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Multi-antigen print immunoassay for seroepidemiological surveillance of bovine tuberculosis on Indian cattle farms

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Summary

Bovine tuberculosis caused by *Mycobacterium bovis* is a zoonotic disease that is responsible for significant economic losses in many countries. The standard diagnostic method, the tuberculin test (TST) that is used in control programmes has serious shortcomings and, given the complex nature and the economic impact of the disease, a number of other diagnostic methods have been examined. The authors have attempted to characterise antibody response using the multi-antigen print immunoassay (MAPIA). A total of 511 serum samples were collected from farms in India on which bovine tuberculosis was prevalent and on farms with low incidence. These were tested using the MAPIA against a panel of five defined *M. bovis* recombinant antigens and two purified protein derivatives (bovine PPD and avian PPD) to study the seroprevalence of the disease on Indian cattle farms. Results indicated that the fusion protein of antigen CFP-10:MPB83 showed a positive response in 142 out of 298 serum samples from tuberculosis-prevalent farms, thereby indicating the serological dominance of the proteins post infection. The antigen selected could be used further in the development of a simple, rapid and accurate serological diagnostic test, paired with TST, for use in bovine tuberculosis control programmes.

Keywords

Bovine, Diagnosis, India, MAPIA, Multi-antigen print immunoassay, *Mycobacterium bovis*, Serology, Tuberculosis.

Multiantigen print immunoassay per la sorveglianza sieroepidemiologica della tubercolosi bovina in allevamenti bovini, India

Riassunto

La Tubercolosi bovina causata da Mycobacterium bovis *è una zoonosi responsabile di notevoli perdite economiche in molti paesi. Il metodo diagnostico standard, test della tubercolina (TST) utilizzato nei programmi di controllo, presenta gravi lacune e, data la complessità e l'impatto economico della malattia, sono stati esaminati altri metodi diagnostici. Gli autori hanno cercato di caratterizzare la risposta anticorpale con il* multi-antigen print immunoassay *(MAPIA). Un totale di 511 campioni di siero sono stati prelevati in India da aziende agricole in cui la tubercolosi bovina era prevalente e in altre a bassa incidenza. I campioni sono stati testati con MAPIA contro un panel di cinque antigeni ricombinanti definiti di* M. bovis *e due ricombinanti derivati proteici purificati (PPD bovina e aviaria) per studiare la sieroprevalenza della malattia negli allevamenti bovini indiani. I*

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risultati indicano che la proteina di fusione dell' antigene CFP-10: MPB83 ha evidenziato una risposta positiva per infezione tubercolare in 142 su 298 campioni di siero provenienti da allevamenti in cui la tubercolosi è prevalente, indicando così la dominanza sierologica delle proteine post infezione. L'antigene selezionato potrebbe essere utilizzato ulteriormente nello sviluppo di un esame sierologico diagnostico semplice, rapido e accurato, unitamente al TST, per i programmi di controllo della tubercolosi bovina.

Parole chiave

Bovino, Diagnosi, India, MAPIA, *Multi-antigen print immunoassay*, *Mycobacterium bovis*, Sierologia, Tubercolosi.

Introduction

Mycobacterium bovis, the causative agent of bovine tuberculosis, belongs to the *Mycobacterium tuberculosis* complex (MTC) of bacterial strains and is known to infect many vertebrates including cattle, goats, pigs and humans (35). Bovine tuberculosis is a chronic zoonotic disease that can incur substantial economic losses (29) in developing countries (3). The current prevalence rate of bovine tuberculosis in India is not studied thoroughly as is the case in other countries such as Brazil, Ethiopia, New Zealand and the United Kingdom (17, 21, 25, 32, 42) because of the absence of an organised farming system and also due to uncontrolled animal movements which make the implementation of bovine tuberculosis control programmes extremely difficult.

The diagnostic tests most widely used for the detection of bovine tuberculosis include the measurement of delayed-type hypersensitivity (i.e. intradermal skin testing) to purified protein derivatives (PPDs) (23) and/or *in vitro* assays for interferon gamma produced in response to mycobacterial antigen stimulation (31, 36, 43). These tests rely on early cell-mediated response, a hallmark of bovine tuberculosis immunopathogenesis (40). However, intradermal skin testing has low diagnostic accuracy (27) and it affects the immune status of animals subjected to repeated testing (5). Recent bovine tuberculosis reviews suggest that routine bovine tuberculosis diagnostic methods (tuberculin skin testing and *in vitro* interferon gamma) lack sensitivity and specificity because of a cross-reactive immune response to T-cell and B-cell epitopes conserved on orthologous molecules present in non-pathogenic mycobacteria and *Mycobacterium avium* subsp. *paratuberculosis* (4, 26, 30). Therefore, alternative diagnostic methods are required for the early detection of infected cattle. Serological assays are generally simple, rapid and inexpensive, but the development of improved serodiagnostic assays requires a thorough understanding of the bovine tuberculosis humoral immune mechanism as is characterised by highly heterogeneous antigen recognition (17). Bovine tuberculosis serology using the enzyme-linked immunosorbent assay (ELISA) was extensively compared with tuberculin skin testing (TST) (14, 42) and has been suggested as an ancillary parallel test for TST-negative cattle in herds with confirmed chronic infection (4) for the detection of anergic animals (15) or simply as complementary tests to detect and remove animals with an advanced disease condition (21, 30).

Recent studies indicate that serum antibody response to bovine tuberculosis has been used to detect animals in different stages of infection, such as early and latent phases of tuberculosis infection (1, 2, 38, 39). Single antigen-based assays never achieve satisfactory serodiagnostic performance in bovine tuberculosis as it has been shown that antigen recognition in the presence of tuberculosis is highly heterogeneous and hence the use of multiple antigens in serological assays is attempted. Serological assays utilising cocktails of antigens should effectively cover the diversity of immune responses and provide more accurate tools for immunological diagnosis of tuberculosis (7).

