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# ANTEMORTEM AND POSTMORTEM EXAMINATIONS OF THE CATTLE CALF NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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A male cattle calf was detected as subclinically and naturally infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by a series of antemortem and postmortem tests. The MAP infection was identified by strong antibody and cell-mediated immune (CMI) response by a commercial ELISA kit and an intradermal Johnin test, respectively, in the initial antemortem examination. The antemortem status of the calf was further confirmed by MAP-specific interferon gamma (IFN-γ) response. For detection of IFN-γ response, MAP-specific IFN-γ release assays (IGRAs): (a) immuno capture ELISA (IC-ELISA) and (b) ELISPOT was employed. In addition, the presence of intracellular cytokine IFN-γ was detected by flow cytometry. For all cytokine assays, MAP-specific recombinant antigens HSP65 and 35 kDa were employed to overcome the poor sensitivity and specificity resulting from the use of Johnin, the crude protein purified derivative of MAP. Postmortem examination of the MAP-infected/suspected cattle calf did not reveal any pathognomonic gross lesions in the gastro-intestinal tract. Histopathological examination of multiple organs showed the presence of epithelioid cells/macrophages and edematous lesions in the mesenteric lymph nodes suggestive of MAP; however, no granulomas were observed in the intestinal tract. The necropsy samples of rectum and mesenteric lymph nodes were positive for isolation of MAP by culture in the BACTEC™ MGIT™ 960 system, and acid fast bacilli were demonstrated by fluorescence microscopy confirming the infection. Due to differential and complex expression patterns of MAP antigens reported in literature, a combination of assays such as those based on IGRAs and antibody detection is essential. Therefore, the current experimental evidence confirms the efficacy of the approach adopted. However, further studies will be needed to understand the optimal combination MAP-specific antigens for use in IGRAs or antibody assays that can be used for detecting MAP infection in every stage of the disease.

**Keywords:** paratuberculosis, MAP diagnosis, bovine IFN-γ, ELISA, ELISPOT, flow cytometry, MAP culture, histopathology

## Introduction

Johne's disease (JD) or paratuberculosis is a chronic, progressive, and incurable intestinal disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in domestic and wild ruminants [1]. The disease causes severe economic losses due to reduced milk production and premature culling [2]. Infected cattle with clinical signs shed the organism in feces [3, 4] and milk [5], resulting in greater risk for other animals as well as human exposure. The histopathological and clinical similarities between JD and Crohn's disease (CD) in humans lie at the basis of controversy on the implication of MAP in the development of CD [6]. Animals are most susceptible during first year of life; fecal–oral route is the primary route of infection [7]. The young calves get infection from contaminated birthing environment, infected colostrums/milk, and rarely in the uterus [8]. Calves become infected soon after birth but rarely show clinical signs during the first 2 years of life whereas clinical signs are more obvious in 2 to 6 years of age. Gross pathological lesions are usually absent in subclinical stage and may also be absent or minimal in symptomatic animals. This poses serious problems in diagnosing the disease in calves infected naturally with MAP. Histologically, granulomatous inflammatory responses with abundance of lymphocytes, epithelioid macrophages, and giant cells in intestinal and associated mesenteric lymph nodes/tissues has been observed from mild to advanced stage of JD [9–11].

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The speed of progression of MAP infection to different stages of disease varies depending on the immune status of individual animals. Thus, no single diagnostic test can be applied to detect MAP infection at every stage. The control of JD was severely hampered due to lack of early diagnostics and vaccines; there are many commercially available tests for JD each with their own advantages and limitations. While most tests perform well at the herd level, for the identification of individually infected animals, it is a prerequisite to use a combination of complementary tests, and repeated sampling can increase the diagnostic sensitivity for efficient JD control programs.

Humoral immune response does not confer protection against the pathogen but the presence of MAP antibodies in milk or serum samples during the preclinical and clinical stages aids in disease diagnosis. Development of a cell-mediated immune (CMI) response by interferon gamma (IFN-γ) producing T-cells of the Th1 subset is essential to the host in defense against intracellular MAP by restricting its replication. This is elicited at an early stage of MAP infection, hence, valid for early and rapid diagnostic interventions to control of JD [12].

Diagnosis of the MAP is a challenge due to the chronic nature and prevalence of four stages of disease, *viz*., stage I, "silent" infection; stage II, unapparent carrier animals; stage III, clinical disease; and stage IV, advanced clinical disease [13]. Diagnosis in stage III and stage IV is not complicated because of obvious clinical signs and shedding of MAP whereas diagnosis is complicated in stages I and II due to no clinical signs and low shedding of MAP. Currently practiced antemortem testing for JD in cattle includes agar gel immunodiffusion [14] and commercial ELISA tests; polymerase chain reaction [15, 16], fecal culture, and single intradermal Johnin tests (SIJT); and interferon gamma release assays (IGRAs) such as ELISA [17, 18] and enzyme-linked immunospot assay (ELISPOT) [19]. Postmortem testing includes gross pathology, histopathology [9, 10], and extraintestinal organ/tissues culture. The SIJT is a practical field test generally used for antemortem diagnosis of JD while bacterial isolation from fecal samples is considered as a gold standard for definitive diagnosis, but the test is time consuming. The IGRAs, which detect the cell-mediated immune (CMI) response by measuring IFN-γ release from sensitized lymphocytes, have been used as an early diagnostic test for MAP infection [17, 20]. Since the IGRAs use crude johnin purified protein derivative (PPDj), the test is not widely accepted in diagnostic laboratories because of low sensitivity [21], poor predictive value [22], absence of standardized protocol, and variation in PPDj strains [23] as well as sampling time [24]. To overcome these problems, identification of immunogenic MAP-specific protein candidates recognized only by MAP-infected animals in different stages of disease and their subsequent application in diagnosis of MAP has been recommended [12].

In the present case study of the naturally infected cattle calf with MAP, various antemortem and postmortem diagnostics assays were performed in combination to analyze the efficacy of investigation of JD.

