Tuberculin response in guinea pigs with recombinant proteins cocktail prepared from Indian strain of Mycobacterium bovis (3/86 Rv)

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The bovine tuberculosis caused by Mycobacterium bovis is a serious disease among cattle worldwide resulting in considerable economic loss. There is a need for a diagnostic test that can discriminate M. bovis infection from BCG vaccination and NTM sensitization in animals. In this study, we intended to find out the potential use of recombinant antigens from Indian strain of Mycobacterium bovis (3/86Rv) for the intradermal tuberculin test of cattle. Immunodominant proteins MPB64, MPB83 and ESAT6 from M. bovis (3/86 Rv) Indian strain were recombinantly overexpressed, purified and immunologically characterized (rMPB64, rMPB83 and rESAT6). Four different cocktail combinations viz., cocktail I of protein antigens contained rMPB64, rMPB83, rESAT6, rCFP10 with protein concentration of 0.5 µg each; cocktail II contained 0.5 µg of each of rMPB64, rMPB83, rESAT6; cocktail III with 1 µg of each rESAT6, rCFP10; and cocktail IV contained rMPB64 and rMPB83 with 1 µg concentration of each protein, were administered at a dose of 0.1 mL. The DTH response was measured in heat killed M. bovis and non-tuberculous mycobacteria (NTM) sensitized, bacille Calmette-Guerin (BCG) vaccinated and control guinea pigs. The first cocktail of rMPB64, rMPB83 and rESAT6 containing 1.5 µg showed almost similar to cocktails II and III but stronger DTH response even at lower individual protein concentrations (each 0.5 µg) than the rESAT6 and rCFP10 protein of third cocktail with higher individual protein concentration (each 1 µg). The fourth cocktail with rMPB64 and rMPB83 elicited less DTH response as compared to the all other formulated cocktails. Cocktail I of four protein antigens elicited highest response at 24 h. Guinea pig model sensitized with heat killed M. bovis was found to be an efficient model for evaluating DTH response elicited by recombinant proteins cocktails. None of the cocktails elicited positive erythematous reaction in NTM sensitized and BCG vaccinated guinea pigs. A diagnostic test based on above cocktails could discriminate M. bovis infection from BCG vaccinated and NTM sensitized cattle.

Keywords: Bovine tuberculosis, Delayed type hypersensitivity

The bovine tuberculosis (BTB), a disease of domestic and wild animals is caused principally by Mycobacterium bovis, a member of the M. tuberculosis complex (MTBC)¹. The disease has been reported in 176 countries of the world as one of the important bovine diseases causing great economic loss². Control strategies for BTB are mainly based on test and slaughter policy accompanied with abattoir surveillance. According to World Organization for Animal Health (formerly OIE), the tuberculin skin test (TST) is recommended test for international trade. Cross-reactive response to tuberculin purified protein derivative (PPD) due to the presence of non-tuberculous mycobacteria (NTM) infections is an additional dispute that makes the test less specific³. Attempts to improve the TST using high doses of defined recombinant antigens resulted in an increased specificity but compromised sensitivity with a longer time required to induce DTH response compared to the PPD based test⁴. Alternatively, the combined use of recombinant proteins cocktail has shown a comparable sensitivity and specificity even at low protein doses with a reduced time requirement for DTH elicitation pointing at their effective earlier diagnostic implications⁵. Studies on the combination of recombinant proteins for the tuberculosis (TB) diagnostics in animals and human are not uncommon⁶–⁸.

It is important to develop the diagnostic reagent that overcomes the demerits of presently used PPD tuberculin test along with added capabilities to distinguish BCG vaccinated animals from infected, an essential prerequisite for control and extermination of TB in animals. In this context, it is convincing to hypothesize that expressed and purified MPB64, MPB83 and ESAT6 proteins from the Indian strain of M. bovis (3/86 Rv) can be an ideal candidate to evaluate DTH response prompted by recombinant proteins.

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cocktail by skin test in *M. bovis* sensitized guinea pig model. It is pertinent here to mention that BCG is not administered in domestic animals indeed; the proposed work utilized guinea pig model immunized with BCG to understand the performance of cocktail as DIVA reagent *i.e.* diagnosis discriminating between BCG vaccination and *M. bovis* infection.