One such novel method for a cocktail-based serological diagnosis is the multi-antigen print immunoassay (MAPIA). MAPIA is used to diagnose infectious diseases, such as tuberculosis, which uses a cocktail of antigens to cover the diversity of the heterogeneous

antibody response in low-technology settings (18, 20). Compared to ELISA-based antibody detection in bovine tuberculosis, MAPIA may enhance the sensitivity of the diagnosis of bovine tuberculosis due to the incorporation of multiple antigens in the assay (8).

In our study, we have optimised MAPIA for bovine tuberculosis serodiagnosis using five defined recombinant *M. bovis* antigens and two purified protein derivatives to characterise antigen recognition profiles of immunoglobulin G (IgG) antibody responses in cattle serum ($n = 511$) obtained from farms with low incidence ($n = 3$) and farms on which the disease is prevalent ($n = 5$). In this study, farms where classified as low incidence farms and prevalent farms based on the TST method recommended by the World Organisation for Animal Health (*Office International des Épizooties*: OIE) and European Union for the diagnosis of tuberculosis (www.oie.int/) and also by the gold standard tuberculosis culture method recommended by the Centers for Disease Control and Prevention (CDC) (34). Results indicated the diagnostic potential of a tuberculosis-specific fusion protein using MAPIA methodology and its further use in the serological diagnosis of bovine tuberculosis on Indian cattle farms.

Materials and methods

Immunochemicals and reagents

Bovine purified protein derivative (BoPPD) was purchased from the Indian Veterinary Research Institute (IVRI) at Izatnagar in Uttar Pradesh and was used for TST. Nitrocellulose membrane was purchased from Amersham Biosciences (San Francisco, California), skimmed milk powder from Difco, protein A/G HRPO from Thermo Scientific (Rockford, Illinois), AEC (3-amino-9 ethylcarbazole) substrate and protease cocktail inhibitor from Sigma (St Louis, Missouri) and Bovigam® interferon gamma (IFN-γ) ELISA kit, bovine and avian PPD from Prionics^{AG} (Schlieren, Zurich) were used for interferon gamma release assays (IGRA) and MAPIA, respectively.

Codon optimised synthetic genes of ESAT-6, CFP-10, MPB70 and MPB83 for bacterial expression were obtained from GeneArt AG (Regensburg). Bacterial expression plasmid pRSET A was obtained from Novagen (Madison, Wisconsin). *Escherichia coli* strains XL Gold and BL21 plys were obtained from Stratagene (Santa Clara, California) and Invitrogen (Grand Island, New York), respectively, and were used for the propagation of plasmids and expression of the recombinant proteins, respectively. T4 DNA ligase used for ligation and isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from New England Biolabs (Ipswich, Massachusetts) and Sigma, respectively. Ni-NTA agarose was obtained from Qiagen (Valencia, California) and was used for affinity purification of the recombinant proteins. His-probe (Pierce, Rockford, Illinois) was used for Western blotting. Luria-Bertani (LB) broth, LB agar, ampicillin (sodium salt) and chloramphenicol were obtained from HiMedia (Mumbai).

Farms and disease status

Various cattle farms ($n = 8$) were selected in this study to examine the occurrence of bovine tuberculosis using MAPIA. Animal populations on each farm varied from 100 to 5 000 crossbred cattle. Farms were screened for bovine tuberculosis by routine TST and culture test and categorised as either tuberculosis-prevalent or farms with a low incidence of disease. Farms on which routine TST were performed once every three months, followed by the culling of positive reactors, were categorised as low incidence and farms with high percentage skin reactors were categorised as tuberculosis-prevalent farms.

Single intradermal tuberculin test

Single intradermal tuberculin tests (SITT) of cattle were performed using bovine tuberculin PPD as described by Mukherjee (24). A total of 100 µl of bovine tuberculin PPD was injected intradermally and a delayed-type hypersensitivity reactivity to PPD was recorded 3 days (72 h) post injection. An animal was declared positive if there was an increase in

skin thickness equal to or exceeding 4 mm (www.oie.int).

Serum from farm animals

Tuberculosis-positive reference sera were obtained from animals which were TST, interferon gamma (IFN-γ ELISA, Bovigam) and culture-positive, whereas tuberculosis-negative reference serum was obtained from animals which were TST, interferon gamma (IFN-γ ELISA, Bovigam) and culture-negative for the optimisation of the assay and used as positive and negative controls, respectively, in the MAPIA.

A total of 298 serum samples were obtained from tuberculosis-prevalent farms ($n = 5$), such as farm 4 ($n = 184$), farm 5 ($n = 32$), farm 6 ($n = 26$), farm 7 ($n = 26$) and farm 8 ($n = 30$) where skin reactors were observed and tuberculosis organisms were isolated and 213 serum samples were obtained from low incidence farms ($n = 3$), such as farm 1 ($n = 72$), farm 2 ($n = 42$) and farm 3 ($n = 99$) for testing by MAPIA where routine TST is performed to cull the skin reactors every three months. All serum samples were collected from different farm animals ($n = 8$) prior to TST for MAPIA.

Culture and identification of field isolates

Milk samples (approximately 5 ml), were collected in sterile Falcon tubes and transported on ice to the laboratory. Nasal, lachrymal and vaginal swabs were collected in 2 ml of sterile 7H9 broth and shipped to the laboratory on ice. Milk samples were decontaminated and processed for culture as described by Gao *et al.* (6). Sterile swabs in 7H9 broth were treated using the modified Petroff method as described by Somoskovi *et al.* (33). A total of 100 µl of inoculum from the above was used to inoculate BACTEC™ *Mycobacteria* growth indicator tube (MGIT™) 7 ml culture tubes and cultured in BACTEC™ 960 in accordance with the instructions of the manufacturer (BD Diagnostic Systems, Franklin Lakes, New Jersey). Cultures that showed a positive result with the BACTEC™ 960 instrument were verified by fluorescence microscopy for acid-fast bacilli using the tuberculosis quick stain kit.

