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Designing Polymerase Chain Reaction (PCR) Technique for the Detection of Specific Causes of Tuberculosis (TB) in Dairy Cattle and Human

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Abstract

Tuberculosis (TB) in cattle and human is caused by *M. bovis*, *M. tuberculosis* and *M. avium* subsp. var *Paratuberculosis* (*M. paratuberculosis*). Selected dairy cattle (N=700) and human (N=20) were tested using tuberculin tests and X-ray imaging. Aspiration biopsy of prescapular lymphnodes from tuberculin test positive cattle and urine, cough, pleural and peritoneal fluid from suspected human was further tested using Ziehl Neelsen staining. Genomic DNA from tuberculin test positive cattle (N=23) and suspected humans (N=20) were tested using multiplex polymerase chain reactions (PCR) targeting 16srRNA (1030bp, 180bp). Results showed that 23 cattle and 11 human were infected with TB. To detect infectivity due to *M. bovis* and *M. tuberculosis* selected cattle (N=11) and humans (N=11) samples were tested in uniplex PCR targeting MPB83 (600bp) and H37Rv Rv3479HP (667bp) genes respectively. Results of PCR showed that all of the bovine and seven human samples generated MPB83 gene specific 600bp amplicon. Two bovine and seven human DNA generated H37Rv Rv3479HP gene specific 667bp amplicons. Seven cattle were infected with *M. bovis* and two with *M. tuberculosis*. Two cattle were co-infected with *M. bovis* and M. paratuberculosis. Four humans were infected with *M. bovis* and seven with *M. tuberculosis*. The MPB83 gene is invariably shared by *M. bovis* and *M. tuberculosis*. The PCR protocol designed targeting fragment of H37Rv Rv3479HP (667bp) gene is selective for *M. tuberculosis*. Results of sequencing showed point mutation in 16srRNA, MPB83 and H37Rv Rv3479HP genes. Phylogenetic analyses of the selected genes of *M. bovis* and *M. tuberculosis* showed that the organisms were belonging to Lineage 1. Intradermal tuberculin test and smear microscopy unable to differentiate infectivity due to *M. bovis* or *M. tuberculosis*. The PCR technique designed appear specific for *M. tuberculosis*, can be used to detect causes of TB in mammals and designing future preventive strategies accordingly.

Keywords: *M. bovis*; *M. tuberculosis*; *M. paratuberculosis*; Tuberculin test; Bovine; PCR; Phylogeny

Introduction

Tuberculosis (TB) is an ancient infectious disease of man, animals and birds [1,2,3]. Human and bovine TB caused by *M. bovis* is a member of *M. tuberculosis* complex (MTBC) that includes: *M. tuberculosis*, *M. africanum*, *M. bovis* and the *Bacillus Calmette–Guérin* strain, *M. microti*, *M. canettii*, *M. caprae*, *M. pinnipedii* and *M. mung* [4]. Infectivity due to *M. bovis* and *M. tuberculosis* are common in dairy cattle and humans [1]. TB caused by *M. tuberculosis* is a specific infectious cause of death in human. In 2013, 9.0 million people in the world developed active TB and 1.5 million die [5,6]. These numbers are increasing globally, especially in Africa, China, India, Eastern Europe and the former Soviet Union [7]. Infectivity in cattle due to *M. bovis* related to heavy economic loss in dairy industry throughout the world due to carcass condemnation, trade restriction and potential zoonotic threat. It has been estimated that globally more than 50 million cattle were infected with *M. bovis* and the resulting economic losses are approximately $3 billion per year [8,9]. In developed countries pasteurization of milk and control of bovine TB (bTB) by test and slaughter have dramatically reduced the transmission of *M. bovis* from cattle to humans but the incidence is poorly defined in developing countries.