## Materials and methods

*Immunochemicals and reagents*

Johnin purified protein derivative (PPDj) was purchased from I.V.R.I., Izatnagar, India, and used for single intradermal johnin testing (SIJT). Paratuberculosis ELISA kit was purchased from Institut Pourquier$^{R}$ 34057 Montpellier Cedex 5, France. Roswell Park Memorial Institute (RPMI) 1640, horse serum, antibiotic, and other antimycotics were purchased from Gibco (Grand Island, New York, USA). Concanavalin-A (Con-A) was purchased from Bangalore-Genei (Bangalore, Karnataka, India). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, Missouri, USA). Lymphoprep™ solution was purchased from Axis-Shield PoC (RodelØkka, Oslo, Norway). Bovine IFN-γ ELISPOT kits were purchased from MABTECH (Nacka Strand, Sweden). Ninety-six-well ELISA plates and vacutainer tubes containing sodium heparin were purchased from BD Biosciences (Franklin lakes, New Jersey, USA). All plastic supplies were purchased from NUNC (Rochester, New York, USA). All molecular biology reagents and fine chemicals were obtained from Invitrogen (New York, USA) and Merck (Mumbai, Maharastra, India), respectively. Codon-optimized synthetic genes of HSP65 and 35 kDa for bacterial expression were obtained from GENEART (Darmstadt, Hesse, Germany). Bacterial expression plasmid pRSET A was obtained from Invitrogen (New York, USA). *Escherichia coli* strains XL Gold and BL21 pLys were obtained from Stratagene (La Jolla, California, USA) and Invitrogen (New York, USA), respectively. T4 DNA ligase and isopropylβ-D-1-thiogalactopyranoside (IPTG) were obtained from New England Biolab (Ipswich town, Suffolk, UK) and Sigma (St. Louis, Missouri, USA), respectively. The plasmid isolation kits, gel extraction kit, and Ni-NTA agarose were obtained from Qiagen (Valencia, California, USA). His6-probe was obtained from Pierce (Rock ford, Illinois, USA). Luria Bertani (LB) broth, LB Agar, ampicillin (sodium salt), and chloramphenicol were obtained from Hi-media (Mumbai, Maharastra, India). Lipopolysaccharide (LPS) removal Sartobind® Q 75-Catridge was procured from Sartorius Stedin Biotech (Blenheim, Epsum Survey KT199 QQ, UK). The limulus amebocyte lysate (LAL) test reagents were procured from Associates of Capecode Incorporated (East Falmouth, MA, USA). The *E. coli* host cell protein (HCP) detection kit was procured from Cygnus Technologies (Southport, NC-28461, USA). DNA molecular weight markers and protein molecular weight markers, acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS), Tris, glycine, ammonium persulphate, TEMED ($N,N,N',N'$-tetramethylethylenediamine), Coomassie brilliant blue R-250, and IPTG (isopropyl β-D-

1-thiogalactopyranoside) were procured from Bio-Rad, USA. The Hybond-C nitrocellulose membrane was procured from Amersham Biosciences, USA.

*Selection of farm*

The study farm with animal population varying from 500 to 1000 crossbred cattle calf of age group 6–12 months was selected for this study to examine the occurrence of JD. The selected farm was screened for JD by routine SIJT and serology for MAP-specific antibodies. The screening was performed once every 3 months followed by culling of positive skin reactors and seropositive animals, and the farm is categorized as JD low incidence farm. In the present study, all the experiments were performed with blood samples obtained from this selected farm.

*Single intradermal Johnin test (SIJT)*

Animals from the selected farm were subjected to SIJT using purified protein derivative of MAP (PPDj) as per World Organisation for Animal Health (OIE), 2010. One hundred microliter of PPDj was injected intradermally on the side of the middle third of the neck, and delayed type hypersensitivity reactivity to PPDj was recorded after 72 h post injection. The animals were kept under observation for the period of 72 h, and the increase in the skin thickness diameter was measured using vernier callipers (Associate agencies, Ahmedabad, Gujarat, India). Animals were declared positive if there was an increase in skin thickness diameter equal to or above 4 mm diameter.

*Sero-diagnosis*

The serological assay was performed with MAP antibody ELISA kit (Institut Pourquier$^{R}$ ELISA Paratuberculosis kit) according to manufacturer's instructions. The presence of MAP-specific antibodies was determined based on the ratio of absorbance of experiment sera samples to absorbance of positive control sera sample (S/P %). Interpretations of sera samples with S/P % less than or equal to 45% are classified as negative for MAP antibodies and S/P % greater than or equal to 55% are classified as positive for MAP antibodies whereas the S/P % 45 to 55 were classified as suspected as MAP antibodies.

*Reference sera*

Ten MAP culture positive and ten MAP culture negative sera were procured from Department of Primary Industries (ATTWOOD, Australia) for testing in the MAP antibody ELISA kit (Institut Pourquier$^{R}$ ELISA Paratuberculosis kit) as reference serum to confirm the MAP antibody ELISA kit sensitivity and specificity.

*Selection of the experimental cattle calf*

During routine JD screening in the selected farm, one of the young crossbred male calves of 8 to 10 months old was turned to be serologically as well as SIJT positive. The suspected cattle calf was segregated from the other animals and kept in the quarantine shed for the clinical observations during the experimental period. The blood samples were collected from the suspected cattle calf in heparinized vacutainer tubes and immediately transferred to laboratory and processed within 2 to 4 h for various MAP diagnostic assays. A seronegative as well as SIJT non-skin reactor animal from the same farm was also included in the study as negative control animal.