Cultures positive by BACTEC™ 960 and staining were sub-cultured on Lowenstein Jensen (LJ) medium with pyruvate or glycerol. Those 4-8 week old cultures on LJ/culture that were positive by BACTEC™ 960 were confirmed as belonging to the MTC group of organisms using the commercial fast plaque tuberculosis assay (Biotech Laboratories Ltd, Ipswich).

Cloning, expression and purification of ESAT-6, CFP-10, MPB83, MPB70 and CFP-10:MPB83 fusion proteins in pRSETA

The 288 bp-sized ESAT-6 gene, 306 bp-sized CFP-10, 666 bp-sized MPB83, 585 bp-sized MPB70 and the 972 bp-sized fusion protein CFP-10:MPB83 (obtained by cloning of CFP-10 and MPB83 in tandem) were cloned into pRSETA using *BamH*I and *Hind*III restriction sites to obtain the bacterial expression vector pRSETAESAT-6, pRSETACFP-10, pRSETAMPB70, pRSETAMPB83 and pRSETACFP-10:MPB83. All of the above bacterial expression vectors were transformed separately into *E. coli BL21 pLys S* cells and plated on LB-agar plates containing 0.01% ampicillin and 0.0033% chloramphenicol. The plates were incubated at 37°C overnight. Single colonies were inoculated into 2 ml of LB broth containing 0.01% ampicillin and 0.0033% chloramphenicol (LB-Amp-Chl), grown at 37°C overnight and used as starter cultures to reinoculate in 2 l of LB-Amp-Chl media. The cultures were grown at 37°C at 200 rpm until the optical density 600 (OD_{600}) reached 0.6 and induced using 1 mM IPTG for 4 h at 37°C in a shaker set at 200 rpm. Sets of uninduced cultures were used as controls. The cultures were pelleted at 5 000 rpm (~7 000 × g) for 5 min and analysed for expression of ESAT-6, CFP-10, MPB70, MPB83 and CFP-10:MPB83 fusion proteins on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to further purification.

ESAT-6 and MPB83 were expressed as insoluble proteins, whereas CFP-10, MPB70 and the fusion CFP-10:MPB83 were expressed as soluble proteins. All the induced cell pellets were resuspended in buffer A (50 mM

phosphate buffer, pH 8.0 with protease cocktail inhibitor and sonicated for 30 min (amplitude of 33% with on/off cycles of 30 sec each). The cell lysates were separated into soluble and insoluble fractions by centrifugation at 10 000 × g at 4°C for 30 min and processed further for Ni-NTA agarose (immobilised metal affinity chromatography) based purification in the presence of the proteins in either of the fractions. For soluble proteins, the column was equilibrated with 10 column volumes of buffer A, the protein sample was passed onto the column and further washed with 20 column volumes of buffer A containing 50 mM imidazole. Elution was performed using buffer A containing 300 mM imidazole (buffer B). The purity of the protein was analysed in SDS-PAGE and Western blotting. Insoluble fractions were solubilised using buffer A containing 8M Urea (buffer C) and passed onto a Ni-NTA agarose affinity chromatographic column equilibrated with 10 column volumes of buffer C. The protein sample was passed onto the column which was washed with 20 column volumes of buffer C containing 50 mM imidazole. The protein samples were eluted using buffer C containing 300 mM imidazole and dialysed against four changes of buffer A with each change being made every 24 h. Finally, the protein was dialysed in phosphate buffered saline (PBS) pH 7.4. The purity of the protein was analysed in SDS-PAGE and Western blotting and quantitated using the bicinchonic acid (BCA) method.

Standardisation of antigen concentration for multi-antigen print immunoassay

Antigens at different concentrations ranging from 5 µg to 62.5 ng in 4 µl of PBS were adsorbed on nitrocellulose membrane as 11 mm narrow bands using a slot blot apparatus (Amersham Biosciences, San Francisco, California) under vacuum (10 psi) for 15 sec. Following adsorption of all seven antigens viz. ESAT-6, CFP-10, MPB83 and CFP-10:MPB83, MPB70, bovine PPD and avian PPD in different concentrations on the membrane, the membrane was cut perpendicular to the antigen bands into strips 3 mm wide. The membranes adsorbed with different concentrations of antigens were blocked for 30 min with 5% of non-fat skimmed milk in PBS containing 0.05% Tween 20 (PBST). The membrane was incubated for 90 min with two-fold dilutions of tuberculosis-positive and tuberculosis-negative reference serum samples ranging from 1:5 to 1:40, with a final dilution of 1:50 in PBST. After washing the nitrocellulose membrane strips five times with PBST, the strips were incubated for 60 min with protein A-G horseradish peroxidase (HRP) diluted with 1:20 000 in PBST and were washed again five times with PBST. Enzyme activity was visualised by incubating the strips for 8 min with AEC substrate prepared in accordance with the manufacturer's instructions. The strips were rinsed extensively in running tap water to stop the colour reaction. The entire assay was performed at room temperature with 400-500 rpm shaking.

Data analysis of control serum of multi-antigen print immunoassay

A visible band of any intensity was read as a positive reaction whilst the absence of a band was indicative of a negative reaction. The optimal antigen concentration and serum dilution were decided by titrating the antigen with known tuberculosis-negative and tuberculosis-positive reference serum samples.

