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Research Article

Detection Of *Mycobacterium Tuberculosis* Complex Infection In Apparently Healthy Mithun (*Bos Frontalis*) Using Serum PCR As An Alternate Diagnostic Tool

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ABSTRACT

Conventional methods for the diagnosis of *Mycobacterium infections* have several limitations but are still the most applicable for implementation of tuberculosis control programme. To determine efficacy of IS6110 based polymerase chain reaction (PCR) in detection of *Mycobacterium* DNA in serum samples collected from 702 mithun from North-Eastern Hilly Region of India, including those at the positive farm were tested. Samples were classified with reference to age, sex, strain of mithun and place. *Mycobacterium tuberculosis* complex specific amplicon could be detected in serum of 3 (1.28%) serum samples of mithun with PCR and specificity of amplicon was confirmed through sequencing. All three serum samples were from Nagaland strain and from the same farm in Nagaland, where cases were diagnosed with intra-dermal test a few years back. The study indicated that *Mycobacterium tuberculosis* complex infection may also be detected with PCR using DNA template extracted from serum, and may be instrumental in early diagnosis of the disease.

INTRODUCTION:

Mithun (*Bos frontalis*), cattle of hilly region, dwelling in North-Eastern hills (NEH) of India and tropical rain forest of China and neighbouring states has an important role in the social, cultural and economic life of the local tribal populations. The total population of mithun in India is 2, 98,255; maximum in Arunachal Pradesh followed by Nagaland, Manipur and Mizoram. All the four mithun strains found in four different states viz., Arunachal, Nagaland, Manipur and Mizoram strains are superior in dressed meat percentage (58.82%) than cattle (55.96%) and are primarily reared for meat.[1]

Bovine tuberculosis, caused by *Mycobacterium bovis*, is an important zoonotic disease also affecting other domesticated, semi-domestic and wild animals and is endemic in India.[2] Tuberculosis is characterized by the formation of nodular granulomas and is commonly defined as a chronic debilitating disease.[3] Bovine tuberculosis is often sub clinical animals showing no apparent clinical signs and if any are not specific and distinctive. Bovine tuberculosis is usually diagnosed on the basis of delayed hypersensitivity reactions using various tuberculin tests such as single intradermal, comparative intradermal, short thermal and Stormont tests.[4] Disease is often diagnosed during meat examination or after death during necropsy examination through gross and histopathological mean. The disease is confirmed through isolation of the bacterium. Molecular tests like polymerase chain reactions and spoligotyping have also been used for confirmation of the suspected cultures or clinical samples often not available in case of animals.[5, 6] Utility of IS*6110* PCR in diagnosis of extrapulmonary tuberculosis has been acclaimed and is of much utility because of lack of specific clinical samples and has been found superior in sensitivity than conventional tests.[6]

Attempts to identify circulating DNA in blood and or serum for identification of pathogens started long back specifically in cases with obscure aetiology.[7-11] Diagnosis of tuberculosis using PCR on blood was also attempted simultaneously. The IS6110 PCR assay correctly identified 39 of 41 patients with proven pulmonary tuberculosis with overall sensitivity and specificity of 95% and 89%, respectively.[12] However, later,[13] very low sensitivity of blood sample based PCR was reported, out of 96 cases, 60 (62.5%) were culture positive and only 14 (14.5%) cases were diagnosed correctly with PCR. In another study using real time PCR and primers from mpt*64* gene on blood samples, diagnostic sensitivities for pulmonary, extra-pulmonary and disseminated TB were 26.9%, 45.4% and 50%, respectively, the sensitivity increased a bit when plasma was also included for isolation of template DNA from white blood cells. [14] Using patient serum as starting sample, PCR for tuberculosis [15] was found comparable any other method for diagnosis of tuberculosis. Serum PCR identified correctly 92% patients with proven tuberculosis, and 83% patients with suspected tuberculosis. None of the specimens from the healthy control group were positive in their study.