*Cloning, expression, and purification of HSP65 and 35 kDa proteins*

The complete HSP65 and 35 kDa DNA sequences were codon-optimized for *E. coli* expression and obtained from GENEART, Germany, as pGA4-HSP65 plasmid and pGA18–35 kDa plasmid. The synthetic plasmids and expression vector (pRSET A) were digested, ligated, and then transformed into *E. coli* XL Gold cells. Plasmid DNA was isolated from transformed colonies and subjected to restriction digestion analysis. The expression of recombinant clones was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot. Medium scale expression of rHSP65 and r35 kDa proteins is checked by SDS–PAGE and Western blot. The purification of inclusion bodies was performed as per published literature [25]. Refolding was done by dialysis with lowering dilutions of urea ranging from 6 to 2 M at 4 °C. Final dialysis was done in PBS pH 7.4 at 4 °C. The protein was estimated using Bradford method, and purity of the protein was analyzed by SDS–PAGE. LPS was removed from the purified JD proteins using Sartobind Q 75-Catridge, according to the manufacturer's instruction. The host cell protein (HCP) contamination in the *E. coli* expressed JD proteins (HSP65 and 35 kDa proteins) was tested using the Cygnus Technologies *E. coli* HCP ELISA kit as per manufacturer's instructions.

*Whole blood assay and estimation of bovine IFN-gamma (BoIFN-γ) cytokine*

Blood samples were collected from seronegative and SIJT negative cattle calves ($n$ = 49) from the selected farm in heparinized vacutainers for whole blood stimulation with JD-specific antigens such as PPDj, HSP65, and 35 kDa in different concentrations to achieve the cutoff or basal level JD-specific IFN-γ response. Briefly, 0.2 ml of heparinized blood samples was stimulated with different concentrations, 15 µg to 0.5 µg per well in serial dilutions of HSP65, 35 kDa, and mixture of HSP65 and 35 kDa antigens. Con-A (1 µg per well) was used as mitogen/

positive stimulant to know the functional viability of the blood cells and 50 µl RPMI media as a negative stimulant in a 96-well plate. The samples were incubated at 37 °C in 5% $CO_2$ incubator for 22 to 24 h. Supernatants were collected after incubation by centrifugation at 500 × *g* for 7 min at 22 ± 2 °C. The JD-specific bovine IFN-γ secreted from stimulated bovine blood samples was estimated using in-house developed and validated bovine IFN-gamma (BoIFN-γ) IC-ELISA using commercial bovine IFN-γ MAb pair (AbD Serotec, UK) (unpublished data). Stimulation index (SI) was calculated as ratio between OD of the antigen stimulated well and the OD of the negative stimulated wells as per published literature [26]. The cutoff was calculated as mean of the SI of SIJT nonreactor animals ($n$ = 49) added to twice the standard deviation of SI of the skin test negative animals in different concentration of the JD antigens used.

*ELISPOT assay to detect JD-specific IFN-γ in selected animal peripheral blood mononuclear cell (PBMCs)*

Peripheral blood samples from selected seropositive and seronegative animals were collected before SIJT, and PBMCs isolation was performed by density gradient centrifugation using lymphoperp™ solution with density 1.077 ± 0.001 grams/ml and cryopreserved as per published literature [27]. BoIFN-γ ELISPOT assay was performed as per published literature with slight modifications using JD-specific antigens such as HSP65, 35 kDa, mixture of HSP65 and 35 kDa, and PPDj. Briefly, the ELISPOT plates were coated with 100 µl per well bovine IFN-γ capture MAb (7.5 µg/ml) and incubated for overnight at +4 °C. $1 \times 10^6$ PBMCs were seeded per well (seropositive animal PBMCs and seronegative animal PBMCs) and stimulated with HSP65, 35 kDa, and mixture of HSP65 and 35 kDa at 0.5 µg/well. Con-A at 50 µl per well (10 µg/ml) and RPMI media alone at 50 µl per well were used as the positive and the negative stimulant, respectively. The ELISPOT plates seeded with PBMCs and antigens were incubated at 37 °C in a 5% $CO_2$ incubator for 24 h. The plates were washed six times with PBS and incubated with 100 µl of detection antibody (0.25 µg/ml) per well for 2 h at 22 °C ± 2 °C. The plates were washed six times with PBS, and 100 µl of streptavidin–alkaline phosphate conjugate (diluted 1:1000 in PBS with 0.5% fetal bovine sera) was added per well and incubated for 1 h at 22 °C ± 2 °C. The plates were washed six times with PBS, and 100 µl of NBT/BCIP substrate was added per well and incubated at 22 °C ± 2 °C for 9 min. The reaction was stopped by washing the plate with 200 µl per well of ultra-pure distilled water for six times. The ELISPOT plate was then dried and subsequently scanned for the development of spots using Immunospot® Series 5 UV Reader (CTL, USA). The results were analyzed on an Immunospot® Series 5 UV Reader (CTL, USA) using its automated software features Immunocapture® and Immunospot® for user-independent setting of counting parameters (SmartCount®) and the gates (Autogate®) for scanning and counting the spots. The corrected spot forming units were calculated by subtracting the spots generated in the media stimulated well from the spots generated in the antigen stimulated well.