Validation of multi-antigen print immunoassay

A single set of reagents with tuberculosis-positive and tuberculosis-negative reference serum were used by three different personnel on three different days to test repeatability. Antigen stability was checked on the membrane at six different temperatures (42°C, 37°C, 22°C, 4°C, –20°C, and –70°C) for a period of six weeks using positive and negative serum samples by MAPIA.

Screening of serum samples in multi-antigen print immunoassay

Serum samples from different farms with low incidence of tuberculosis ($n = 213$) and tuberculosis-prevalent farms ($n = 298$) with tuberculosis-positive animal serum as positive

controls and tuberculosis-negative animal serum as negative controls were tested for antibodies using the MAPIA.

Data analysis of the farm samples

MAPIA results were scored qualitatively by three independent operators with no prior knowledge of the disease status of the farm, with a visible band of any intensity being read as a positive reaction and the absence of a band being considered as a negative reaction. The scoring of three independent operators were analysed by taking the majority of results as test results and documented for the samples. Percentage of samples turning positive and negative in tuberculosis-prevalent and low incidence farms, respectively, were estimated from MAPIA data analysis.

Results

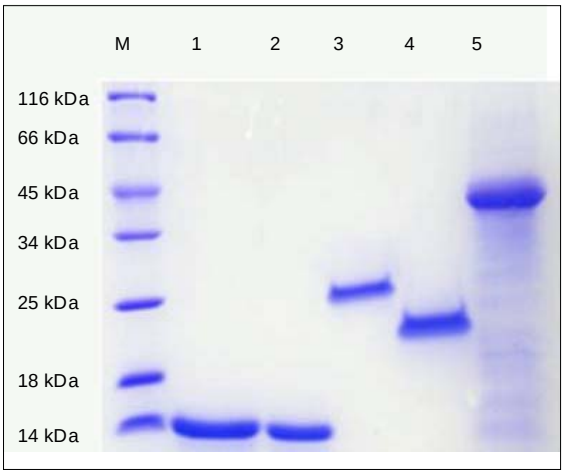
Expression and purification of the recombinant ESAT-6, CFP-10, MPB83, MPB70 and CFP-10:MPB83 fusion proteins

The recombinant proteins were expressed and purified to 95% homogeneity. A total of 12% SDS-PAGE and Western blot analysis using His_6 probe gave a single band of ~11 kDa for ESAT-6, ~12 kDa CFP-10, ~26 kDa MPB83, ~22 kDa MPB70 and ~38 kDa CFP-10:MPB83 fusion (Figs 1A and 1B).

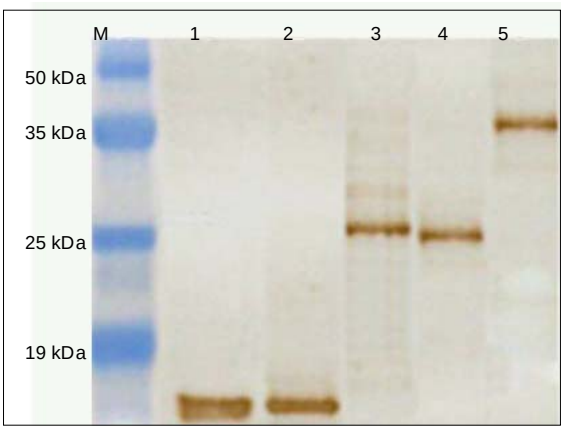
Farm selection

Farms 1, 2 and 3 were selected as farms with incidence of tuberculosis based on the removal of TST reactors for a period of five years. Farms 4, 5, 6, 7 and 8 were selected as tuberculosis-prevalent farms where TST reactors were recorded. In tuberculosis-prevalent farms, 66, 75 and 22 milk samples from farms 4, 7 and 8, respectively, and 58 nasal swabs from farm 5 were processed for bacterial isolation by culture. A total of 6 isolates: 1 and 4 milk samples from farms 4 and 7, respectively, and 1 nasal swab from farm 5 were detected positive by culture that revealed the presence of growth using the BACTEC™ MGIT™ 960 system, LJ media and subsequently fluorescent staining for acid-fast bacilli. The TST and culture test results of low incidence and prevalent farms are presented in Table I.

A. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of purified ESAT-6 (lane 1), CFP-10 (lane 2), MPB83 (lane 3), MPB70 (lane 4) and fusion protein CFP-10:MPB-83 (lane 5)



B. Western blot analysis of purified ESAT-6 (lane 1), CFP-10 (lane 2), MPB83 (lane 3), MPB70 (lane 4) and fusion protein CFP-10:MPB-83 (lane 5)



ESAT-6 early secretory antigenic target-6
CFP-10 culture filtrate protein-10
MPB83 mycobacterial protein-83
MPB70 mycobacterial protein-70
BoPPD bovine purified protein derivative
AvPPD avian purified protein derivative

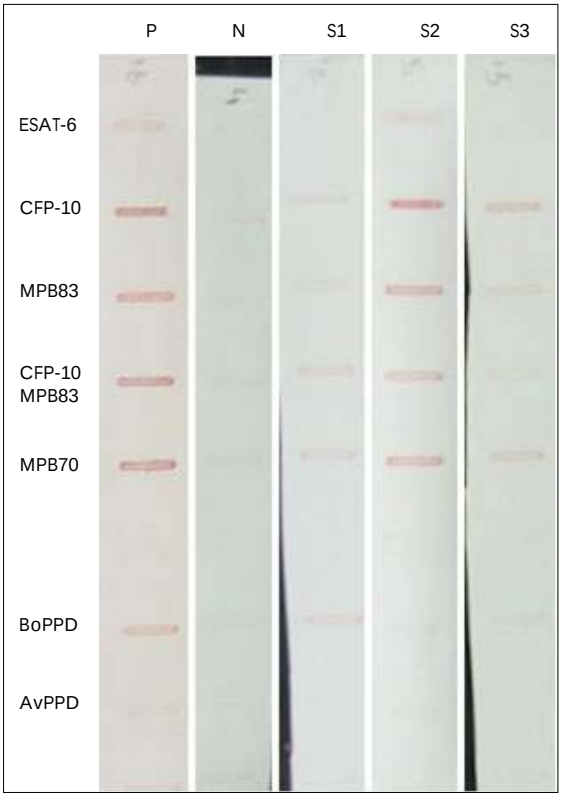
Figure 1
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of recombinant tuberculosis-specific proteins