M. bovis and *M. tuberculosis* are now predominantly an occupational zoonosis with potential risk for workers on farms, in abattoirs, and in zoos of developing countries [6,10]. Bangladesh is one of the highest TB burden countries; total human case notification in 2013 was 190891 and ranked seventh in terms of global TB distribution [6]. The national tuberculosis control programme (NTP) in Bangladesh has successfully treated 100254 (92%) of the 109012 smear positive cases registered in 2009 and more than 3.7% patients had died due to treatment failure [11]. However, survey is lacking about the prevalence of TB in dairy cattle of Bangladesh. Traditionally, intradermal tuberculin tests and Ziehl Neelsen staining were used in Bangladesh to identify TB in dairy cattle. In human, impression smear staining and chest X-rays are commonest techniques used in field level. Intradermal tuberculin test, Ziehl Neelsen staining and X-rays although detect TB in common but cannot differentiate infectivity due to *M. tuberculosis* and *M. bovis* [12]. These tests were used in preliminary screening of TB in farm animals and human. It is necessary to detect and differentiate TB in cattle and human due to *M. bovis* and *M. tuberculosis* in order to design efficient control measure. Now a day the incidence of human TB due to *M. bovis* is rising. TB in cattle and human due to *M. bovis* is generally drug resistance. Polymerase chain reaction (PCR) techniques targeting various genes although said to be sensitive for the detection of specific cause of TB [13] but is inconclusive [14,15]. This study was, therefore, adapted and designed PCR technologies to identify specific causes of TB in dairy cattle and human and compared its efficacy with the traditional methods. Selected genes of *M. tuberculosis* and *M. bovis* were sequenced and analyzed to identify the level of mutation, possible origin and phylogenetic lineages of the organisms.

Methodology

Intradermal tuberculin tests

Single intradermal tuberculin tests (SITT) were used on to 700 dairy cattle as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [3]. Randomly selected 100 dairy cattle from

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each of the dairy farm were tested using SITT during the period from January 2011 to June 2014. The test reactor (N=23) were further tested using comparative intradermal tuberculin tests (CITT). The tuberculin was obtained from Synbiotics Europe, Lyon – France (Figure 1a) and tests were restricted to animals more than six months of age. The SITT was done in the caudal fold of dairy cattle using a short needled syringe (McLintock® preset syringe and tuberculin testing equipment, Glasgow, G81 1NH) in the Government and private cattle farm of Mymensingh, Sirajganj, Bogra, Sylhet, Chittagong, Dhaka and Tangail Districts, Bangladesh. The syringe was loaded with bovine type tuberculin (Synbiotics Europe, Lyon – France) and 0.1ml of the content was injected per site. The test reaction in the injection sites were measured using calipers following 24, 48 and 72 hours of injection. A case was considered positive while the skin reaction with bovine type tuberculin was 4mm 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. The SITT reactors were isolated and tested for CITT in the neck region within 10 days of caudal fold SITT.

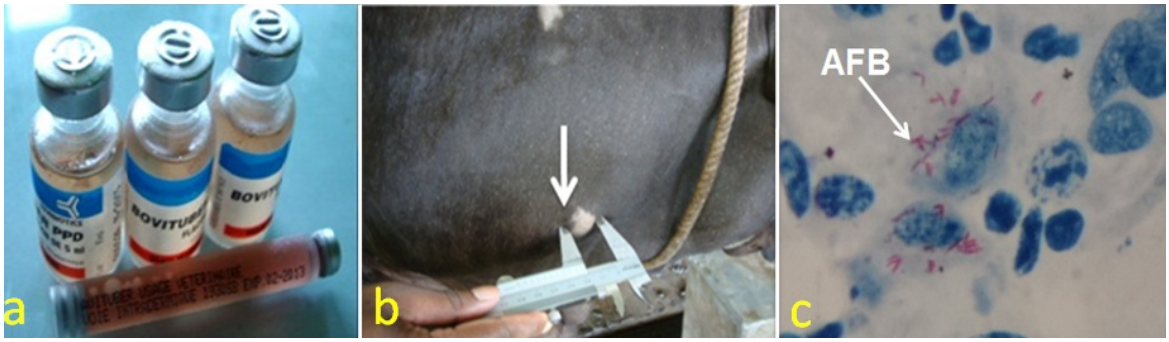

Figure 1: Bovine and avian PPD (a) used in this study. There was a swelling of skin at neck region (b, arrow) following 48hours of intra dermal tuberculin tests of a cow. Ziehl Neelsen staining of smear prepared from the cough of a suspected human showed pink color AFB (c, arrow) in the cytoplasm of respiratory epithelium and epitheloid cells (100X).