**Materials and Methods**

**Bacteria and purified protein derivatives (PPDs)**

*M. bovis* (3/86 Rv) strain was obtained from Mycobacteria Laboratory, Division of Bacteriology and Mycology, IVRI, Izatnagar. It was a strain isolated from the mediastinal lymph node of cattle in 1986 and the culture was maintained on Lowenstein-Jensen medium. *M. avium*, *M. smegmatis* and *M. fortuitum* were obtained from the Tuberculosis Research Centre, Chennai. The *M. bovis* BCG strain was purchased commercially. Bovine and avian PPD (PPDb and PPDa) were prepared as done earlier.

**Expression and purification of recombinant proteins**

*E. coli* BL21 (DE3-pLysS) was used as expression host strain. The pET32b (+) vector (Novagen Inc., USA) cloned with *mpb64, mpb83* and *esat6* genes (pET32b-mpb64, pET32b-mpb83 and pET32b-esat6) from *M. bovis* Indian strain (3/86 Rv) in our laboratory were used for expression. The recombinant expression constructs were already confirmed for the presence of specific gene of interest by vector specific primers with NCBI accession numbers of KJ614484, KJ614485 and KJ614483. Further, all three plasmids were confirmed by double restriction digestion with *BamHI* and *HindIII* (Thermo Scientific, USA).

For expression, *E. coli* BL21 (DE3-pLysS) transformed with pET32b-mpb64, pET32b-mpb83 and pET32b-esat6 plasmid constructs were grown in LB medium containing suitable antibiotics and induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma-Aldrich, USA) in mid-log growth (A_{600nm} of 0.4 to 0.8) phase. Fractionation was done at 2 h intervals up to 8 h to check the expression level induced within the cells and analyzed by SDS-PAGE on 12% acrylamide resolving gel. The recombinant proteins were purified under denaturing conditions by affinity chromatography using Ni-NTA Superflow cartridges (Qiagen, Hilden, Germany) as per standard procedure. The expression level of rMPB64, rMPB83 and rESAT6 was calculated by estimating the protein concentration.

**Immunoblotting**

rMPB64, rMPB83 and rESAT6 were transferred onto nitrocellulose membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, USA) and detected using rabbit anti-His monoclonal antibody Purified (Sigma, USA) and goat anti-rabbit HRPO conjugate (Sigma, USA) as primary and secondary antibodies, respectively. A second western blotting was performed with *M. bovis* (strain 3/86 Rv) polyclonal hyper immune guinea pig serum as primary antibody and goat anti-guinea pig IgG-HRPO conjugate (Sigma, USA) as secondary antibody. Both protein-antibody reactions were detected by incubating the membrane in the dark with di-amino-benzidine (Sigma, USA) substrate.

**Recombinant proteins cocktails**

Extensive dialysis of eluted rMPB64, rMPB83 and rESAT6 proteins using SnakeSkinTM Pleated Dialysis Tubing (Thermo Scientific, USA) with 10000 MWCO and 3500 MWCO (molecular weight cutoff) for overnight at 40°C was performed against PBS. The Limulus Amebocyte Lysate PYROGENTTM kit (Lonz, Walkersville, USA) was used as an *in vitro* end-product endotoxin level detection test as per the manufacturers guidelines. The rMPB64, rMPB83 and rESAT6 proteins with rCFP1013 were used in different formulations in endotoxin free sterile PBS (pH 7.4) to obtain four proteins cocktails. Cocktail I contained 0.5 µg of each of rMPB64, rMPB83, rESAT6 and CFP10. Cocktail II was made up of 0.5 µg of each rMPB64, rMPB83, and rESAT6 with protein concentration of 1.5 µg in a dose of 0.1 mL. Cocktail III contained with 1 µg of each rESAT6 and rCFP10 and cocktail IV contained rMPB64 and rMPB83 with 1 µg concentration of each protein.

**Animal testing**

The study protocol for using animals was approved by the standing Institute Animal Ethics Committee (IVRI, Izatnagar). Twenty four apparently healthy adult Dunkin-Hartley (200-300 g) guinea pigs (*Cavia porcellus*) of either sex obtained from the Laboratory Animal Resource Section (IVRI, Izatnagar) were used in the study and maintained under standard laboratory conditions of housing, food and water. Animals were divided into four groups with 6 animals in each group. In the first group, guinea pigs were sensitized by deep intramuscular injection with 0.5 mL suspension of liquid paraffin containing 4 mg/mL heat-killed *M. bovis* (3/86 Rv) bacilli. The second group was
immunized by subcutaneous injection with 0.1 mL volume containing approximately 5 × 10⁴ CFU of the BCG vaccines. A third group was sensitized with 0.5 mL of a suspension in liquid paraffin containing 4 mg/mL of heat-killed NTM (heat killed suspension of M. avium, M. smegmatis and M. fortuitum in equal volume), while the last group was mock sensitized with intramuscular injection of PBS. Thirty five days after sensitization, intradermal injection was performed as per Mantoux technique. Four cocktails with PPDb (10 IU), PPDa (10 µg) and PBS as control were tested in 0.1 mL of injection volume in the flank region of guinea pigs. The injection sites were checked for the extent of DTH responses after 24, 48 and 72 h and recorded.

