Bovine tuberculosis in India: Potential basis for zoonosis

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\textbf{Summary} Our laboratory has designed a specific nested-PCR (N-PCR) assay, based on the hupB gene of \textit{Mycobacterium tuberculosis} (Rv2986c) and \textit{Mycobacterium bovis} (Mb3010c) as a method to differentiate these closely related species. The present paper deciphers the utility of this assay for identification of pathogenic Mycobacteria in clinical samples. Extra-pulmonary clinical samples obtained from cattle and humans were investigated. Pre-dominance of \textit{M. tuberculosis} (15.7%) and \textit{M. bovis} (26.8%) was seen in humans and cattle, respectively. However, more importantly, both mycobacterial pathogens (mixed infection) were identified in a number of samples. In humans 8.7% of the samples and 35.7% in cattle were classified as mixed infection. The detection of mixed infection with the mycobacterial pathogenic duo in humans and bovines denotes the prospect of potential transmission of these pathogens from humans to cattle (zoonosis) and vice versa (reverse zoonosis).

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Introduction

Infection with *Mycobacterium bovis* and *Mycobacterium tuberculosis* have been known to cause bovine and human tuberculosis, respectively.\(^1\)–\(^3\) The reported increase in incidence of infection by these two species in domesticated animals and humans has become the subject of investigation. However, there are reports that these mycobacterial pathogens are not limited to these susceptible hosts. In fact infection with *M. tuberculosis* and *M. bovis* has shown to occur across a wide spectrum of species.\(^4\)–\(^5\) In developing countries, increase of *M. bovis* infection in humans has manifested into a grave public health problem.\(^6\)–\(^8\) This crisis has been related to the fact that in developing countries domesticated animals and humans share the same habitat.\(^9\) The problem has been further exacerbated by reports of: (i) transmission to animals from humans shedding infectious tubercle bacilli\(^10\),\(^11\); and (ii) the association of HIV with *M. bovis* besides *M. tuberculosis*.\(^12\)–\(^14\)

The disease caused by *M. bovis* is indistinguishable to that caused by *M. tuberculosis*.\(^15\) The bacteriological, biochemical and genetic similarities of these two species have made it difficult to differentially identify them in clinical samples/cultivated isolates. Presently, a battery of tedious tests (microbiological, biochemical, etc.) has been described for detecting and differentiating *M. tuberculosis* and *M. bovis*. In the recent past there have been reports of molecular biological techniques such as multiplex-PCR and spoligotyping for differentiating these two mycobacterial species.\(^16\)–\(^21\)

Our laboratory has designed a reliable and specific, PCR-RFLP and nested-PCR (N-PCR) assays to differentiate and detect these species in clinical isolates and samples.\(^22\)–\(^24\) The assay exploits the 27bp difference in the C-terminal part of the *hupB* gene between *M. tuberculosis* and *M. bovis* (Rv2986c in *M. tuberculosis*; Mb3010c in *M. bovis*).\(^22\) Further, the utility of the PCR target in identifying mixed infection with these closely related pathogens namely *M. tuberculosis* and *M. bovis* in an individual sample has been demonstrated.\(^23\),\(^24\) The degree of correlation between the gold standard culture-based identification techniques and PCR-RFLP assay has been shown to be 99.0% (\(p<0.001\)).\(^23\) The present paper summarizes some of our work on the detection and differentiation of the two pathogens in extra-pulmonary samples of bovine and human origin.

Materials and methods

Animals

Fifty-six cattle of a single herd were segregated into two groups. One group consisting of 29 cattle with signs and symptoms of tuberculosis (TB category) and the second, 27 healthy animals clinically free of tuberculosis (NTB category). Clinical examination of the cattle was carried out at Central Military Veterinary Laboratory, Meerut. Samples were processed in the laboratories of HKP for N-PCR for detection of pathogenic Mycobacteria and VMK for cultivation and characterization by standard techniques. After approval by the animal ethical committee the study was initiated.

Patients

Patients clinically suspected of tuberculosis, attending various OPD clinics or admitted in wards (Neurology, Gastro-enterology, Surgery, Obstetrics and Gynecology) at the All India Institute of Medical Sciences were included in the study. Patients included were both adults and children. Total numbers of samples collected were 331, which included 192 tissue biopsies and 139 body fluids. All tissue samples were processed for routine histology and evaluation as apart of the on going patient investigation. After approval by the institutional ethical committee the project was initiated. For the study only extra-pulmonary samples from cattle and patients were included. All the samples were stored at \(-20\) °C till further processing.