Antigens and serum concentration achieved by multi-antigen print immunoassay

To determine the optimal concentration of antigen and antibody to be used in the MAPIA, titration was done using varying concentrations of antigens ranging from 5 µg to 62.5 ng and serum dilutions ranging from 1:5 to 1:50. Results indicated that the optimal concentration of ESAT-6, CFP-10, MPB83, MPB70 and CFP-10:MPB83 fusion protein, bovine PPD and avian PPD to be used were 1 µg, 1 µg, 125 ng, 125 ng, 750 ng, 1 µg and 1 µg, respectively. The optimum serum dilution to be used was found to be 1:20. Figure 2 represents the MAPIA performed with optimal parameters, along with positive, negative control sera and field serum samples.

Validation of multi-antigen print immunoassay

MAPIA enabled a high level of repeatability of results with positive and negative serum samples with three different operators on three different days. Antigens adsorbed on membranes were stable on the membrane for 6 weeks at 22°C, 4°C, –20°C, and –70°C but unstable beyond one week at 37°C and 42°C.



ESAT-6 early secretory antigenic target-6
CFP-10 culture filtrate protein-10
MPB83 mycobacterial protein-83
MPB70 mycobacterial protein-70
BoPPD bovine purified protein derivative
AvPPD avian purified protein derivative

Figure 2
Multi-antigen print immunoassay (MAPIA) showing the antigen recognition patterns by antibodies from test sera samples
Strip P is MAPIA performed with positive reference sera from tuberculosis culture positive animal
Strip N is MAPIA performed with negative reference sera from tuberculosis culture negative animal
Strips 1, 2 and 3 are the MAPIA performed with samples collected from tuberculosis prevalent farm

Table I
Tuberculin skin testing and culture test results from farms with low incidence of tuberculosis and on farms on which tuberculosis was prevalent

Farm and number of animals tested by TST and MAPIA	No. of skin reactors (%)	Confirmed *Mycobacterium bovis* isolation on farm*
Farm 1 (*n* = 72)	0.00	0
Farm 2 (*n* = 42)	0.00	0
Farm 3 (*n* = 99)	0.00	0
Farm 4 (*n* = 184)	54.30	1
Farm 5 (*n* = 32)	100.00	1
Farm 6 (*n* = 26)	68.75	Not performed
Farm 7 (*n* = 26)	33.30	4
Farm 8 (*n* = 30)	85.00	0

TST tuberculin skin testing MAPIA multi-antigen print immunoassay
* confirmed by culture in BACTEC™ *Mycobacteria* growth indicator tube (MGIT™) 960 and fluorescent acid-fast bacilli staining

Seroepidemiology of bovine tuberculosis on tuberculosis-prevalent farms and on farms with low incidence of disease

MAPIA results of 298 serum samples from five tuberculosis-prevalent farms and 213 serum samples from three low incidence farms are presented in the Figures 3A and 3B. Among the five defined mycobacterial proteins used in the MAPIA, the fusion protein (CFP-10: MPB83) showed highest percentage positivity of 54.04% followed by CFP-10 (47.02%), MPB83 (24.78%), ESAT-6 (0.768%) and MPB70 (0.728%) in serum samples from tuberculosis-prevalent farms ($n = 298$). Antigen-wise reactivity of the individual farm included in this study is presented in Table II as well as in Figure 4. None of the samples reacted with avian PPD which indicated the MAPIA specificity. Four culture positive samples out of six were also tested by MAPIA and all animals reacted to the fusion protein (CFP-10:MPB83) as well as to the CFP-10 protein. The other two positive culture samples were not tested by MAPIA.

Discussion

Serological studies following infection by MTC demonstrate that inadequate knowledge of humoral immune response has generally led to the development of serodiagnostic methods that have poor sensitivity and specificity (7) due to the complex nature of the disease. It has been repeatedly observed by many researchers that profile of the antibody response varies among animals; the reaction to single recombinant antigen is sometimes transient, fluctuating and lacks satisfactory sero-diagnostic performance (2, 17, 42) which indicates that antigen recognition in clinical tuberculosis is highly heterogeneous.

To date, no detailed study on the humoral response by *M. bovis* has been conducted in cattle and, therefore, the development of a sensitive rapid and inexpensive serodiagnostic test, like MAPIA, would complement current methods of bovine tuberculosis diagnosis and possibly reduce diagnosis costs. Wild animals are implicated in the maintenance and transmission of bovine tuberculosis. Moreover, the routine diagnostic method (TST) is impractical for use in many wild animals. Thus, antibody-based assays are attractive as they imply minimal handling of animals and the advantage of storage of samples prior to further processing. Recently MAPIA methodology was adopted to determine antigen recognition patterns in white-tailed deer (*Odocoileus virginianus*), reindeer (*Rangifer tarandus*) (37, 38) European badgers (*Meles meles*) (9), cervids (*Cervus elaphus*) (11) and