Statistical analysis

Data recorded was analyzed by PROC GLM in SAS version 9.3 (SAS Institute Inc., Cary, North Carolina, USA) software. Differences in DTH responses to different proteins cocktail combinations with PPDb and PPDa in all experimental animal groups at three time points (24, 48 and 72 h) were compared by two-way ANOVA with repeated measures, with application of Tukey HSD as post analysis test. Differences were considered significant if \( P < 0.05 \).

Results

Expression of recombinant MPB64, MPB83 and ESAT6 proteins

Purified expression plasmids after restriction enzyme digestion with BamHI and HindIII released specific insert DNA of 695 bp, 611 bp and 296 bp from pET32b recombinant construct of mpb64, mpb83 and esa6 genes, respectively. The pET32b-mpb64, pET32b-mpb83 and pET32b-esa6 transformed E. coli BL21 (DE3-pLysS) cells expressed polyhistidine tagged recombinant proteins of approximately 42, 39, 29 kDa, respectively at an IPTG concentration of 1.0 mM (MPB63), 0.5 mM (MPB83) and 1.0 mM (ESAT6) (Fig. 1). These proteins showed a molecular weight higher than natural proteins from M. bovis due to expression of an additional 19 kDa tagged fusion protein from the vector sequences. The expression kinetics was studied with maximum expression observed 4 h (MPB64), 6 h (MPB83) and 2 h (ESAT6) of IPTG induction. Ni-NTA affinity chromatography efficiently purified rMPB64, rMPB83 and rESAT6 proteins (Fig. 2) with an average yield of 20.0, 22.1 and 25.8 mg/L, respectively.

Immunoblotting

The rMPB64, rMPB83 and rESAT6 proteins in western blotting with anti-His monoclonal antibody generated specific band in both induced bacterial cell lysate and purified recombinant protein (Fig. 3). In case of MPB83 faster moving bands with degradation products of lesser molecular weight were found. Such bands were not found in all other expressed proteins. In the second immunoblot, guinea pig anti-M. bovis
antiserum reactivity towards expressed proteins were visualized as a specific band at the expected molecular weight size confirmed immunogenicity and specificity of rMPB64, rMPB83 and rESAT6 as mycobacterial antigen (Fig. 3).

**DTH response to cocktail formulations**

Endotoxin content in dialyzed recombinant proteins was less than 1.2EU/mg which is acceptable for performing in vivo experiment. All four formulated cocktails elicited positive DTH response at 24, 48 and 72 h after injection, as defined by an erythematous reaction on skin of guinea pigs. The DTH response for a cocktail containing 0.5 μg of each rMPB64, rMPB83 and rESAT6 was 8.32±0.15 mm (mean response ± standard deviation) after 24 h. Further, addition of another 0.5 μg of rCFP10 in rMPB64, rMPB83 and rESAT6 cocktail significantly increased ($P <0.05$) erythema diameter. We have also recorded interesting observation that erythema produced by rMPB64, rMPB83 and rESAT6 containing cocktail was not significantly different ($P >0.05$) to that produced by rESAT6 and rCFP10 cocktail. A cocktail containing 1 μg of rMPB64 and rMPB83 showed less skin erythema diameter of 7.54±0.24 after 24 h of injection. The positive response measured at the 24 h time point were generally significantly ($P <0.05$) stronger than that at 48 h for all the groups. By 72 h, all the skin reactions were found to decrease (Table 1).

**Performance and specificity of cocktail as DIVA reagent**

Six guinea pigs vaccinated with BCG showed a positive skin reaction of 11.56±0.29 mm to PPDb. Positivity was also observed for PPDb. All the four cocktails (I, II, III and IV) from rMPB64, rMPB83, rESAT6 and rCFP10 were unable to recognize this group strengthening our DIVA hypothesis (Table 1). None of the formulated antigenic cocktails elicited positive erythematous reaction in NTM sensitized guinea pigs indicating their high specificity toward detection of $M. bovis$ infection (Table 1). The naïve control group was also tested with all the protein cocktail formulations and showed negative DTH response as expected (Table 1).