Smears microscopy and X-ray imaging to detect TB

Aspiration biopsy from the prescapular lymphnodes of tuberculin test positive cattle (N=23) were collected. Smears of sputum, pleural, peritoneal fluids and urine from 20 suspected humans were collected. The aspiration biopsy, pleural fluids, peritoneal fluids and urine sample were spin down at 5000 g for 10 minutes, supernatant were discarded leaving 20 µl solution per tube. About 5 µl sediment per sample were smeared on to clean slides. A loop full volume of cough was smeared onto clean slide; air dried and fixed on flame. The smears on to the slides were stained with Ziehl Neelsen staining [16]. The stained slides were air dried and observed under oil immersion microscopy. Rest of the samples was snap frozen and used in DNA extraction. The suspected human (N=20) being were subjected to X-ray imaging of their chest and identifying nodular lesions in lungs.

Extraction of genomic DNA

Most of the mycobacterium is localized in macrophages of effected lymph nodes, urinary sediment, pleural and peritoneal fluids, cough and macrophages of these sites served as the unique source of mycobacterial DNA. Lungs tissues from infected cattle at necropsy were used in genomic DNA extraction and PCR detection of diseases. The infected lungs of cattle, aspiration biopsy and cells in the sediments were crushed in liquid nitrogen, suspended in cell lysis buffer and extracted DNA using the phenol–chloroform–isoamyl (PCI) alcohol and ethanol precipitation method [15]. The quality and quantity of the extracted DNA was measured by using agar gel electrophoresis and spectrophotometry (A260 and A280). Approximately 70-100 ng DNA/µl was dissolved in TE buffer and stored at -20°C until PCR was carried out.

PCR detection of TB

The 16SrRNA gene of *M. tuberculosis* complex (MTBC) was targeted to amplify in multiplex PCR (mPCR) using MYCGEN-F1, MYCGEN-R1, MYCAV-R2, MYCINT-F2, TB 1-F3 and TB1-R3 primers [17,18]. Amplification of 1030bp, 850bp, 372bp and 180bp fragments in mPCR indicated the presence of the genus mycobacterium, *M. intracellulare*, *M. tuberculosis* complex and *M. avium* subsp var. *paratuberculosis* respectively [19]. The DNA from these samples was further tested for specific detection of *M. bovis* and *M. tuberculosis* in uniplex PCR (uPCR). The uPCR protocols adapted [13] targeting fragment of MPB83 genes of *M. bovis*. The forward (5'-CAGGGATCCACCATGTTCTTAGCGGGTTG-3´) and reverse (5'-TGGCGAATTCTTACTGTGCCGGGGG-3´) primers were used in uPCR to generate 600bp amplicons of mature protein gene MPB83 of *M. bovis* chromosomal DNA [19]. The PCR was unable to differentiate infection in cattle and human due to *M. bovis* and *M. Tuberculosis*. A more specific uPCR was, therefore, designed to identify selectively H37Rv Rv3479HP gene of *M. Tuberculosis*. The uPCR protocol used the forward (5′-GAACTCACCGTCGGTGGTGA3′) and reverse (5′-CCTTGCTCGATCTCTGCGTC3′) primers expected to generate 667bp amplicon specific for infectivity due to *M. tuberculosis*. Each uPCR and mPCR was performed in a total of 25 µl reaction volume consisting of 2x PCR master mix (Promega® Inc, USA), 20pmol primer in each and 150-200 ng DNA template. The cycling parameter for the PCR identification of all three genes in a thermal cycler (Master Cycler Gradient, Eppendorf, Germany) was an initial denaturation at 98°C for 5min. A total of 30 cycles of PCR amplification reactions were carried out. The uPCR identification of MPB83 gene in a thermal cycler included the denaturation at 95°C for 1min, annealing at 56°C for 1min and extension at 72°C for 1min. The cycling parameter for the uPCR identification of H37Rv Rv3479 strain of *M. Tuberculosis* was

the denaturation at 94ºC for 30sec, annealing at 57ºC for 1.5min and extension at 72ºC for 3min. The final extension for uPCR and mPCR were set at 72ºC for 10min and the reactions were held at 4ºC. The amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose gel, stained with ethidium bromide and examined under UV light using an image documentation system (Cell Biosciences, Alphalmager HP,USA).