Processing of samples for isolation of target DNA for N-PCR

(i) Minced tissue biopsies suspended in inhibitor removing solution (IRS) was disrupted using a bead beater (Biospec, USA).\(^25\) The supernatant was transferred and centrifuged at 12,000g for 15 min. The pellet was washed with sterile water, suspended in 100\(\mu\)l of lysis buffer (10% Chelex-100 suspension, 0.3% Tween-20 and 0.03% Triton X-100), heated in a dry bath at 90 °C for 40 min and centrifuged at 10,000g for 10 min. The supernatant was used as target DNA.

(ii) Body fluids were filtered and treated with IRS and lysis buffer as described. The lysate was centrifuged at 12,000g and the supernatant was used as target DNA.
N-PCR for *hupB* DNA target (international patent application no. PCT/IN03/00302)

The two step PCR was carried out as follows: The primers N & S were used to amplify *hupB* gene as described (Fig. 1A). The N & S PCR product was used in the second step to amplify the C-terminal part of the gene using F & R primers (Fig. 1A). The N-PCR products were analyzed on 10% polyacrylamide gel. The expected size of amplicon for *M. tuberculosis* and *M. bovis* was ~116 and 89 bp, respectively. The 27 bp difference in the *hupB* gene of *M. tuberculosis* and *M. bovis* have been shown in Fig. 1B.

Results

Detection and differentiation of *M. bovis* and *M. tuberculosis* in cattle by N-PCR assay

Bovine samples obtained from the two categories of cattle viz. tuberculous (TB) and not categorized as tuberculous (NTB), were processed for N-PCR as described in methods. The amplified products obtained in representative bovine samples have been shown in Fig. 2. The mycobacterial pathogens in the cattle were identified as *M. tuberculosis* and *M. bovis* based on gel analysis. The molecular weights of the N-PCR products obtained for standard *M. tuberculosis* and *M. bovis* corresponded to 116 and 89 bp, respectively (Fig. 2, Lanes 4 and 5). N-PCR product of cattle # 91 (Lane 3) and cattle # 119 (Lane 7), matched with that of *M. tuberculosis*, were identified to be *M. tuberculosis*. Where as N-PCR product derived from cattle # 105 (Lane 6) matched with that of *M. bovis*, hence predicted to be *M. bovis*.

Ninety six point five percent (28/29) of the animals clinically diagnosed as TB were found to be infected with pathogenic mycobacteria by N-PCR (Table 1A). Where as in animals, NTB 85.2%, (23/27) were found to be infected (Table 1A). Two types of infection with the mycobacterial pathogens were discovered by N-PCR. Infection with a single species
of pathogenic mycobacteria was seen in 31/52 (59.6%) cattle (Table 1C). *M. tuberculosis* and *M. bovis* in an individual animal was considered to be mixed infection. It was detected in 20/52 (35.7%) animals (Table 1C).

**Detection and differentiation of *M. bovis* and *M. tuberculosis* in cattle by culture**

Mycobacteria was isolated and identified by classical standard culture techniques, in 17 of the 56 cattle examined (Table 1A). Of these 9 were identified to be infected with *M. tuberculosis* and 7 with *M. bovis* (Table 1C). In samples derived from one animal, clinically classified as NTB, both *M. tuberculosis* and *M. bovis* (mixed infection, Table 1C) was isolated. Pathogenic mycobacteria was isolated from both categories of animals namely TB and NTB. However as expected, the percentage positivity in detection was found to be higher in case of animals belonging to the TB group (44.8%), compared to 14.8% of the NTB group (Table 1A). Similar trend was seen on examining the N-PCR results. However, the percentage of cattle positive for mycobacterial pathogens were higher compared to culture. In the TB group 96.5 and in the NTB 85.2% of the animals, *M. tuberculosis* and/or *M. bovis* was detected.