In animals, for diagnosis of tuberculosis use of PCR started simultaneously using several types of clinical and nonclinical samples including faeces, milk, tissues and organ aspirates etc. But there is hardly any report of using blood or serum as sample.[16] However, recently serum sample based PCR has been used in animals for diagnosis of *Brucella*[17] and *Bordetella bronchiseptica* infections[18] goats and dogs, respectively. Serum has been reported superior than blood as sample for *brucellosis* by PCR[8] and also better results have been reported for tuberculosis in human beings[12], probably due to co-purification of PCR inhibitory substances with pathogen DNA.[19] Information of use of PCR based diagnosis for tuberculosis in mithun with any kind of sample is still more scares. Therefore, this study was undertaken to evaluate the efficacy of PCR in diagnosis of tuberculosis infection in mithun using serum samples.

MATERIALS AND METHODS

A total of 702 serum samples including 104 samples from the farm where tuberculin reactors were identified a few years back (Annual report 2010-2011)[20] but at present no reactor was detected were collected. Besides, samples were also collected from mithuns without any history of tuberculin testing from different places in North Eastern States of India (Table. 1). All the samples were from apparently healthy mithuns. Of the 702 mithun sampled, 104 from Medziphema Mithun Farm and 84 from Mithun Farm, Porba, Nagaland were tested negative for tuberculosis with single intradermal tuberculin test, and other animals in the study were never been tested with any of the test for tuberculosis. Genomic DNA was extracted from 200 µL of serum sample using QIAamp DNA Mini

Kit (Qiagen, Germany) following the recommended protocol. The concentration and purity of the DNA extracted from the serum was measured by Nano drop (Thermo Scientific, USA) and DNA samples with desired purity, indicated by $260/280_{nm}$ reading ratio of 1.6 to 1.8 were used as template for PCR reaction. The DNA extracted from the serum samples was stored at -20°C till used in PCR. Using IS6110 sequence specific custom synthesized (Eurofin Pvt. Ltd., India) primers. The primers used in the study were designed to give 445 bp product and their sequences were: F5' GACCACGACCGAAGAATCCGCTG 3' and R5' CGGACAGGCCGAGTTTGGTCATC 3'. PCR reactions were carried out. PCR reaction was optimized in 25µl reaction volume using 5 µl of DNA template, 1 µl each of 10 pMol forward and reverse primers, 12.5 µl of PCR master mix (Qiagen, Germany) and 5.5 µl of nuclease free water. The amplification of PCR was carried out in a thermal cycler with an initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min, extension at 72°C for 1 min, followed by final extension step at 72°C for 10 min. Amplicon of 445 bp in PCR product was analysed under UV-gel documentation system (Alpha Innotech Co., USA) after electrophoresis on 1% agarose gel (IBI Scientific, Peosta Lowa) containing 0.2 mg/ ml ethidium bromide at 80 volts using 0.5× TBE electrophoresis buffer (Bio Basic Inc. USA). As marker 100 bp DNA ladder (GelPilot DNA Molecular Weight Markers, Qiagen India Pvt. Ltd., New Delhi, India, Cat. no. 239035) was used. Purified amplicon was submitted for sequencing to Eurofin Pvt. Ltd, India. For sequencing of the PCR product PCR products from all positive and control samples were submitted paid sequencing to Eurofin Pvt. Ltd, India.