Sequencing of PCR amplicons

Fragments of 16SrRNA, MPB83 and H37Rv Rv3479HP genes of Mycobacterium sp were amplified in PCR and visualized in 1.5% soft gel. The selected bands in the agarose were gel purified (Promega INC, USA) and performed sequencing from AIT biotech, Singapore. The nucleic acid sequences of 16SrRNA (Genbank accession no KP321958), MPB83 (Genbank accession no KP321969) and H37Rv Rv3479HP (Genbank accession no KP321971) genes were analyzed using Basic local alignment system (BLAST) and deduced amino acid sequences were plotted. The amino acid sequences were aligned with other related sequences retrieved from the GenBank. Sequence editing, alignment and phylogenetic tree construction (Clustal V method) were carried out with the "Lasergene" software package (Modules-EditSeq and MegAlign; DNASTAR Inc., USA). Phylogenetic analysis was carried out using the neighbor-joining method based on the principle of parsimony [20]. The sequence information of the genus, species, strain and genes of the Mycobacterium species were tested in BLAST and identify further the specific causes of TB in human and cattle.

Results and Discussion

Globally TB has been a major cause of morbidity and mortality of men and animals. The genomes of *M. bovis* and *M. tuberculosis* showed 99.90% identity at the nucleotide level. Therefore, the most of the genome based technology, intradermal tuberculin test, virulence and host tropism could not differentiate infectivity in hosts due to *M. bovis* and *M. tuberculosis*. Indeed, *M. bovis* has shown increased virulence upon infection, widely zoonotic and drug resistance. Due to the presence of saprophytic mycobacterium, high genome sequence homology among species of mycobacterium and the lack of species-specific genes for each of the *M. bovis* and *M. tuberculosis*, distinctive protocols are needed to detect and differentiate these bacilli. It takes 6-8 weeks to grow mycobacterium in culture, fastidious mycobacterium often contaminate the culture; therefore, cultural identification is not a feasible option. Literature available indicated that the occurrence of bovine TB due to *M. bovis* and *M. tuberculosis* is common in many developing countries [21] but the information is lacking in Bangladesh. The traditional tests protocols such as intradermal tuberculin test, Ziehl-Neelsen staining of smears and tissue section, and X-ray imaging are infrequently used in cattle and human in Bangladesh. The efficacy of these tests is variable on farms and in the laboratories, requires validation.

Tuberculin tests

Out of 700 selected dairy cattle tested using tuberculin test, a total of 23 (3.29%) appeared positive to bovine PPD (Figure 1b). Two cattle appeared positive to both bovine and avian PPD. Holstein Frisian (n=21) cross breed cattle appeared as highest sensitive to tuberculin test than red Chittagong (n=01) and Sahiwal cross breed (n=01) cattle. Tuberculin tests are routinely used to detect TB in cattle. The use of tuberculin tests was also effective on farms of western India, the prevalence of bTB reported were between 0.65% and 1.85% [22]. A comparative study onto the prevalence and pathology of bTB was conducted on 5,424 cattle (2,578 zebus, 1,921 crosses, and 925 Holsteins) in the central highlands of Ethiopia, a highest prevalent zone of TB in the world. Using comparative intradermal tuberculin test, necropsy and bacteriology the overall prevalence of bTB reported was 13.5%; prevalence was higher in Holsteins (22.2%) than zebus (11.6%) [23]. The occurrence of bTB has been almost eradicated from Canada [24] following test and slaughter of test reactors. The bTB has almost been eradicated from Australia by using sound technologies that ran more than 25 years [25]. In Japan, the bovine tuberculin reactor rate was 4,463 per 100,000 dairy cows in 1903, which then declined to 30 per 100,000 dairy cows in 1967 and more recently, the prevalence further declined to about zero [26,27]. In Korea, the prevalence of bTB has significantly been reduced [28]. Eradication of bTB due to *M. bovis* in USA is a great challenge owing to the presence of a wildlife reservoir. In the northern Lower Peninsula there is an apparent active transmission of bTB among wildlife and cattle with prevalence of less than 0.1% in cattle herds. The rest of the state represents areas with apparent bTB prevalence also less than 0.01% in cattle herds [29]. The non-slaughter cattle movement within the state was not predominantly the determinants of bTB transmission rather than wild life.