**Comparison of N-PCR and culture technique**

Differences in the detection of pathogenic mycobacteria in cattle by N-PCR and culture technique

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**Table 1**

(A) Detection of pathogenic mycobacteria in cattle

<table>
<thead>
<tr>
<th>Category</th>
<th>Total no.</th>
<th>N-PCR (%)&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Culture (%)&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Total no. positive by either method (%)&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB</td>
<td>29</td>
<td>28 (96.5)</td>
<td>13 (44.8)</td>
<td>28 (96.5)</td>
</tr>
<tr>
<td>NTB</td>
<td>27</td>
<td>23 (85.2)</td>
<td>4 (14.8)</td>
<td>24 (88.8)</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>51 (91.1)</td>
<td>17 (30.3)</td>
<td>52 (92.8)</td>
</tr>
</tbody>
</table>

(B) Correlation of detection of pathogenic mycobacteria by N-PCR and culture in cattle categorized as tuberculous and non-tuberculous

<table>
<thead>
<tr>
<th>Category</th>
<th>N-PCR &amp; culture</th>
<th>N-PCR alone (%)</th>
<th>Culture alone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive by both assays</td>
<td>Negative by both assays</td>
<td>Incomplete correlation&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>TB (29)</td>
<td>12</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NTB (27)</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>13 (23.2)</td>
<td>4 (7.1)</td>
<td>3 (5.3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Category</th>
<th>Total no. of cattle infected</th>
<th>N-PCR</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>M. tb</td>
<td>M. bovis</td>
</tr>
<tr>
<td>TB</td>
<td>28</td>
<td>6 (21.4)</td>
<td>11 (39.3)</td>
</tr>
<tr>
<td>NTB</td>
<td>24</td>
<td>10 (41.7)</td>
<td>4 (16.7)</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>16 (30.8)</td>
<td>15 (28.8)</td>
</tr>
</tbody>
</table>

<sup>+</sup>Nested PCR assay for detection and identification of members of the MTB complex.

<sup>†</sup>Identification of cultivated mycobacterial isolates by standard tests (CDC, Atlanta).

<sup>‡</sup>Percentage.

<sup>§</sup>Correlation was seen in terms of detection and not speciation viz. by culture the animal is infected with *M. tuberculosis* whereas by N-PCR *M. bovis* or vice-versa.
were observed. N-PCR results matched with those obtained by the culture techniques in 13/56 (23.2%) animals (Table 1B). Limited correlation was seen in 3 animals, in that N-PCR and cultivated isolates of samples from these animals established infection with mycobacterial pathogens. However, differences in the speciation of the pathogen was seen with the two assays (Table 1B). No correlation was seen in 64.3% (36/56) of the animals. N-PCR alone was positive in 35 cattle (62.5%, Table 1B). One animal positive by culture was negative by N-PCR (Table 1B).

The sensitivity of detection of pathogenic mycobacteria in cattle by N-PCR was found to be 2-fold or more (96.5%) compared to culture (44.8%, Table 2). However specificity of N-PCR (14.8%) was lower than culture (85.1%). This difference could be attributed to the detection of mycobacterial pathogens in 23 of the 27 non-tuberculous animals, by N-PCR (Tables 1A and 2).

**Detection and differentiation of M. tuberculosis and M. bovis in human extra-pulmonary samples by N-PCR**

Tissue biopsies and fluids collected from patients suspected for extra-pulmonary tuberculosis was processed as described in methods. The amplified products separated on polyacrylamide gel in representative samples have been shown in Fig. 3. N-PCR product of Gl biopsy # 50 (Lane 2) and ascitic fluid # 24 (Lane 5) matched with M. bovis (89 bp, Lane 4), hence identified to be M. bovis. In Gl biopsy # 49 (Lane 1) dual amplified products of both M. tuberculosis and M. bovis were observed. Hence, considered as mixed infection.

Of the 331 samples analyzed, in 115 (34.7%) pathogenic mycobacteria were detected by N-PCR (Table 3). M. tuberculosis was identified in 15.7%, M. bovis in 10.3% and mixed infection was observed in 8.7% of the samples investigated (Table 3). The proportion of M. bovis and M. tuberculosis infection...
identified in human samples varied. Predominance of *M. bovis* in fluids (18.7%) and *M. tuberculosis* in biopsies (25.5%) was seen (Table 3).