RESULTS AND DISCUSSION

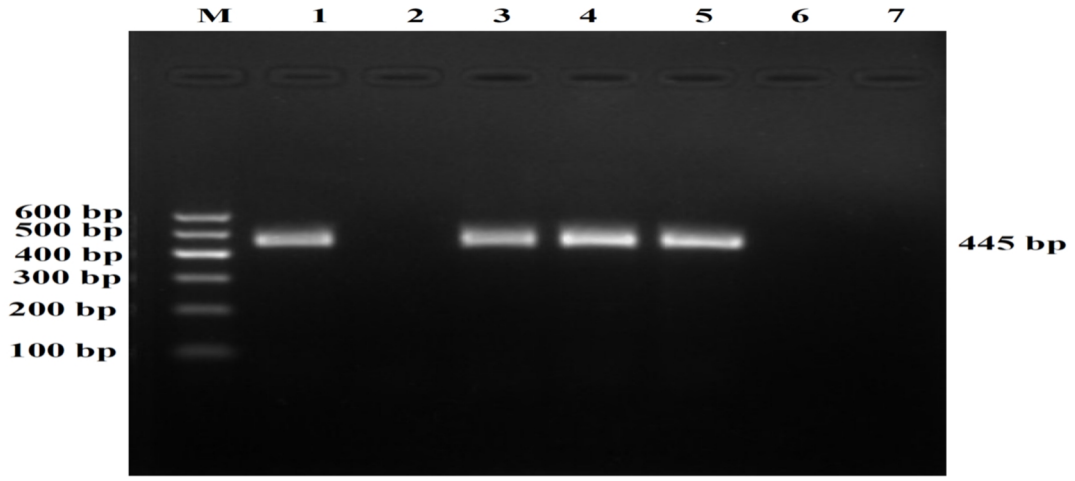
The average genomic DNA concentration in serum of mithun ranged between 0.3 ng μL^{-1} and 28.8 ng μL^{-1} of serum. Out of 702 serum samples, 3 (0.43%) samples were positive for *Mycobacterium tuberculosis* complex with PCR targeting IS*6110* gene giving sequence specific amplicon of 445 bp (Figure. 1). Sequencing results indicate complete homology of PCR product from positive samples with that of control strain's IS6110 sequence. All the three serum samples were from mithun of Nagaland strain and also from Nagaland region (Table. 1). Of these two were male (3 years and 5 years of age) and one female (5 years of age). Both the male were non tuberculin reactors but from the same farm on which positive tuberculin reactors were detected three years ago. One female animal was from a small herd of 62 animals in Phek village. Use of IS6110 based PCR is the most widely used and accepted[21] and specific in diagnosis of tuberculosis in human and animal samples[12, 16] but its utility has not yet been tested on mithun samples. The study concluded that serum samples can be used to diagnose tuberculosis infection even in those mithun which are non-reactors in tuberculin test and results may probably be extrapolated for diagnosis of tuberculosis using serum samples of other animals.

Table 1. Details of mithun serum samples collected from different places in North Eastern Hilly region of India

S. NO.	Places of sampling	Number of samples collected	Number of animal positive with PCR
1	Medziphema, Nagaland	217	0
	Mithun farm Medziphema, Nagaland	104	2 (1.92%)
2	Phek, Nagaland	62	1 (1.61)
3	Kohima, Nagaland	37	0
4	Longidang, Wokha, Nagaland	11	0
5	Longleng, Nagaland	11	0
6	Mithun Farm, Porba, Nagaland	84	0
7	Wokha, Nagaland	10	0

8	Zunheboto, Nagaland	27	0
9	Tuensang, Nagaland	17	0
10	Champhai, Mizoram	27	0
11	Chandel, Manipur	14	0
12	Churchunpur, Manipur	17	0
13	Senapati, Manipur	14	0
14	Lower Dibang Valley , Arunachal Pradesh	17	0
15	Pasighat, Arunachal Pradesh	17	0
16	Papumpare, Arunachal Pradesh	16	0
	Total	702	3

Fig. 1. Mycobacterium tuberculosis specific PCR using IS6110 specific sequence. As marker 100 bp DNA ladder (GelPilot DNA Molecular Weight Markers, Qiagen India Pvt. Ltd., New Delhi, India, Cat. no. 239035) was used.



Lane M : Marker
Lane 1 : Positive control
Lane 2 : Negative control
Lane 3-5 : Positive samples

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