**Discussion**

Previous attempts to increase the specificity of BTB diagnostics using PPD have utilized comparative tuberculin testing with PPDb and PPDa. However, the RD encoded proteins have attracted a great deal of interest as next generation diagnostic reagent for BTB. Advances in comparative genomics identified that a total of 16 regions of difference (RD) are present in virulent MTBC strains, but are deleted from $M. bovis$ BCG and most of the NTM. ESAT6 is a highly explored RD1 encoded secretory protein, considered to be a potential diagnostic candidate for BTB. The RD2 region encodes MPB64, a major protein secreted by actively...
dividing *M. bovis* and reported to have effective immunodiagnostic potential. Both of these antigens elicit DTH response in guinea pig models sensitized with *M. tuberculosis*, but sensitivity was reported to be significantly lower than PPD. Yet another antigen, MPB83, a cell surface lipoprotein highly expressed by *M. bovis* and considerably less abundantly in some BCG strains, has found to induce T-cell immune responses. These proteins have been used individually to evaluate DTH eliciting potential for development of specific diagnostics for TB.

There is an emergent concern regarding use of recombinant proteins in cocktail formulations for TB diagnostics. A number of studies have suggested that a combination of several purified antigens may elaborate a stronger skin reaction that can substitute present tuberculin PPD used. Hence, we presented here different formulations of recombinant proteins to develop a DTH response based diagnostic cocktail reagent that could explicitly detect not only *M. bovis* infection but also differentiates it from BCG vaccination and NTM sensitization. In the present study, we used guinea pigs which is one of the most established animal model for tuberculosis diagnostic due to its extreme susceptibility to the infection, similar immunopathology, DTH response, and verified BCG protection. Hence, we presented here different formulations of recombinant proteins to develop a DTH response based diagnostic cocktail reagent that could explicitly detect not only *M. bovis* infection but also differentiates it from BCG vaccination and NTM sensitization. In the present study, we used guinea pigs which is one of the most established animal model for tuberculosis diagnostic due to its extreme susceptibility to the infection, similar immunopathology, DTH response, and verified BCG protection. We used guinea pigs which is one of the most established animal model for tuberculosis diagnostic due to its extreme susceptibility to the infection, similar immunopathology, DTH response, and verified BCG protection. We used guinea pigs which is one of the most established animal model for tuberculosis diagnostic due to its extreme susceptibility to the infection, similar immunopathology, DTH response, and verified BCG protection. We used guinea pigs which is one of the most established animal model for tuberculosis diagnostic due to its extreme susceptibility to the infection, similar immunopathology, DTH response, and verified BCG protection. We used guinea pigs which is one of the most established animal model for tuberculosis diagnostic due to its extreme susceptibility to the infection, similar immunopathology, DTH response, and verified BCG protection.