### Demonstration of the absence of PCR inhibitors in sample extracts

To rule out the presence of PCR inhibitors in the extracted DNA template from various samples, spiked N-PCR was performed. The results of spiked N-PCR of representative tissue biopsies have been shown in Fig. 4. The template DNA of the samples, which were negative by N-PCR (Lanes 3, 5, 7, 9, 11 and 13), was spiked with *M. tuberculosis* DNA (TB research material, NIH, USA) and N-PCR was performed. All the six samples showed the expected 116 bp product (Lanes 2, 4, 6, 8, 10 and 12) specific for *M. tuberculosis* (Lane 1). This showed the absence of PCR inhibitors in the processed clinical samples.

### Discussion

Bovine tuberculosis, caused by *M. bovis*, has been on the increase in developed countries and continues to occur in developing countries. The epidemiological impact of *M. bovis* infection in humans has not been assessed and is a major lacuna in developing countries. *M. bovis* has been known to spread to humans from infected cattle (zoonotic TB) by aerosol or by consumption of contaminated dairy products (Fig. 5). *M. bovis* is clinically indistinguishable from that caused by *M. tuberculosis*. *M. bovis* has been shown to predominate in extra-pulmonary forms of tuberculosis. This predominance has been attributed to infection by *M. bovis* occurring via the oral route. The utility of the PCR-RFLP and N-PCR assay based on *hupB* (*Rv2996c*) gene of *M. tuberculosis* has been earlier shown in our laboratory to differentiate *M. tuberculosis* from *M. bovis*. The assay differentiates *M. tuberculosis* and *M. bovis* from other members of the TB complex, NTB mycobacteria and unrelated species. The N-PCR assay developed was found to be sensitive. The assay has been used to detect and differentiate *M. tuberculosis* and *M. bovis* in samples derived from animals and humans. Infection by *M. bovis* in our studies have been shown in 28.8% of the cattle and 10.7% human samples, respectively, (Table 1C, Table 3). Human disease caused by *M. bovis* has
been confirmed in several reports, as evidence of transmission of *M. bovis* from infected animals to humans (Zoonosis).\(^3,6,10,26\) There have been no reports from India.\(^27\) Moreover to the best of our knowledge no survey has been done in India till date on the prevalence of *M. bovis* infection in animals/humans.

In the present study 15–28% of the animals (Table 1C), were discovered to be infected with *M. tuberculosis* as assessed by N-PCR and culture. *M. tuberculosis* is known to be a human pathogen. The occurrence of *M. tuberculosis* has been as a result of human-to-cattle transmission, an example of reverse zoonosis (Fig. 5).\(^3,10,11\) Animals infected with *M. tuberculosis* potentially constitute a grave public health hazard as virulent bacilli can be transmitted to humans.\(^11\) Mixed infection, i.e. presence of both *M. tuberculosis* and *M. bovis* in the same sample, was observed in some of the cattle and human samples investigated (Table 1C). Kidane et al. had also reported the presence of both *M. tuberculosis* and *M. bovis* in an individual sample of tuberculous lymphadenitis patient.\(^16\)

The constrained observation of TB caused by *M. bovis* in humans in developing countries has been attributed to: (i) the use of direct smear microscopy as the method for diagnosis of suspected TB, which does not differentiate between species of the *M. tuberculosis* complex; (ii) the poor growth and isolation rates of *M. bovis* from clinical samples on Lowenstein-Jensen medium, the standard culture media used routinely;\(^28\) (iii) the limited bacillary load in extra-pulmonary samples;\(^16,29\) and (iv) the zoonotic aspect of *M. bovis* which remains largely uninvestigated, inadequately reported and the lack of coordination among public health agencies.\(^1,15\)

The identification of infection with *M. bovis* and *M. tuberculosis* in human samples as determined by N-PCR assay would suggest re-defining the existing control and prevention policies for tuberculosis. The disease surveillance programs in humans especially in areas where risk factors are prevalent need to be modified. As, zoonotic TB represents a significant risk in rural communities and areas where domesticated animals and humans share a common environment. The situation is critical in developing countries as tuberculosis caused by *M. bovis* has been reported in AIDS patients.\(^12-14\)

Hence, the present study along with other limited reports in developing nations on the co-occurrence of the two pathogens in the environment, requires the coordinated efforts of medical and veterinary health professionals to define and implement unified control measures for prevention of both human and bovine tuberculosis.

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