*Intracellular cytokine staining of MAP-specific IFN-γ by flow cytometry*

Cryopreserved PBMCs of seropositive/SIJT skin reactor and seronegative/SIJT non-skin reactor were thawed as per published literature [27]. The cells were washed in cRPMI with DNase (0.02 mg/ml) and centrifuged at 1300 rpm (340 × *g*) for 6 min in room temperature (RT). Cell pellet resuspended in cRPMI and $1 \times 10^6$ cells/well were seeded in 24 well plate. HSP65 (5 µg, 10 µg, and 15 µg), 35 kDa (5 µg, 10 µg, and 15 µg), mixture of HSP65 and 35 kDa (5 µg, 10 µg, and 15 µg), and PPDj (10 µl, 25 µl, and 50 µl) were used for antigen-specific cytokine secretion in 100 µl of cRPMI, and the final volume was adjusted to 900 µl using cRPMI. Plate is incubated in 5% $CO_2$ at 37 °C for 2 h. Phorbol myristate acetate (PMA) (50 ng) and ionomycin (1 µg) were added for positive stimulation, and RPMI media was used as negative control. Golgistop containing Brefeldin A (1 µl per million cells) was added in all wells except in assay blank well. Plate was further incubated at 5% $CO_2$ at 37 °C for 4 h. The samples were transferred to a 2-ml tube and centrifuged at 211 × *g* for 6 min in RT. The pellet was resuspended in 100 µl Pharmingen™ stain buffer (BD Biosciences, San Diego, CA) and transferred to a 96-well "V" bottom plate (BD Biosciences, NJ, USA); volume was made up to 300 µl using stain buffer and centrifuged. Finally, pellet was resuspended in 100 µl stain buffer. The surface staining antibodies mouse anti-bovine CD4-FITC (MCA1653F, AbD Serotec) and mouse anti-bovine CD8: Alexa Fluor 647 (MCA837A647, AbD Serotec) were added to all wells except the blank. Plate was incubated in a $CO_2$ incubator at 37 °C for 30 min, and all sample volume was made up to 300 µl using stain buffer and centrifuged. The pellet was resuspended in 100 µl of cytofix/cytoperm™ (BD Biosciences, San Diego, CA) and incubated at 4 °C for 15–20 min. After incubation, 200 µl of cytofix/cytoperm™ was added to all wells and centrifuged, and the pellet was resuspended in 300 µl of cytofix/cytoperm™ and centrifuged. The pellet was resuspended in 100 µl of cytofix/cytoperm™, and the intracellular staining antibody mouse anti-bovine IFN-γ: RPE (MCA1783PE, AbD Serotec) was added and incubated in dark for 30 min at RT. Cells were then washed with 400 µl of perm/wash buffer, and the cell pellet was resuspended in 200 µl of stain buffer for data acquisition. All washing steps were conducted at 500 × *g* for 8 min at 24 °C. The cells were acquired using FACSCanto II (BD, San Jose, California), a total of 20,000 events were recorded in list mode for all the samples and analyzed with FACS Diva 6.1 software, and the stimulation index was calculated as per published literature [28].

*Postmortem and sample collection of the experimental calf*

The seropositive/SIJT reactor male crossbred calf was euthanized as per institutional animal ethical committee guidelines, and postmortem was conducted. Gastrointestinal tract and lymph nodes were examined for gross pathology. Multiple organs such as intestine/rectum, mesenteric lymph nodes, spleen, liver, and submandibular lymph nodes were collected in 10% formalin for histopathological observation. Mesenteric lymph node, rectum, and fecal samples were collected during necropsy for MAP isolation in BACTEC MGIT culture tubes and cultured in BACTEC 960 as per the manufacturer's instructions (BD Biosciences). Tissues of submandibular lymph nodes, liver, spleen, and mesenteric lymph nodes were processed as per standard protocol, and isolated cells were analyzed for the T-cells by immunophenotyping using bovine-specific CD4 and CD8 fluorochrome-labeled antibodies (AbD Serotec, UK).

*Tissue isolation and tissue stimulation for JD-specific IFN-γ*

The isolated tissue cells were seeded at $1 \times 10^6$ cells per well in 96-well plates and stimulated with different concentrations of 15 µg to 0.5 µg per well in serial dilutions of HSP65, 35 kDa, and mixture of antigens. The samples were incubated at 37 °C in 5% $CO_2$ incubator for 22–24 h. Tissue culture supernatants were collected after incubation by centrifugation at 500 × *g* for 7 min at 22 ± 2 °C. The JD-specific bovine IFN-γ secreted cytokine from stimulated tissue cells was estimated by ELISA as described above.

*MAP isolation from the necropsy material*

Tissue samples collected during postmortem – mainly mesenteric lymph nodes and intestine – were homogenized to make a 2-ml tissue suspension. Each homogenized sample was decontaminated with 4 ml of 4% sodium hydroxide by vortexing the mixture for 15 min followed by centrifugation at 3220 × *g*. The sediment was washed with sterile phosphate buffer and finally suspended in 1 ml of 7H9 broth; 0.5 ml of the suspension was inoculated into MAP-specific commercial media (BACTEC MGIT Para TB tubes Cat. No. 245154). The inoculated MIGT tubes were incubated in BACTEC 960 system as recommended by the manufacturer's instructions for 49 days. The clinical sample turning positive by BACTEC 960 instrument was checked for acid fast MAP bacilli using fluorescent staining kit (BD Biosciences, Sparks, Maryland, USA).

*Molecular identification of the MAP culture*

Genomic DNA from the reference MAP ATCC (strain 19698) and positive and negative flagged samples from BACTEC 960 system were extracted by employing a commercial kit (Tetracore, USA) according to manufacturer's instructions. MAP-specific IS Mav1 gene target was selected because of the specific diagnostic potential of the region [29, 30]. Primers and probes for real-time PCR were designed using software from Genescript. The IS Mav1 gene-based real-time PCR was conducted according to the optimized protocol (unpublished data).

*Histopathology*

The multiple organs collected during postmortem were adequately sliced and preserved in 10% neutral buffered formalin. The stored samples were processed by conventional method. Five micrometer paraffin sections were stained with hematoxylin and eosin and examined under light microscopes. All deviations from normal histology were recorded and compared with corresponding controls.

## Results

*Sero-prevalence and SIJT status of selected farm*

The organized farm was selected for this experiment, which maintains approximately 500–1000 crossbred calves of less than 1 year of age. During our experimental period, sera samples ($n$ = 50) collected from the selected farm and examined using MAP ELISA kit revealed the 2% seroprevalence rate and SIJT revealed that there is 1% skin reactors in the farm.

*Serology of reference sera*

Ten MAP culture positive and ten MAP culture negative sera were procured from Department of Primary Industries (ATTWOOD, Australia) and tested by MAP antibody ELISA kit, showing 100% sensitivity and specificity. The percentage positivity of the sera samples tabulated in *Table 1* indicates the MAP antibody ELISA kit performance in reference culture positive and negative sera samples.