The wild life reserver of bTB is not a major source of TB in cattle in Bangladesh. The IDTT although used in dairy cattle of Bangladesh, the test reactor cattle maintained in the herd for longer period of time; hence the prevalence of bTB is yet to reduce at expected level. The intradermal tuberculin test protocol we adapted appeared sensitive in terms of detecting carrier or infected state of TB in cattle. Based on the result of this investigation it may be concluded that farm operation using Holstein Friesian cross breed cattle, lack of performing routine tuberculin test, lack of interest to dispose tuberculin test reactors promptly, livestock operation allow close contact of human to farms might have posed risk of spreading TB to man and farm animals. Holstein Friesian cross breed cattle was found to infect more with chronic Fascioliosis which may render the animal more susceptible to bTB [30].

Impression smears staining and X-ray imaging

Mycobacterium species are impermeable to certain dyes and stains. Despite this, the bacterium is permeable to carbon fuchsin while heated and are not decolorized in acid alcohol; the acid fast bacilli (AFB) was, therefore, appeared pink in a contrasting background [31]. In this study, out of 23 tuberculin test positive aspiration biopsy samples tested from cattle, five showed the presence of AFB. The urine, sputum, pleural and peritoneal samples tested from suspected human (N=20) showed AFB in two, seven, four and three cases respectively. Two cases showed the presence of AFB in urine, pleural and peritoneal fluid indicating systemic TB. Seven sputum samples showed AFB (Figure 1c) indicating pulmonary form of TB; one of which showed the presence of AFB in pleural and peritoneal fluid. In a case AFB was only detected in urine. Microscopic examination of smears is faster and cheaper than any other method to visualize AFB but this test is unable to discriminate other organisms which shared acid-fast staining properties like Legionella, Nocardia, Rhodococcus, Tsulunnurella, Cryptosporidium, and Cyclospora [32]. Additionally, this method lacks sensitivity and can only reveal the presence of AFB when concentrations are exceeding 104 bacteria/ml [33]. The X-ray imaging only detected pneumonic form of TB in five cases. Out of seven smears (sputum) positive TB, only five of them showed pneumonic lesions as detected by X-rays. Not all of the smears microscopy and X-ray

imaging were case sensitive and species specific. Most of the laboratories preferred to use PCR technologies towards confirmatory detection of TB in cattle and human [13,19,34]. We have adapted culture of AFB in LOWENSTEIN-JENSEN (L-J) MEDIA (data was not shown) but also appeared less sensitive (about 30-35%) compared to PCR detection of TB in cattle and in human.

PCR detection and differentiation of TB in cattle and human

Multiplex and uniplex PCR were applied to identify specific cause of TB in cattle and human. A pair of PCR protocol was standardized and a uniplex PCR protocol was designed to detect and differentiate TB in cattle and human due to *M. bovis* and *M. tuberculosis*. To identify TB in living cattle, aspiration biopsy (N=23) from the enlarged superficial lymphnodes were collected and used in DNA extraction and PCR amplification. Out of 23 tuberculin test positive cattle, only 07 aspiration biopsies showed the presence of TB due to *M. bovis* and one showed *M. tuberculosis*. Multiplex and uniplex PCR were, therefore, used with the extracted genomic DNA from the lungs of Tuberculin test positive cattle and human samples and identify 16srRNA, MPB83/MPT83 and H37RvHP genes of bovine and human TB. The mPCR found to amplify fragment of 16srRNA gene in cattle and human; a total of 11 cattle (Figure 2) and 11 humans (Figure 3) samples were further tested in uPCR to identify their specific etiology. All of the extracted DNA from lungs tissues of tuberculin test positive cattle in mPCR found to generate 1030bp amplicon (Figure 2a) and were specific for the genus Mycobacterium. Short fragment of 16srRNA gene (180bp) was also generated in two cases positive to avian PPD and specific for infectivity due to *M. paratuberculosis*. The uPCR targeting MPB83/MPT83 gene, 600bp amplicons were generated in all 11 cattle and suggestive for infection with *M. bovis* (Figure 3b). Wiker [35] reported the term 'MPB' to a protein (major basic protein) derived from *M. bovis* and hence the term 'MPT' while the protein is derived from *M. tuberculosis*. The MPB83 is highly expressed in *M. bovis* but this study showed that the gene MPT83 is also expressed in *M. tuberculosis*. Out of 11 human sample tested in PCR targeting MPT83 gene, 09 appeared to amplify 600bp fragment (Figure 3a). Detection of the fragment of MPB83/MPT83 gene in PCR appeared as a non-specific technology to detect specific cause of TB in cattle and humans due to *M. bovis*.