### Table 1 — DTH response to different recombinant protein cocktail formulations with PPD 

<table>
<thead>
<tr>
<th>(A) sensitized with heat killed <em>M. bovis</em></th>
<th>Mean±SD (24 h)</th>
<th>Mean±SD (48 h)</th>
<th>Mean±SD (72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M64+M83+E6+10</td>
<td>8.90±0.26</td>
<td>6.66±0.21</td>
<td>5.25±0.17</td>
</tr>
<tr>
<td>M64+M83+E6</td>
<td>8.32±0.15</td>
<td>6.32±0.32</td>
<td>4.86±0.14</td>
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<tr>
<td>E6+C10</td>
<td>8.06±0.15</td>
<td>6.11±0.19</td>
<td>4.73±0.24</td>
</tr>
<tr>
<td>M64+M83</td>
<td>7.54±0.24</td>
<td>5.54±0.27</td>
<td>4.21±0.27</td>
</tr>
<tr>
<td>PPDa</td>
<td>11.91±0.46</td>
<td>10.68±0.36</td>
<td>8.31±0.26</td>
</tr>
<tr>
<td>PBS</td>
<td>8.39±0.24</td>
<td>7.25±0.16</td>
<td>5.45±0.30</td>
</tr>
<tr>
<td>(B) vaccinated with <em>M. bovis</em> BCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M64+M83+E6+10</td>
<td>1.24±1.03</td>
<td>0.17±0.42</td>
<td>0±0</td>
</tr>
<tr>
<td>M64+M83+E6</td>
<td>0.80±0.97</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>E6+C10</td>
<td>0.91±0.13</td>
<td>0.36±0.57</td>
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<tr>
<td>M64+M83</td>
<td>0.89±1.02</td>
<td>0.35±0.55</td>
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<tr>
<td>PPDa</td>
<td>11.56±0.29</td>
<td>10.48±0.30</td>
<td>8.22±0.36</td>
</tr>
<tr>
<td>PBS</td>
<td>8.33±0.20</td>
<td>7.23±0.17</td>
<td>5.46±0.36</td>
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<tr>
<td>(C) sensitized with NTM</td>
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</tr>
<tr>
<td>M64+M83+E6+10</td>
<td>0.85±0.96</td>
<td>0.22±0.54</td>
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<tr>
<td>M64+M83+E6</td>
<td>1.18±0.96</td>
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<tr>
<td>E6+C10</td>
<td>0.84±0.94</td>
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<tr>
<td>M64+M83</td>
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<td>0.35±0.54</td>
<td>0±0</td>
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<tr>
<td>PPDa</td>
<td>8.36±0.27</td>
<td>7.24±0.24</td>
<td>5.52±0.40</td>
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<tr>
<td>PBS</td>
<td>11.52±0.34</td>
<td>10.43±0.36</td>
<td>8.00±0.38</td>
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<tr>
<td>(D) control</td>
<td></td>
<td></td>
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<tr>
<td>M64+M83+E6+10</td>
<td>1.45±0.97</td>
<td>0.43±0.68</td>
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<tr>
<td>M64+M83+E6</td>
<td>1.00±1.17</td>
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<tr>
<td>E6+C10</td>
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<td>M64+M83</td>
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<td>0.17±0.41</td>
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</tr>
<tr>
<td>PPDa</td>
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<td>0.37±0.58</td>
<td>0±0</td>
</tr>
<tr>
<td>PBS</td>
<td>1.13±0.91</td>
<td>0.33±0.52</td>
<td>0±0</td>
</tr>
<tr>
<td>Mean±SD (72 h)</td>
<td>8.00±0.38</td>
<td>5.46±0.36</td>
<td>8.00±0.38</td>
</tr>
</tbody>
</table>

[(M64+M83+E6+10: Cocktail containing 0.5 µg of each rMPB64, rMPB83, rESAT6 and rCFP10; M64+M83+E6: Cocktail containing 0.5 µg of each rMPB64, rMPB83 and rESAT6; E6+C10: Cocktail containing 1.0 µg of each rESAT6 and rCFP10; M64+M83: Cocktail containing 1.0 µg of each rMPB64 and rMPB83; PPDa: Bovine PPD; PPDa: Avian PPD; PBS: Phosphate buffered saline; Different superscripts in same column differs significantly, *P <0.05*)]
In addition health hazard is a major issue of handling these infected animals. Use of killed mycobacteria for experimental animal sensitization is also recommended by the guidelines of OIE\textsuperscript{26}. In our study, we expressed and purified rMPB64, rMPB83 and rESAT6 from Indian \textit{M. bovis} strain (3/86 Rv) and evaluated DTH response elicited by recombinant protein combinations in guinea pig models with different sensitization. It is well established that PPD or recombinant protein antigens act on the macrophages at the inoculation sites to secrete proinflammatory interleukins which in turn convert nativeT-cells into tth1 cells, a basis of elicitation of DTH response\textsuperscript{27}. Characterization of \textit{Mycobacterium bovis} antigens such as CFP10, ESAT6, MPB83 and MPB64 contain cross-reactive epitopes and immunological responses could vary among individual animals. Due to the genetic diversity of the response of \textit{M. bovis} infected cattle to individual antigens, a large cocktail of such antigens will be necessary for the development strong DTH response.