**Table 1.** Ten culture positive and ten culture negative samples S/P % by MAP ELISA kit

| Reference positive samples | | Reference negative samples | |
|---|---|---|---|
| S. No | S/P % | S. No | S/P % |
| 1 | 130.61 | 11 | 2.29 |
| 2 | 180.31 | 12 | 2.58 |
| 3 | 231.12 | 13 | 1.96 |
| 4 | 185.45 | 14 | 1.66 |
| 5 | 171.09 | 15 | 2.70 |
| 6 | 167.76 | 16 | 2.55 |
| 7 | 89.49 | 17 | 8.64 |
| 8 | 219.27 | 18 | 3.99 |
| 9 | 79.48 | 19 | 9.17 |
| 10 | 176.01 | 20 | 2.85 |

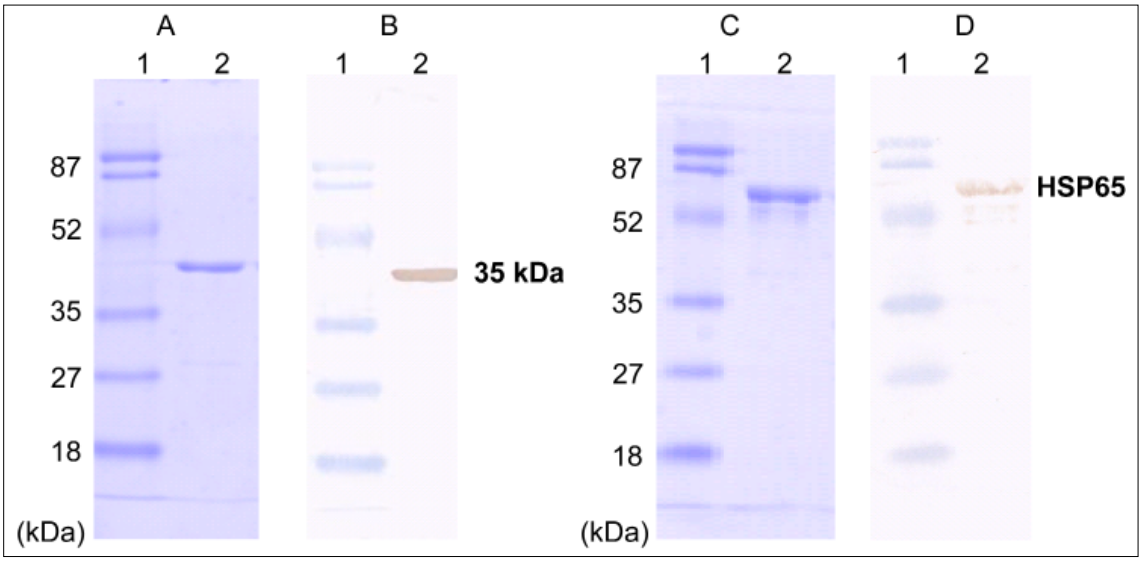


**Fig. 1.** SDS–PAGE (A) and Western blot (B) analysis of 35 kDa protein. Lane 1: prestained protein molecular weight markers; lane 2: 35 kDa protein (0.5 µg loaded onto the gel). SDS–PAGE (C) and Western blot (D) analysis of HSP65 protein. Lane 1: prestained protein molecular weight markers; lane 2: HSP65 protein (0.5 µg loaded onto the gel)

*Serology and SIJT status of the experimental cattle calf*

Out of 50 animals screened, one of the experimental cross-bred male calves of 8 to 10 months old in the selected farm turned serologically positive and was suspected for JD, but no clinical signs were observed. The presence of MAP-specific antibodies was determined by MAP antibody ELISA kit (Pourquier® ELISA paratuberculosis-paratub. serum-S, Institut Pourquier, France) based on the ratio of absorbance of experiment sera samples to absorbance of positive control sera sample (S/P percentage). The S/P % value of the suspected calf was 139.7% and 136.5% for the paired sera samples collected within a month interval indicating a high level of MAP-specific antibodies present