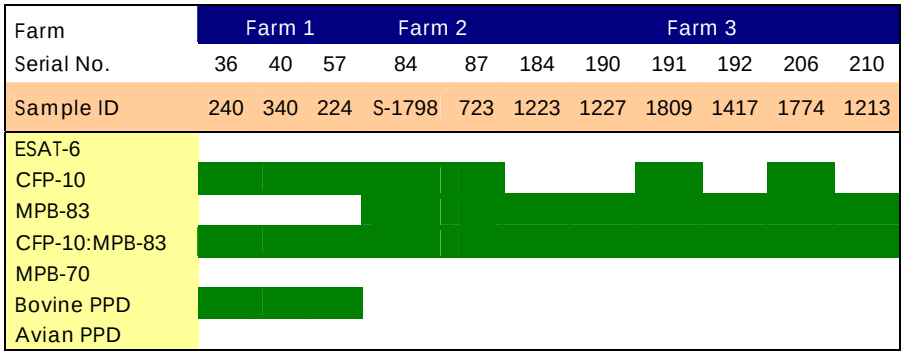


Figure 3A
Heterogeneous tuberculosis antigen recognition by positive sera samples from farms with low incidence of tuberculosis
Each lane represents one serum sample tested by multi-antigen print immunoassay
Dark shading indicates serum reactive to the respective antigen
No shading indicates serum is not reactive with antigens

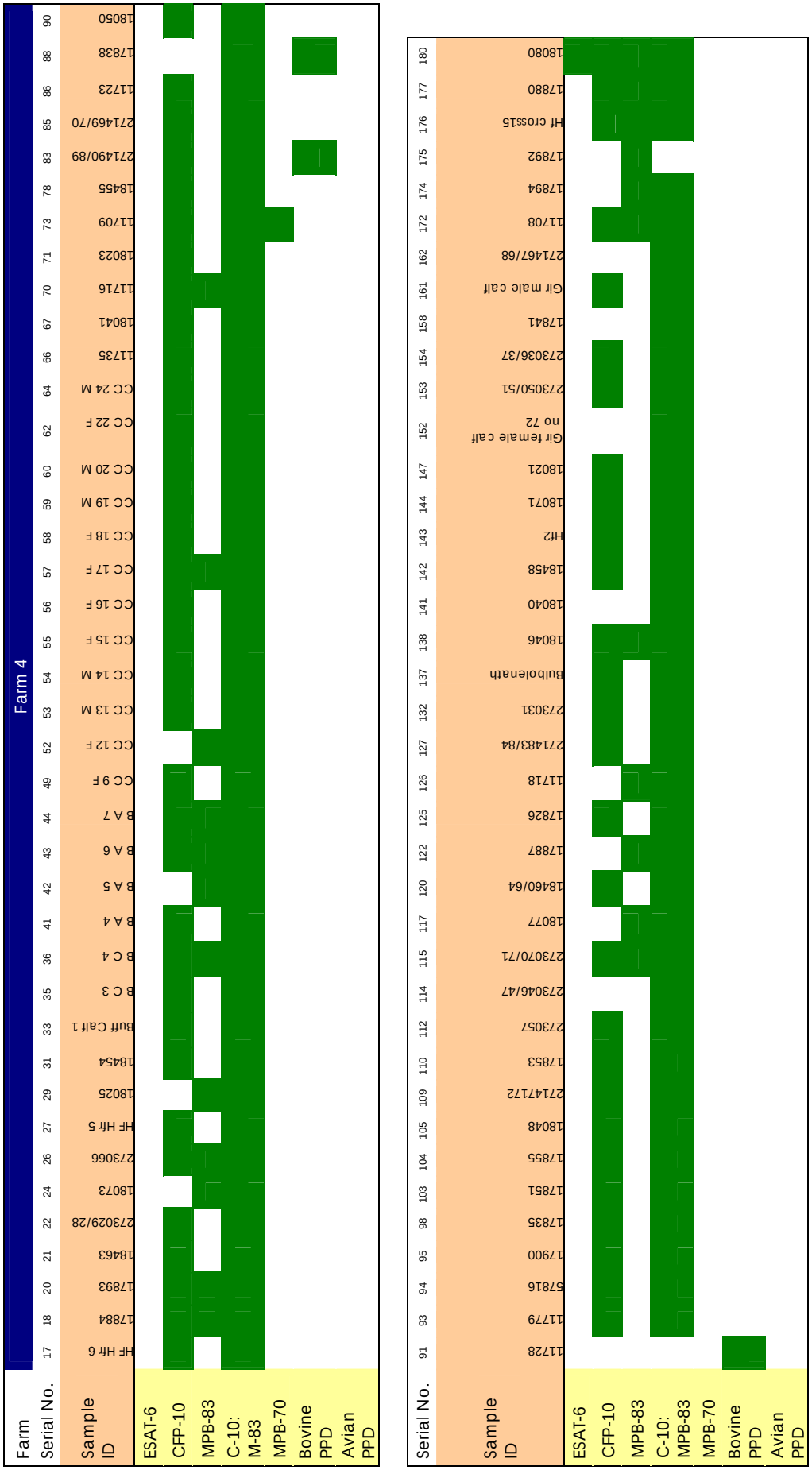


Figure3B
Heterogeneous tuberculosis antigen recognition by positive serum samples from farms on which tuberculosis was prevalent (Farm 4)
Each lane represents one serum sample tested by multi-antigen print immunoassay
Dark shading indicates serum reactive to the respective antigen
No shading indicates serum is not reactive with antigens