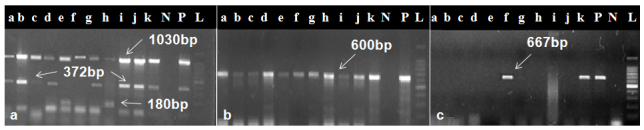

Figure 2: Detection and differentiation of the causal agents of TB in cattle using multiplex and uniplex PCR. Lane L is for 100bp ladder, P is for positive control, N is for negative control and lanes a to k containing test samples. Fragments of 16srRNA gene specific to the genus mycobacterium (1030bp) were generated in all of the tuberculin test positive cattle (a). The tuberculin test positive cattle (b) also generated 600bp amplicon suggestive to infection with *M. bovis*. PCR amplification targeting fragment of H37RV Rv3479HP gene of *M. tuberculosis* showed generation of 667bp amplicon in two (lane f and k) cases (c). Nine cattle were infected with *M. bovis*, two of them were co-infected with *M. bovis* and *M. avium* subsp. var *paratuberculosis*. Two cattle were infected with *M. tuberculosis*.

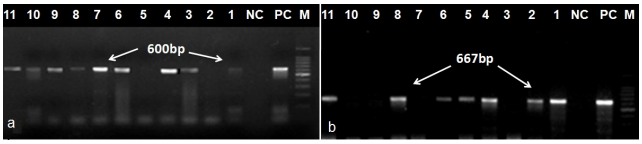

Figure 3: PCR detection and differentiation of the causal agent of TB in human. The lane M is for 100bp ladder, PC is for positive control, NC is for negative control and lane 1 to 11 represent tests samples. Out of 11 sample tested in PCR, 600bp amplicon suggestive for infectivity due to *M. bovis* was generated in nine cases and 667bp amplicon specific for infectivity due to *M. tuberculosis* was generated in seven cases.

To identify specific cause of TB in cattle and human due to *M. tuberculosis*, H37Rv Rv3479HP gene specific PCR was designed with the extracted DNA from 11 cattle and 11 humans. Results of uPCR showed that two cattle (Figure 2c, lane f and k) were infected with *M. tuberculosis* (667bp amplicon). Out of 11 human samples tested in PCR, seven samples appeared to amplify 667bp fragments (Figure 3b) and were infected with *M. tuberculosis*. The uPCR primers designed in this study targeting 667bp fragment of H37Rv Rv3479HP gene is specific for *M. tuberculosis* as the reverse primer was designed from the region of the gene where there was base deletion region in *M. bovis*. Results of mPCR also showed that nine cattle were infected with *M. bovis* of which two (Figure 2a, lane e and h) were co-infected with *M. paratuberculosis*. Co-infectivity in human with this bacterium was not detected in this study. *M. tuberculosis*, *M. bovis* and *M. paratuberculosis* are extremely zoonotic pathogens. It needs to test all of the dairy cattle in the farms by using tuberculin test twice in a year and eliminate the test reactor at early onset to reduce zoonotic threat. The farmers working in infected premises also need to sit for regular testing by tuberculin and PCR technologies. The genomic DNA from the test reactors cattle and human in infested premises can be used in PCR to detect specific causes of TB and understand the distribution of *M. bovis*, *M. tuberculosis* and *M. paratuberculosis*. The PCR amplicons need to test further by sequencing and phylogenetic analysis to know their phylogenetic position, transmission cycle and degree of mutation.

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequence similarities and divergence among the 16srRNA, MPB83/MPT83 and H37Rv Rv3479HP genes of *M. bovis* and *M. tuberculosis* were evaluated. The genus Mycobacterium 16srRNA gene sequences were more conserved; out of 11 cattle and human samples tested in PCR and sequenced, point mutation was seen in a case of cattle (Table 1). The sequence divergence within Lineage 1 of 16srRNA gene sequences ranged from 0% to 0.1% only and between the lineages ranged from 0.1% to 8.5%. Out of two cattle and seven human samples tested targeting H37Rv Rv3479HP gene of *M. tuberculosis*, point mutation was also seen in a case of cattle (Gen bank accession nos. KP321971, Table 1). The sequence divergence of *M. tuberculosis* H37Rv Rv3479HP gene is relatively more conserved; the sequence divergence within and between the Lineage 1 ranged from 0.0% to 0.2%. Result of sequencing of MPB83 genes of cattle (N=09) and human (n=04) showed point mutations in two cattle (Table 2). The sequence divergence of MPB83 gene of *M. bovis* in Lineage 1 ranged from 0% to 0.2% and between the lineages ranged from 0.2% to 0.3%. All others 16srRNA, MPB83 and H37Rv Rv3479HP genes of *M. bovis*