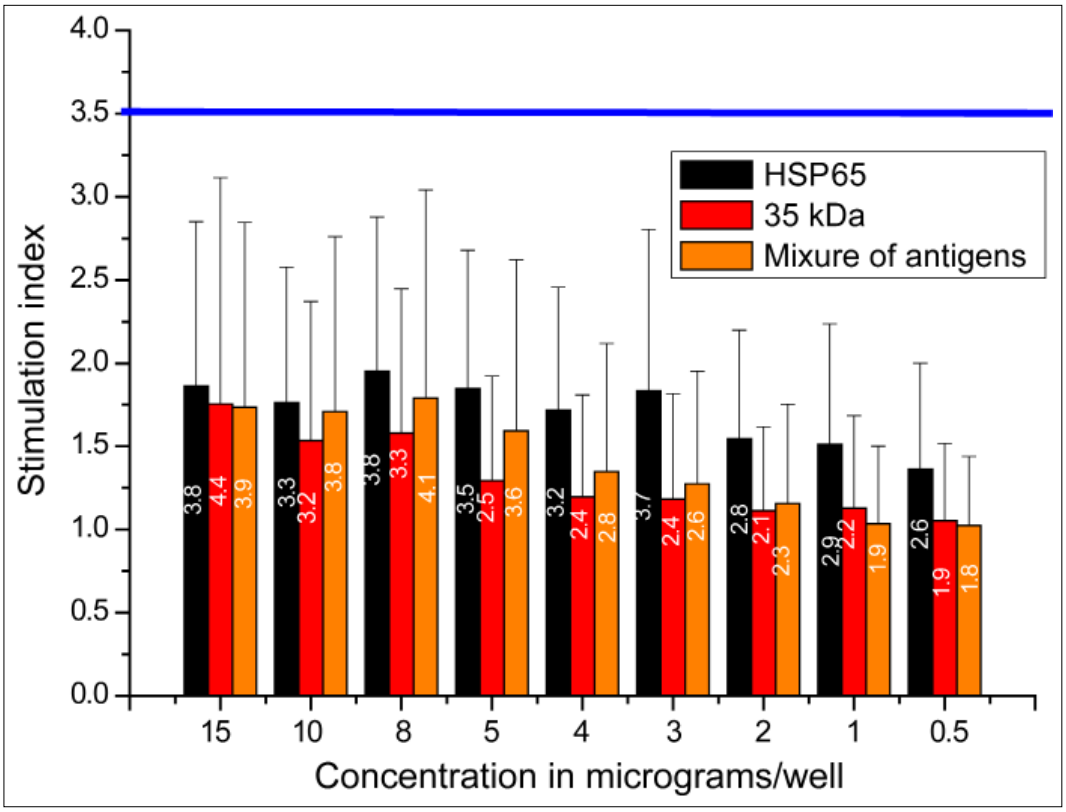


**Fig. 2.** The figure showing the mean and SD of the seronegative/SIJT nonreactors animals ($n = 49$) stimulation index using different concentrations of HSP65, 35 kDa, and mixture of HSP65 +35 kDa. The mean plus 2 SD of the stimulation index was calculated as cutoff values were mentioned in the center of each bar

in the serum. The whole blood samples from the animal were collected for IGRAs before performing the SIJT. The experimental seropositive crossbred male calf also turned to be SIJT reactor with an increase in skin thickness diameter of 6 mm.

*Expression and purification of the recombinant HSP65 and 35 kDa protein*

The codon-optimized HSP65 gene and 35 kDa gene for *E. coli* expression was obtained from GENEART and was cloned into bacterial expression vector pRSETA for protein expression. The recombinant clones were checked by restriction digestion analysis and automated DNA sequencing. The positive recombinant clone was selected for protein expression. The expression of HSP65 (65 kDa) protein and 35 kDa protein was clearly seen in induced culture but not in uninduced culture. The expression of recombinant protein was confirmed by Western blot probed with anti-$His_6$ MAb. The expressing clone was chosen for the medium scale expression and purified to 95% homogeneity. SDS–PAGE using 12% gel and Western blot analysis using anti-$his_6$ MAb gave a single band of protein size 65 kDa and 35 kDa with yields ranging from 4 mg/L of HSP65 culture and 3 mg/L of 35 kDa *(Fig. 1)*. Endotoxin and HCP contamination in the JD proteins were checked and kept minimal to prevent nonspecific stimulation.

*Whole blood assay for JD-specific IFN-γ detection in SIJT nonreactors*

The bovine IFN-γ stimulation index from the SIJT nonreactors animals ($n$ = 49) from JD low incidence farm stimulated with HSP65, 35 kDa, and mixture in different concentration with the mean and SD of the SI is presented in *Fig. 2*. The cutoff was calculated as the mean of the SI of the seronegative/SIJT nonreactor animals ($n$ = 49) added to twice the SD of SI of all 49 animals shown inside the bars in *Fig. 2* in all the concentrations. In all concentrations of JD-specific antigens, the mean basal level of IFN-γ secretion threshold is found to be less than 3.5 SI *(Fig. 2)*.

*Whole blood assay for JD-specific IFN-γ detection in experimental cattle calf*

The SI of the SIJT reactor and seropositive animal was observed to be more than 3.5 in all different concentrations with HSP65, 35 kDa, and mixture, whereas the seronegative animal was observed always less than 3.5 SI in all different concentrations of JD antigens used.

*Bovine IFN-γ ELISPOT assay for the experimental cattle calf*

The corrected spot forming units were calculated in seropositive/SIJT skin reactor animals PBMCs as well as seronegative/SIJT non-skin reactor, showing significantly greater JD-specific corrected spot forming units (SFU) than the seronegative and non-skin reactor animal. The average corrected SFU of 169.6 for HSP65, 120.6 for 35 kDa, and 138 for mixture of antigens observed in the seropositive/SIJT reactor compared to corrected SFU of 8 for HSP65, 12.6 for 35 kDa, and 7.66 for mixture of antigens was observed in the seronegative/non-skin reactor and is shown in *Fig. 3*. The significant >10-fold increase in JD-specific SFU observed in the seropositive/SIJT reactor

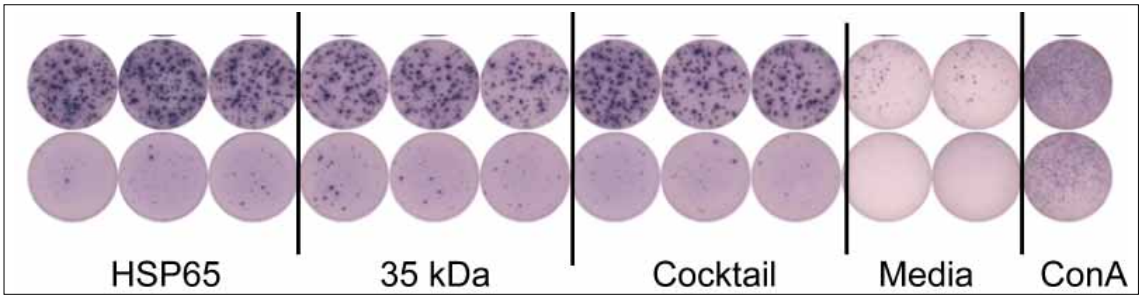


**Fig. 3.** ELISPOT using 35 kDa and HSP65 antigen

**Table 2.** The calculated stimulation index of the IFN-γ secreting cells by intracellular cytokine staining for the seronegative and seropositive animals PBMCs upon stimulation with JD specific antigens

| Antigen | Seronegative/ non-skin reactor | Seropositive/ skin reactor | Seronegative/ non-skin reactor | Seropositive/ skin reactor |
|---|---|---|---|---|
| | IFN-γ CD4 T-cells | IFN-γ CD4 T-cells | IFN-γ CD8 T-cells | IFN-γ CD8 T-cells |
| HSP65 – 10 µg | 0.47 | 6.5 | 1 | 5.6 |
| 35 kDa – 15 µg | 2.7 | 2.6 | 1.2 | 8.8 |
| HSP65 + 35 kDa 15 µg | 1.8 | 4.7 | 0 | 12 |
| Johnin – 25µl | 2.6 | 2.3 | 0 | 4.4 |

animals compared to a seronegative/SIJT nonreactor has encouraged to move further into other *in-vitro* JD diagnostic investigations.

