frozen, extracted DNA for PCR detection of specific of TB in cattle.

PCR detection of specific cause of TB in cattle

Most of the mycobacterium localized in the epithelial cells, macrophages, lymphocytes and neutrophils of infected tissues and these cells serve as the unique source of Mycobacterial DNA. The lungs and lymphnodes showed granulomatous lesions were retrieved from liquid nitrogen and used in DNA extraction. The lungs and lymphnodes were crushed in liquid nitrogen and extracted DNA using the phenol–chloroform–isoamyl (PCI) alcohol and ethanol precipitation method (Labony *et al.*, 2014). The purity and concentration of extracted DNA were determined by agar gel electrophoresis and spectrophotometric analysis. The absorbance of the extracted DNA was measured at A²⁶⁰ and A²⁸⁰. The DNA concentration obtained were ranged between 200ng to 350ng/μl. The extracted DNA was further diluted in nuclease free water at a concentration of 100ng/μl and stored at -20°C until PCR was carried out.

The PCR protocols adapted targeting three important genes to identify and differentiate infectivity due to *M. tuberculosis* and *M. bovis*. DNA from the BCG vaccine strain was used in PCR reactions as positive control for *M. bovis*. Three sets of PCR were carried out targeting MPB83, pncA and oxyR genes (Table 1). The reaction was carried out in 25μl volume in the thin walled PCR tubes. The promega TNC PCR Master Mix Kit (2x) was used to amplify genomic DNA in a programmable thermocycler (Eppendorf, Germany). Three sets of PCR primers 20pol/ reactions was used. A total of 32 cycles of PCR amplification was carried out (Table 2) and the amplicons were electrophoresed in 1.5% agarose gel. The images were captured by using a gel documentation system (Cell Biosciences, Alphamager HP, USA).

Sequencing of MPB83, pncA and oxyR genes to identify specific cause of TB

The fragments of MPB83 (600bp), pncA (185bp) and oxyR (270bp) genes as amplified in PCR were gel cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The PCR product (50μl) was electrophoresed and the band corresponding to the DNA of interest was located against long-wavelength ultraviolet light. Using a sterile scalpel and blade the segment of the gel containing the band of interest was cut

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out, chopped and extracted (according to manufacturer instruction). Purified cDNA (50-75 µl) of each gene was transferred to 0.5ml PCR tube separately for each sample. For each sample forward and reverse primer (10pmol) were used. The cleaned cDNA along with its primers was shipped to AIT Biotech, Singapore for sequencing. The sequences obtained were analyzed with Basic local alignment system (BLAST) and nucleic acid bases at specific location were analysed. The nucleic acid sequences were aligned with other related sequences retrieved from the GenBank and the species of Mycobacterium involved were determined.

Table 1. The PCR primers used to amplify fragments of MPB83, pncA and oxyR genes of *M. tuberculosis* (MTB) and *M. bovis* (BTB)

Primer name	Primer sequences (5'-3')	Amplicon size	References
MPB83 F	cagggatccaccatgttcttagcgggtg	600bp	Jiang <i>et al.</i> , 2006
MPB83 R	tggcgaattc-ttactgtgccgggg		
pncA F (MTB)	atgcgggcgtgatcatcgtc		Monteros <i>et al.</i> , 1998
pncA R		185bp	
MTB			
BTB	cggtgtgccggagaagcgg cggtgtgccggagaagcgg		
oxyR F (MTB)	tggccgggcttcgcgcgt		
oxyR R		270bp	
MTB	gcacgacggtgccaggea		
BTB	tcacgacggtgccaggea		

Table 2. The thermal profiles used to amplify fragments of Mycobacterium genes

Genes targeted	Primers name	Thermal profile				Reaction held
		Initial denaturation	PCR Cycles (N=32)			
			Denaturation	Annealing	Elongation	
MPB83	MPB83 F MPB83 R	98°C, 5min	95°C, 1min	56°C, 1min		4°C
pncA	pncA F pncA R	95°C, 12min	94°C, 1min	67°C, 1min	72°C, 2min	72°C, 10min
oxyR	oxyR F oxyR R			70°C, 1min		