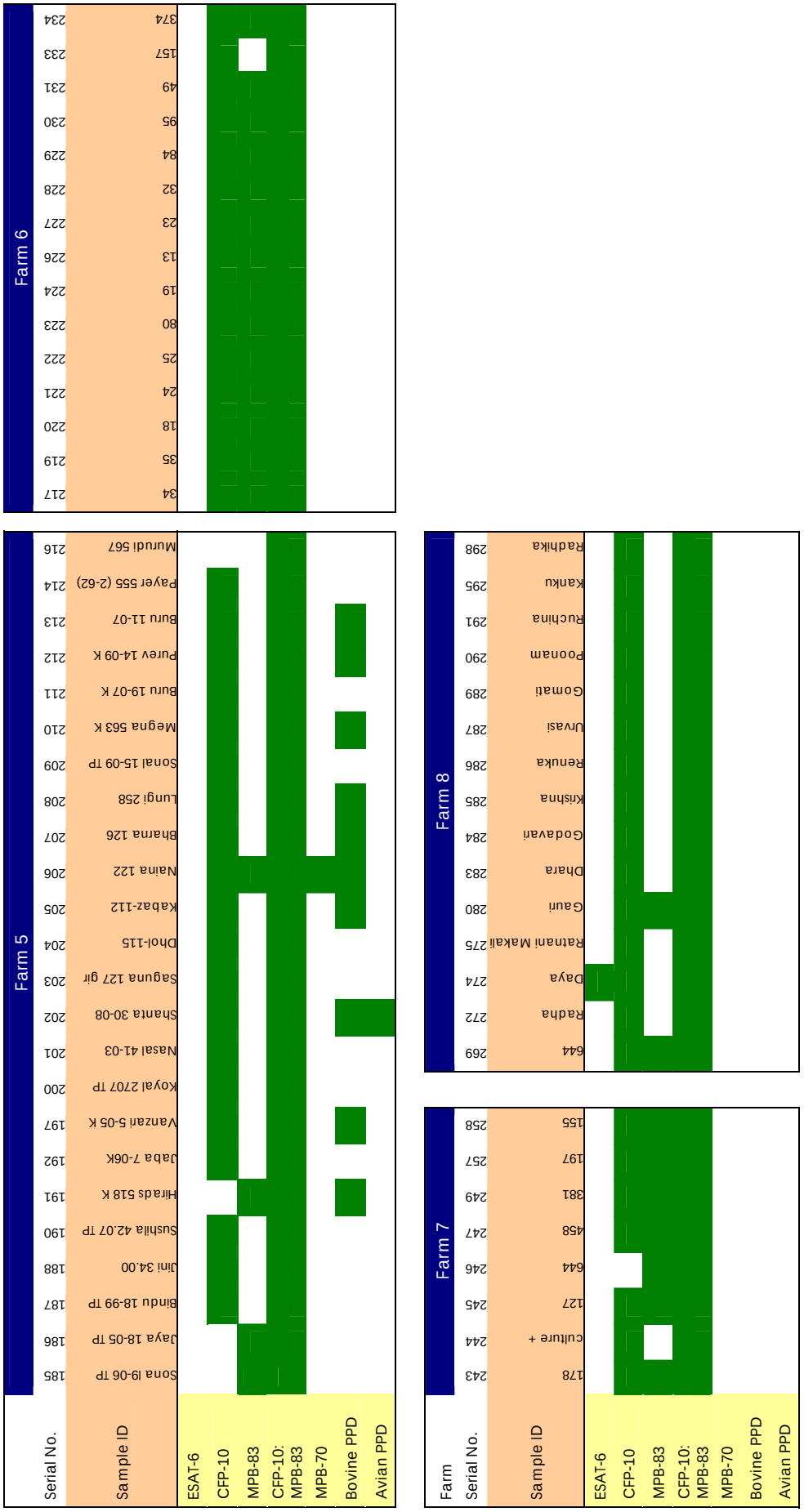
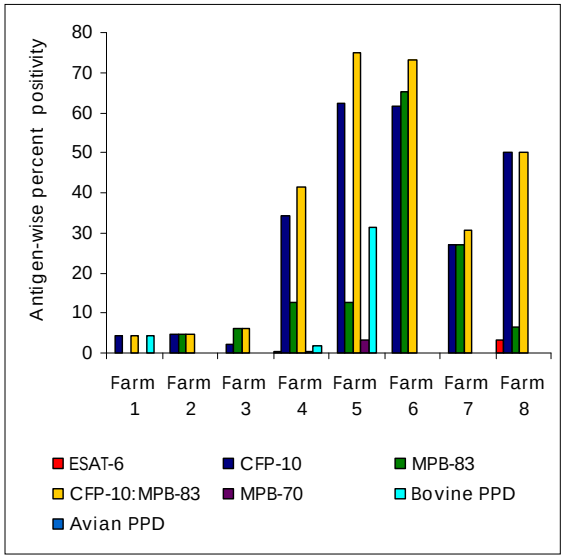


Figure3C
Heterogeneous tuberculosis antigen recognition by positive serum samples from farms on which tuberculosis was prevalent (Farms 5, 6, 7 and8)
Each lane represents one serum sample tested by multi-antigen print immunoassay
Dark shading indicates serum reactive to the respective antigen
No shading indicates serum is not reactive with antigens

Table II
Antigen-wise reactivity of serum samples from farms on which tuberculosis incidence was low and on farms on which tuberculosis was prevalent

Serial No.	Farm No.	Antigen wise reactivity (%)						
		ESAT-6	CFP-10	MPB83	CFP-10:MPB83	MPB70	Bo PPD	Avian PPD
1	Farm 1	0	4.2	0	4.2	0	4.2	0
2	Farm 2	0	4.7	4.7	4.7	0	0	0
3	Farm 3	0	2	6	6	0	0	0
4	Farm 4	0.54	34.2	12.5	41.3	0.54	1.63	0
5	Farm 5	0	62.5	12.5	75	3.1	31.25	0
6	Farm 6	0	61.5	65.4	73.1	0	0	0
7	Farm 7	0	26.92	26.92	30.8	0	0	0
8	Farm 8	3.3	50	6.6	50	0	0	0