*Intracellular cytokine staining for bovine IFN-γ in the experimental cattle calf*

The JD-specific CD4 IFN-γ stimulation index of the seropositive/skin reactor animal is 13.8-fold more than the seronegative/non-skin reactor for HSP65 at 10 μg concentration *(Table 2)*. There was no significant increase with 35 kDa and johnin; however, mixture of antigens showed 2.6-fold increases. The JD-specific CD8 IFN-γ stimulation index of the seropositive/skin reactor animal is 5.6-fold more than the seronegative/non-skin reactor for HSP65 at 10 μg concentration whereas 7.3-fold more than the seronegative/non-skin reactor for 35 kDa at 15 μg concentration; however, synergistic effect has 12-fold increase in mixture of antigens and 4.4-fold increase in PPDj.

*Postmortem details*

At the time of the postmortem examination, the animal was slightly emaciated; the necropsy was performed immediately after euthanizing the animal. There was no prominent pathognomonic lesion in the gastrointestinal tract, lymph nodes, liver, spleen, heart, etc.

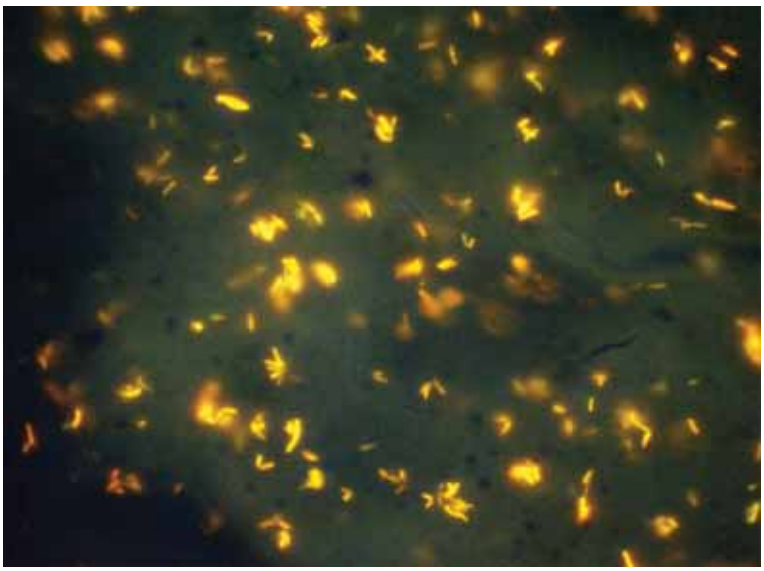

**Fig. 4.** Smear from MGIT culture positive from the lymph node isolate detected by AFB fluorescent staining and magnification is ×100

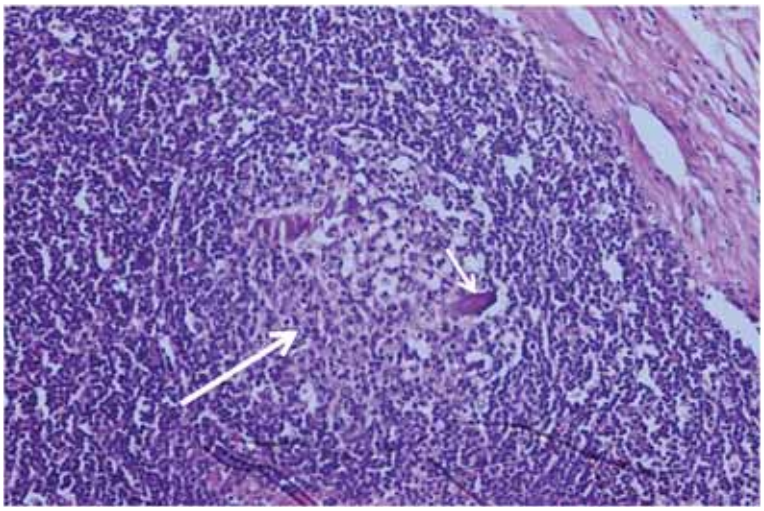

**Fig. 5** Histopathology of mesenteric lymph node. Photomicrograph showing calcification (short arrow) and germinal center in follicle (long arrow) in cortex of lymph node. The stain used is hematoxylin and eosin (H&E) and the magnification is ×200

*Cultural identification of MAP from the necropsy material*

Postmortem samples such as mesenteric lymph node and intestine showed positive identification signals by BD-BACTEC-MGIT 960 system, on day 18 and 20 postinoculation with recorded growth units of 1045 and 543, respectively, whereas the fecal samples were not flagged. Samples confirmed positive by MGIT were examined by fluorescent staining. Translucent rods were observed against dark background under 100 × objective in both the samples *(Fig. 4)*.

*Molecular identification of the MAP culture*

Real-time PCR for MAP conducted by the optimized protocol on DNA templates extracted from mesenteric lymph node and intestine was positive by real-time PCR, since the mean Ct values were 35.21 and 36.89, respectively, and within the cutoff Ct value of 40 (unpublished data).

*Tissue isolation and tissue stimulation for JD-specific IFN-γ*

Tissue-specific IFN-γ release was observed only in the spleen with stimulation index of more than 4 in all different concentrations of JD antigens, but the stimulation index was less than 2 in other organs.