or *M. tuberculosis* sequenced did not show mutation at any time point. The fragments of 16srRNA, MPB83 and H37Rv Rv3479HP genes of *M. bovis* or *M. tuberculosis* showed mutation, used in phylogenetic analysis, they were fallen in Lineage 1 (Figures 4 and 5) and showed sequence similarities with the Indian lineages. The fragment of MPT83 (n=03) and H37Rv Rv3479HP (n=07) genes sequences of human origin did not show mutation at time point. Only a fragment of MPT83 gene showed point mutation and were similar with the sequence information of bovine MPB83 gene (Gen bank accession no KP321969).

Figure 4: Maximum Likelihood evolutionary tree based on nucleotide sequence of the *M. bovis* MPB83 gene (Gen bank accession nos KP321969, KP321959, KP321966, KP321962, KP321968 and KP321960, bovine isolates) using MEGA 5 software. A total of 29 isolates were retrieved from Gen bank and subjected to phylogenetic analysis. Bootstrap values (1000 replication) above 60% are shown next to the nodes. All of the bacterial strains of *M. bovis* and *M. tuberculosis* (red colored fonts) studied were fallen into lineage 1 and showed similarities with the Indian isolates in Lineage 1.

Sequencing and translational analysis of selected genes

Out of 11 cattle samples sequenced, a case of 16srRNA gene, two cases of MPB83 gene and a case of H37RvRv3479HP gene showed point mutation. Only one human MPT83 gene showed point mutation. A 16srRNA gene of Bangladeshi isolate (*M. tuberculosis* H37Rv Savar BD 2014) showed point mutation at base position 989 and the nucleotide base Guanine (G) was replaced by Adenine (A). Results of analyzing MPB83/MPT83 proteins of *M. bovis* or *M. tuberculosis* (Table 2) showed that in a case Arginine (R) was replaced by Proline (P) in the protein lineage of *M. bovis* BAU 447 BD 2014 at position 165 (Gen bank accession no KP321969). In another case, two substitutions of amino acids were noticed in the MPT83 protein lineage of *M. tuberculosis*. Generally one amino acid deletion were noted in 221 position of the protein lineage of *M. tuberculosis* H37Rv Rv3479HP strand (Table 1) but amino acid Glutamic acid (E) was found in position 221 of a Bangladeshi isolate *M. tuberculosis* H37Rv Savar BD 2014 (Gen bank accession no KP321971). Where two amino acid substitutions were noted in the protein lineage of *M. tuberculosis* (H37Rv Mymensingh BD 2014 strain, Gen bank accession no KP321960). At amino acid position 11, Arginine (R) were replaced by Serine (S) and at position 21 Aspartic acid (D) was replaced by Valine (V, Table 2) and fallen in Lineage 1. Mutation was not evident in other Bangladeshi isolates of *M. bovis* or *M. tuberculosis*. The mutation at base point or changes at amino acid level of the tested bacteria could have contributed pathogenicity or drug resistance properties, require further observation.

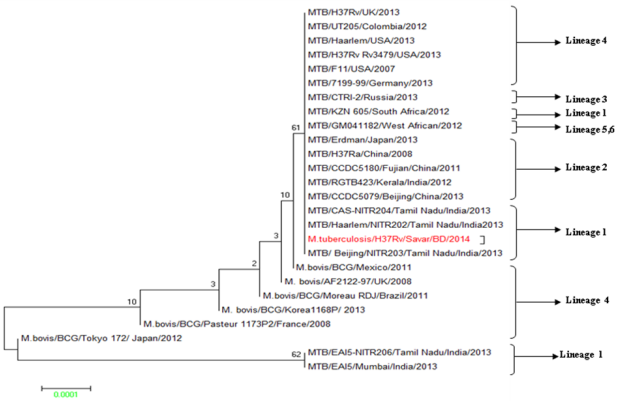

Figure 5: Maximum Likelihood evolutionary tree based on nucleotide sequence of the *M. tuberculosis* H37Rv Rv3479HP gene (bovine isolate) using MEGA 5 software. Out of 11 samples tested, point mutation was seen in a case and was used to develop phylogenetic lineage. A total of 26 isolates were retrieved from Gen bank and included in phylogenetic lineage. Bootstrap values (1000 replication) above 60% are shown next to the nodes. The local isolates (red colored fonts) of *M. tuberculosis* H37Rv Savar BD 2014 strain was fallen into lineage 1 and showed similarities with the Indian isolates in Lineage 1.