ESAT-6 early secretory antigenic target-6
CFP-10 culture filtrate protein-10
MPB83 mycobacterial protein-83
MPB70 mycobacterial protein-70
Bo PPD bovine purified protein derivative



ESAT-6 early secretory antigenic target-6
CFP-10 culture filtrate protein-10
MPB83 mycobacterial protein-83
MPB70 mycobacterial protein-70
BoPPD bovine purified protein derivative
AvPPD avian purified protein derivative

Figure 4
Antigen-wise percentage positivity data for all sera samples (n = 511) from farms on which tuberculosis incidence was low and on farms on which tuberculosis was prevalent

elephants (*Elephas maximus* and *Loxodonta africana*) (10, 19) infected with *M. bovis*. Information is very scarce on the use of antibody-based bovine tuberculosis diagnostic assays in cattle with little or no data available on the seroepidemiological status of cattle herds using defined *M. bovis* antigens (2, 17, 42).

Our paper deals with the serological study on a few organised farms in India using a panel of recombinant tuberculosis antigens and PPDs in MAPIA. The findings indicate that serum antibodies are produced against a variety of *M. bovis* antigens in cattle sera from various farms. The number and type of serologically reactive antigen varied greatly from animal to animal and from farm to farm. No single antigen or groups of antigen reacted with all sera from different farms. However, it was observed that the maximum percentage of antibody response was observed for CFP-10:MPB83 fusion protein on all farms. This concurs with observations made by others that the strong antibody responses were elicited by MPB83, followed CFP-10 against *M. bovis* (10, 39) but not yet tested as fusion protein (CFP-10-MPB83).

Despite the fact that India is an endemic country for bovine tuberculosis, to date no detailed study has been made on the seroepidemiology of bovine tuberculosis on Indian dairy farms. The heterogeneous antigen recognition observed in our study might be due to the expression of different mycobacterial proteins at different stages of bovine tuberculosis on different farms of the country. This kind of heterogeneous antigen recognition variability with respect to the stage

of disease has been reviewed for human tuberculosis (12, 13) but not for bovine tuberculosis.

Our study provides the first report that fusion protein CFP-10:MPB83 appear to be more sensitive than other panels of antigens on all of the tuberculosis-prevalent farms. It was observed that four out of six cultures from animals that were positive using MAPIA and all serum samples reacted to the fusion protein (CFP-10:MPB83). This data supports the observation that fusion protein (CFP-10: MPB83) would be the antigen of choice in the serological diagnosis of bovine tuberculosis. Furthermore, this MAPIA used defined antigens that were highly specific for MTC and could therefore constitute an ancillary test to TST, to avoid false-positive results due to potential cross-reactivity with other paratuberculosis and non-tuberculous bacteria exposed cattle.

In farms with a low incidence of tuberculosis, all antigens responded significantly less (<5%) compared to tuberculosis-prevalent farms, where higher responses to various antigens were observed. However, the fusion protein CFP-10:MPB83 was found to be the serodominant antigen as 142 out of 298 serum samples in tuberculosis-prevalent farms were positive for CFP-10:MPB83 fusion protein. Poor antibody response to ESAT-6 and MPB70 (<1%) on all farm animals was indicative of the subdominant nature of these antigens for bovine tuberculosis. In this study, a total of six isolates were confirmed out of 221 clinical samples from the tuberculosis-prevalent farms by BACTEC™ 960 and fluorescent acid-fast bacilli staining. The tuberculosis culture positivity rate (2.72%) observed in this study was lower because most of farms are far from the laboratory and thus transportation delays might have encouraged the growth of contaminants and non-tuberculosis myco-bacteria (15). Here we used tuberculosis culture tests as a tool to differentiate the farms based on tuberculosis incidence in parallel with TST. Moreover, in developing and resource poor nations, it is difficult to practise tuberculosis culture tests as they are more expensive, require upgraded laboratory infrastructures and skilled and experienced staff (28). Thus, the MAPIA developed in this study using the serodominant antigen as fusion partner (CFP-10-MPB83) might be a more cost-effective, rapid cowside test that could be adapted as an ancillary screening test to TST in bovine tuberculosis control programmes in developing countries like India. The other major problem with the current bovine tuberculosis diagnostic tests is its reduced sensitivity due to significant cross-reactivity with non-tuberculous mycobacteria and the *M. avium* complex. The fusion protein (CFP-10: MPB83) comprising CFP-10 and MPB83 proved to be a highly specific candidate in the detection of bovine tuberculosis than paratuberculosis (22, 41). In addition, the MAPIA is user-friendly as there is no need to revisit a farm and no problem of desensitisation as observed in TST that would also not need high levels of expertise during field application in developing nations.

The MAPIA technology described here can be therefore applied in bovine tuberculosis control programmes and related epidem-iological studies and also for bacillus Calmette-Guérin (BCG) vaccine efficacy studies in cattle. Recent literature reviews suggest that serological tests produce highly variable results in regard to sensitivity and specificity and therefore cannot be recommended as the unique test for tuberculosis diagnosis. However, the World Health Organization policy encourages further research to identify new tests for human tuberculosis diagnosis and/or serological tests with improved sensitivity and specificity (44).

In conclusion, the present study describes the MAPIA for detection of antibody response against various multiple recombinant proteins of *M. bovis* in various tuberculosis-prevalent and low incidence farms. We identified the potent serodominant antigen for diagnosis of bovine tuberculosis as CFP-10:MPB83 fusion protein. The MAPIA developed in this study could be used for initial tuberculosis screening either alone or in combination with TST for improved sensitivity of bovine tuberculosis screening, thereby leading to more successful control programmes in developing countries.

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