RESULTS AND DISCUSSION

Bovine tuberculosis (BTB) is an infectious disease of cattle and one of the biggest challenges facing the cattle farming industry today, particularly in Bangladesh (Ayele *et al.*, 2004). It is caused by mostly due to bacterium *M. bovis* and also due to *M. tuberculosis* (Alam *et al.*, 2012; Hossain *et al.*, 2016). This study was carried out at the BAU Dairy farm and 100 adult cattle were tested using CFTT and CITT. Results of CFTT revealed skin thickening/ hypersensitivity in five cattle. The CIFT positive cattle were further tested with comparative tuberculin tests, none of the cattle appeared positive to avian PPD. Previously Al Mamun *et al.* (2016) used both the CFTT and CITT and none of the test cattle responded to avian PPD. Dairy cattle in Bangladesh were infected with bovine TB (*M. bovis*), human TB (*M. tuberculosis*) and para TB (*M. avium subsp var paratuberculosis*) and causing variable rate of morbidity and mortality (Hossain *et al.*, 2016). By using CITT, infectivity in cattle due to avian TB can be differentiated from TB due to *M. bovis* and *M. tuberculosis* if there is swelling at both the injection sites (Al Mamun *et al.*, 2016; Hossain *et al.*, 2016). However, in this study, the CFTT responder cattle did not respond to avian PPD and the cattle (N=05) were assumed to be infected with either *M. bovis* or *M. tuberculosis*. Molecular test protocols like PCR and sequencing of specific genes are needed to ensure infectivity in cattle due to *M. bovis* and *M. tuberculosis*. The tuberculin test positive cattle (N=05) were, therefore, sacrificed by intravenous administration of saturated magnesium solution and further investigation was carried out.

Detection of specific causes of tuberculosis in the dairy cattle

At necropsy (N=03) the carcasses appeared very thin and paler (N=03), nodular masses was seen in the lungs (Figure 1a) of three tuberculin test positive cattle. Two cattle were appeared apparently healthy and the cattle had localized TB in mesenteric lymphnodes. The prefemoral (N=01) and mesenteric lymph nodes (N=02) were swollen in three cases (Figure 1b and 1c). In a case nodular lesions was seen both in the lungs and mesenteric lymphnodes. Caseous lesion was not seen in the prefemoral and thoracic lymphnodes of tuberculin test positive cattle. Tuberculosis in cattle caused granulomatous lesions in visceral organs (Al Mamun *et al.*, 2016) and such lesions as seen at necropsy is indicative of Mycobacterial infectivity. To support the findings of this study, histopathological and PCR detection of specific causes of TB was carried out.



Figure 1. Lungs (a), prescapular lymph nodes (b) and mesenteric lymphnodes (c) obtained from Tuberculin test positive cattle. Nodular masses were seen in infected lungs (a, black arrow). The prescapular and mesenteric lymphnodes were enlarged and the capsule appeared tensed (b & c).

Formalin fixed tissues sections were, therefore, stained with H&E staining. Tissues sections of lungs and lymph nodes studied under microscope showed accumulation of macrophage and fibrin in lungs. Typical granulomas characterized by central core of caseous necrosis was not seen in lungs (Figure 2a) but were seen in lymphnodes (Figure 2b). The lungs lesions showed accumulation of macrophage, lymphocyte and fibrous connective tissue in the infected areas (Hossain *et al.*, 2016). The gross and microscopic lesions seen in tuberculin test positive cattle were suspected to infect with Mycobacterium. The cause(s) of the granulomatous reaction in tissues was, therefore, detected by using Ziehl Neelsen staining. Tissue sections of lungs and lymphnodes while stained with Ziehl Neelsen staining showed acid fast bacteria (Figure 2c) in intracellular and extracellular state. The detection of acid fast organism in tissue section of lungs and lymphnodes is suggestive for Mycobacterial infectivity and may be due to *M. bovis* or *M. tuberculosis* (Alam *et al.*, 2012; Hossain *et al.*, 2016; Michel *et al.* 2007).