Isolate/lineage	Nucleotide position in the sequences of 16srRNA gene				
	986	987	988	989	990
Consensus	A	T	A	G	C
Lineage 1	.	.	.	.	.
M. tuberculosis H37Rv Savar BD 2014	.	.	.	A	.
Lineage 2	.	.	.	.	.

Lineage 4	.	.	.	.	.
Lineage 5,6	.	.	.	.	.

Isolate/lineage	**Nucleotide position in the sequences of *M. tuberculosis* H37Rv Rv3479HP gene (1-221)**			
	1	**115**	**221**	
Consensus	N		A	X
Lineage 1				
M. tuberculosis H37Rv Savar BD 2014	.		.	E
MTB EAI5/NITR206 Tamil Nadu, India 2013			T	.
MTB EAI5 Mumbai, India 2013			T	
Lineage 2	.		.	.
Lineage 3	.		.	.
Lineage 4				
MTB H37Rv UK 2013	X			.
Lineage 5,6	.		.	.

Table 1: Analysis of lineage-specific nucleotide sequences of 16srRNA and H37Rv Rv3479HP genes of Mycobacterium. Residues identical to the consensus are indicated by dots. The nucleotide bases in the DNA strand were analyzed compared with the sequences retrieved from Genbank and substitution points were marked. Out of 11 cattle sample tested, point mutation was seen in a case of 16srRNA (Genbank accession no KP321958) and H37RvRv3479HP (Genbank accession no KP321971) genes of *M. tuberculosis* .

Isolate/lineage	**Amino acid substitution in the protein strand of *M. bovis* MPB83 (11-165)**		
	11	**21**	**165**
	R	**D**	**R**
Lineage 1	.	.	.
M. bovis BAU 429 BD 2014	.	.	.
M. bovis BAU 447 BD 2014	.	.	P
M. tuberculosis H37Rv BAU 600 BD 2014	.	.	.
M. tuberculosis H37Rv Savar BD 2014	.	.	.
M. bovis Sabok BD 2014	.	.	.
M. tuberculosis H37Rv Mymensingh BD 2014	S	V	.

Table 2: Lineage-specific amino acid (AA) substitutions on to the protein strands of MPB83/ MPT83 genes of *M. bovis* or *M. tuberculosis*. Out of 11 samples investigated, aa replacement was seen in two cases and the sequences (Genbank accession nos KP321969 and KP321970) were submitted in Genbank.

Conclusions

This study showed that the intradermal tuberculin tests protocols adapted were sensitive to detect TB in cattle. Acid fast staining of smears or tissue sections although detect AFB but were less sensitive (≤ 50%). Specific cause of TB can best be detected by using PCR. The PCR technologies designed in this study targeting 667bp fragment of H37Rv Rv3479HP gene is highly specific for the detection of *M. tuberculosis*. Use of PCR targeting MPB83 gene although detect infectivity due *M. bovis*; *M. tuberculosis* was also found to share the gene (MPT83). There are point mutation in the genomic fragments of 16srRNA, MPB83 and H37RvRv3479HP genes in cattle and human; the mutation rate is higher in cattle TB. The dairy cattle investigated in this study were infected with *M. bovis*, *M. tuberculosis* and *M. avium* subsp var *paratuberculosis*. Human being was also infected with *M. bovis* and *M. tuberculosis*. Human TB derived from cattle is usually drug resistance. These organisms are extremely zoonotic and possess zoonotic threat. The people working in the farm require identifying the existence of specific cause of TB and level of drug resistance properties (study in progress). It is recommended that, all of the tuberculin test positive cattle should be disposed immediately after testing and identify the organism involved by using PCR to design future preventive and control strategies.

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