A cocktail containing rMPB64, rMPB83 and rESAT6 proteins even at lower final protein concentrations (1.5 μg) induced statistically similar but stronger DTH response than rESTA6 and CFP10 proteins cocktail at a higher concentration (2μg). Our study reported that cocktail with rMPB64 and rMPB83 elicited a significantly lesser skin erythematous diameter even at a higher concentration of each protein (1 μg) when compared with other cocktails. The cocktail with MPB64 and MPB83 could elicit a significantly less skin diameter than the cocktail with CFP-10 and ESAT6, because the latter is a potent DTH-inducing antigens combination in guinea pig model. This observation reconfirms the previous study where MPT64 suppresses TNF-α production within the granuloma which is crucial for its formation\textsuperscript{28}. Added to this, some non-responder animals to MPB64 antigen have been reported in previous studies\textsuperscript{18}. According to Vordermeir \textit{et al.}\textsuperscript{29} when the frequency of response for ESAT-6, MPB64 and MPB83, in a cohort of 18 field reactors tested, the proliferative responses were induced in 66, 33 and 50% of the animals, respectively. The authors contended that only a subset of animals will respond to any given antigen. In this context, although ESAT-6 was the dominant antigen, MPB83 and MPB64 were recognized by some animals that did not recognize ESAT-6. One of the interesting observation reported by Whelan \textit{et al.}\textsuperscript{30} that a cocktail of the recombinant antigens (ESAT-6, MPB83 and MPB64) induced DTH responses in guinea pigs however this cocktail failed to stimulate measurable DTH in infected calves. Thus, they reported that while the differences in sensitivity between guinea pig and cattle (and human) DTH remain unclear, their data suggested that caution should be used when attempting to extrapolate quantitative DTH data between these species. Whelan \textit{et al.}\textsuperscript{30,31} tested a cocktail of the \textit{M. tuberculosis} complex recombinant proteins ESAT-6, CFP-10, MPB70 and MPB83. The cocktail elicited DTH skin test response in 78% naturally infected tuberculin-positive cattle. This cocktail did not induce any skin response in BCG-vaccinated cattle despite them being sensitized for strong tuberculin response. It has also been suggested that inclusion of Rv3615c (Mb3645c) enhanced skin test sensitivity in naturally infected cattle without compromising specificity. It is important to note here that in our study none of the cocktail combinations were found to give DTH response in NTM sensitized and BCG vaccinated guinea pigs, yet a positive DTH response was observed for PPDb and PPDa testing in both these animal models. The data of our study suggested that DIVA strategy \textit{i.e.} diagnosis discriminating between BCG vaccination and \textit{M. bovis} infection can be achieved with stronger skin reactions induced by a combination of different antigens. We did not notice any kind of adverse reactions in guinea pigs injected with cocktails. Meanwhile, the skin reaction was found significantly (P <0.05) maximum after 24 h of antigenic cocktail injection and followed by 48 and 72 h. The two cocktails containing \textit{M. bovis} recombinant proteins: cocktail 1 (C1): ESAT-6, CFP-10 and MPB83 and cocktail 2 (C2): ESAT-6, CFP-10, MPB83, HspX, TB10.3, and MPB70. C1, C2, and PPD-B showed similar response by DTH in \textit{M. bovis}-sensitized guinea pigs\textsuperscript{32}. Direct comparison of pre- and post-skin test blood samples revealed that the SICCT test induced significant boosting of the gamma interferon response in \textit{M. bovis}-infected animals to both the ESAT-6–CFP-10 and Rv3615c peptide cocktails that comprise the DIT, which persisted for the ESAT-6–CFP-10 reagent for at least 14 days. Importantly, no similar boosting effects were observed in non-infected BCG vaccinates, suggested that DIVA blood testing after a recent skin test would have minimal impact on test specificity\textsuperscript{33}.

In conclusion, we reported the expression and purification of rMPB64, rMPB83 and rESAT6 of...
M. bovis (3/86 Rv) and subsequent evaluation of DTH response by cocktail formulations in different guinea pig models. In vivo experiment revealed potential of all the cocktails in eliciting DTH response only in M. bovis sensitized but not in BCG vaccinated and NTM sensitized guinea pig model pointing at DIVA implications of these cocktails. The experimental observations showed that cocktail combination containing all four recombinant proteins viz., rMPB64, rMPB83, rESAT6 and rCFP10 was superior in eliciting DTH response in M. bovis sensitized guinea pigs on comparison with other cocktails tested. Present study utilised a guinea pig model sensitized with heat killed mycobacterial suspension having advantage over the use of hazardous live mycobacteria sensitized animal models. Experimental data suggests that rMPB64, rMPB83, rESAT6 and rCFP10 proteins cocktail can be used as DTH based diagnostic strategy having DIVA feature making a way forward for development of specific diagnostics for TB in animals.

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