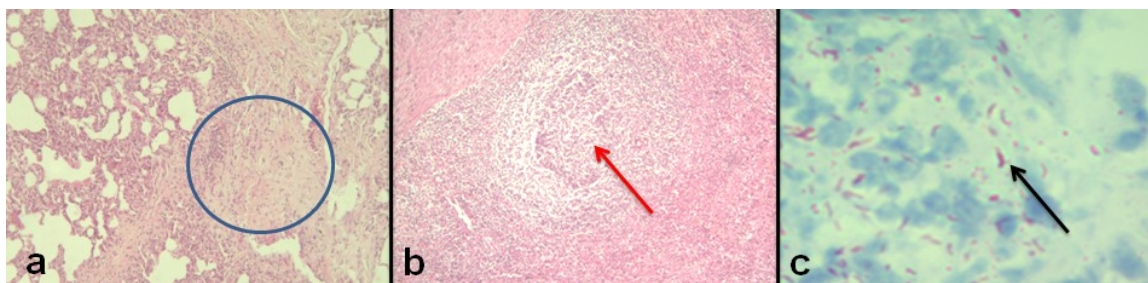


Figure 2. Sections of lungs (a) and lymphnode (b) obtained from tuberculin test positive cattle and stained with H&E (10x). Accumulation of macrophage, lymphocytes and fibrin in tissue section of lungs of was seen. Sections of lymphnodes (b, arrow) showed granulomas consisting of central core caseous necrosis and peripheral infiltrations of monocytes and lymphocytes. Sections of lungs obtained from tuberculin test positive cattle and stained with Ziehl Neelsen stain showed pink color acid fast bacilli in the tissues (100x).

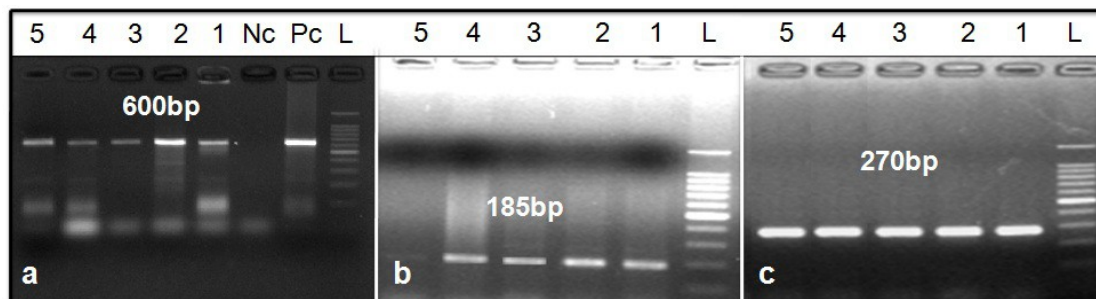


Figure 3. PCR detection of MPB83 (a), pncA (b) and oxyR (c) genes of Mycobacterial organisms. DNA extracted from the lungs and lymphnodes tuberculin test positive cattle showed the amplification of 600bp, 270bp and 185bp fragments of MPB83 (a), pncA (b) and oxyR (c) genes of Mycobacterium species respectively. The lane L is for 100bp ladder, Pc is for BCG vaccine DNA and Nc is for negative control. The lanes 1, 2, 3, 4 and 5 represent the test samples.