*Histopathology*

Histopathology revealed hyperplasia and characteristic infiltration of macrophages and

epithelioid cells either in the paracortical or the corticomedullary area of mesenteric lymph nodes, as well as focal calcification. However, granulomas, necrosis, and multinucleate giant cells were absent. The histological profile taken together possibly indicated MAP lesions *(Fig. 5)*.

## Discussion

In the development of JD control programs, it has frequently been assumed that young calves rarely become infected and cattle of more than 2 years are infectious and shedders. However, this assumption is in contrast with observed fecal shedding of MAP in young calves [31–33], and even in the present study, MAP was isolated and confirmed in a cattle calf aged 8 to 10 months suggesting that cattle calf might have picked up the infection either from contaminated birthing environment or via contaminated colostrums/milk from the infected dam. The dam of this naturally infected calf would not be traced as the calf was procured from the local market and introduced in the organized farm. In the present case study, antemortem and postmortem analysis of the naturally MAP-infected cattle calf was carried out with various diagnostic assays. CMI-based diagnostics test mostly employed in JD diagnosis because IFN-γ is considered as the essential cytokines that play an important role in achieving protective immunity against mycobacterial infection [34, 35]. Even though humoral immune response against mycobacterial infection has been considered nonproductive, the active role of B-cells and antibodies in mycobacterial infection was proven in the last decade [36–39]. Thus, in the present case study, both CMI and humoral response assays were adopted; moreover, in JD, it is proven that antibody response appears late in infections and prominent during subclinical and clinical stage. MAP antibody detection by ELISA was recommended in large scale JD control programs in dairy herds because of its speed, less cost, and high throughput. Currently, five commercial MAP ELISA kits were available for MAP diagnosis, and in the present study, MAP antibody ELISA kit (Institut Pourquier$^{R}$ ELISA Paratuberculosis kit) for the antibody detection has been used. MAP antibody ELISA kit showed 100% specificity and sensitivity in reference sera samples. Thus, the same MAP antibody ELISA kit was applied in the selected farm for JD screening, and one of the cattle calves showed significant S/P % of 139%, indicating the strong MAP humoral response in the animal, and the same animals showed positive skin reaction to PPDj by SIJT testing posing suspicion for MAP infection. The advantage of MAP antibody ELISA kit is that it has serum preadsorption with *Mycobacterium pheli* to prevent nonspecific binding with cross reacting antibodies [20] and observed to have significant diagnostic sensitivity and positive predictive value [40]. In the present study, recombinant MAP antigens such as HSP65 and 35 kDa were used for the IGRAs in combination with crude antigens/PPDj as there are reported low specificity problems for the immunological diagnosis of JD using crude antigens [41–44]. As reported in tuberculosis (TB) diagnosis that use of RD1 antigens (ESAT-6 and CFP-10) was proved as diagnostic potential, numerous studies have been currently on-going and future studies are recommended to identify new MAP antigenic target for immunodiagnostics tests [12, 34, 45] both for humoral and CMI responses. During the course of infection, cattle are dependent on the development of CMI responses in the protection against intracellular MAP, thus, CMI detectable early in the infection. The PPDj is an undefined mixture of antigens used in the skin test to measure delayed type hypersensitivity and is proven to be of low specificity. Research suggests early MAP-specific CMI responses can also be measured using the IFN-γ test. The IFN-γ ELISPOT assay is a sensitive test and has potential to improve the JD diagnosis which is comparable or better rate than IFN-γ ELISA. Thus, in the present study, both IGRAs (ELISPOT and ELISA) were carried out in the seropositive/SIJT reactor as well as in seronegative/SIJT nonreactor animal. In ELISPOT, more than 10-fold increase in the MAP-specific IFN-γ SFU was detected in seropositive/SIJT reactor than seronegative/SIJT nonreactor animal PBMCs. To augment the specificity of the IGRAs, it is necessary to attempt well-defined and MAP-specific antigens to recall CMI responses; some of the MAP-specific antigens such as secreted antigens [21, 46, 47], cell wall and membrane antigens [34], lipoproteins [48, 49], heat shock proteins [50], and various other hypothetical proteins [51] are under study. The optimal combination of novel MAP antigens is to be included in the future for detection of MAP-specific IGRAs to diagnose the JD in all stages of infection. However, in the present study, two MAP-specific antigens HSP65 and 35 kDa, and mixture of antigens were compared with crude PPDj in IGRAs, indicating that both antigens were potent in detecting MAP infection in the skin reactor cattle calf. The MAP culture is currently regarded as definitive JD diagnostic method, MAP is extremely fastidious organism and requires the longest incubation periods of all the mycobacteria cultured to date (6 to 16 weeks) [52, 53]. Fecal culture does not give true indication of JD infection status because the harsh chemical decontamination of fecal samples is required to suppress growth of competitive microorganism, which can reduce the sensitivity of fecal MAP culture. Thus, even in the present study, the fecal samples collected from the cattle calf turned negative might be due to the harsh decontamination process adopted to remove the complex microflora to prevent the overgrowth during the extended incubation period. Even though the fecal samples turned negative in the present study, the MAP was isolated in the mesenteric lymph node and rectum samples were collected during postmortem confirming the MAP infection in the case study. Biopsy of sections of intestine and regional lymph nodes for culture and histopathology may provide a definitive diagnosis, suggesting MAP isolation. In the current studies, histopathological lesion in the mesentric lymph nodes possibly indicated the MAP infection [9, 10, 54].

The indirect economic losses are caused by premature culling of the young calves' results in unrealized future income, loss of genetic value of progeny, and reputation of the farm. Thus, the effective disease control programs depend on early diagnosis of infection which requires optimized combination IFN-γ inducers antigen and through understanding of management sources of infection, routes of transmission, and MAP pathogenesis by veterinarians as economic importance of this disease is usually downplayed by farm owners. The present study indicated that calves can acquire infection from infected mothers or farm environment and remain undetected. Improved tests like IGRA using recombinant antigens and ELISPOT are likely to add value in establishing a confirmative diagnosis in suspected MAP infection.

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