Tuberculin test reactivity, gross and microscopic changes in tissues and Ziehl Neelsen staining of tissue sections represent the cause of illness as *Mycobacterium species*, mostly due to either *M. bovis* or *M. tuberculosis*. The specific cause of tuberculosis in cattle was, therefore, confirmed by using species specific PCR. Initially a PCR was adapted to amplify fragment of MPB83 gene specific for *M. bovis* (Sreedevi *et al.*, 2003; Jiang *et al.* 2006; Taylor *et al.* 2007). The extracted DNA (N=05) including BCG vaccine DNA found to amplify 600bp fragment in uniplex PCR (Figure 3a). MPB83 gene is critical for *M. bovis* but may also be shared by *M. tuberculosis* (Hossain *et al.*, 2016). To differentiate infectivity of cattle TB due to *M. tuberculosis* and *M. bovis* two more PCR were adapted targeting pncA and oxyR genes (Table 1). The extracted DNA (N=05) while used in PCR found to generate 185bp and 270bp amplicons (Figure 3b and 3c) suggestive for infectivity due to either *M. tuberculosis* or *M. bovis*. The specific cause of cattle TB due to *M. tuberculosis* and *M. bovis* was not confirmed by amplifying pncA and oxyR genes. In order to confirm further the specific cause of TB in cattle, the fragments of MPB83, pncA and oxyR genes were sequenced. Mutation in the fragments of MPB83 gene (600bp) was not seen at any time point and all the cattle samples tested in PCR found to contain similar base sequences in MPB83 gene. The polymorphisms in pncA and oxyR genes at specific loci are good indicator of specific infectivity due to *M. bovis* and *M. tuberculosis* (de los Monteros *et al.*, 1998). Point mutation in the pncA (169 base point) and oxyR (285base point) genes are the indicator of infectivity due to either *M. bovis* or *M. tuberculosis*. Results of sequence analysis of pncA gene showed replacement of nucleic acid base (guanine to cytosin) in position 169 in a case (cattle No. 5). Similarly, sequence analysis of oxyR gene of the studied isolates showed replacement of nucleic acid base (adenine to guanine) in position 285 in the same case (cattle No. 5). The nucleic acid bases in pncA and oxyR genes of *Mycobacterium species* in cattle No. 1, 2, 3 and 4 differ greatly from cattle No. 5. Analysis of the polymorphisms in the sequence of pncA and oxyR genes, it was confirmed that the cattle No. 5 of BAU dairy farm was infected with *M. Tuberculosis* and cattle 1, 2, 3 and 4 were infected with *M. Bovis*. The tuberculin tests, necropsy, histopathology, acid fast staining and PCR were successfully applied onto the cattle and samples collected from the cattle of BAU dairy farm. About 4% cattle were infected with *M. bovis* and 1% with *M. tuberculosis*. The PCR protocols and sequencing system adapted accurately identified infectivity due to *M. bovis* and *M. tuberculosis*.

CONCLUSIONS

To identify mycobacterial infectivity in cattle (N=100) intradermal tuberculin tests were carried out and five cattle appeared positive to both single intradermal tuberculin tests and comparative intradermal tuberculin tests but none of the cattle tested positive to avian PPD. At necropsy, tuberculous nodules in lungs and enlargement of lymphnodes (N=03) was seen but were not consistent in all cases. In a case tuberculous nodule in lungs and enlargement of lymphnodes were seen. Two tuberculin test positive cattle only showed enlargement of lymphnodes and lacking nodular lesion in lungs. The PCR protocols adapted amplified fragments of MPB83, pncA and oxyR genes. Mutation was not seen in the fragment of MPB83 gene sequenced and sequencing of MPB83 gene could not contribute specific detection of the cause of TB. The sequence analysis of pncA and

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oxyR genes indicated that four cattle were infected with *M. bovis* and one with *M. tuberculosis*. Both the *M. bovis* and *M. tuberculosis* are extremely zoonotic pathogens and differential infectivity in cattle can be achieved by amplifying and sequencing fragments of *pncA* and *oxyR* genes. Cattle of BAU dairy farm were infected with both the *M. Bovis* and *M. Tuberculosis*. It needs to test all the elderly dairy cattle with tuberculin tests twice in a year, disposed test positive cattle before spreading TB in other cattle and farmers working in the farm. Genomic DNA from the lungs or lymphnodes of tuberculin test positive cattle require to be tested in PCR, sequencing and sequence analysis for the detection of specific cause of TB and design future preventive strategy.

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