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## Serodiagnostic potential of his 17.8 and his 20.8 kDa proteins in ELISA and LFA in paratuberculosis

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**Abstract**

Johne's disease or paratuberculosis is a chronic progressive granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *Paratuberculosis* (Map). It causes significant economic losses in livestock industries worldwide. In the United States, Map-positive herds experience economic losses of almost US \$100 per cow and a disease cost of US \$200 to 250 million annually. ELISA based assay is ideal and sensitive for the screening of sera from infected animals, but it requires equipment and skilled technicians. The development of rapid, simple and specific assay is crucial for detection and surveillance of Map infection in animals. Therefore in the present study, the recombinant proteins from *E. coli* harboring pQE862 C+ and pQE1637 N+ clones respectively encoding 17.8 kDa and 20.8 kDa proteins were purified with the aim of developing ELISA based test as well as lateral flow assay for detection of paratuberculosis in infected animals. The recombinant antigens purified by affinity chromatography were used for conjugation with gold nanoparticles, spotted as test line and purified recombinant antibody raised in guinea pigs used as control line on nitrocellulose membrane. Five positive and five negative commercial ELISA kit tested samples were used in this experiment. Both antigens found efficacious in ELISA and LFA for diagnosis of paratuberculosis. Though a large number of samples are required for proper validation yet Overall, the developed test will be useful to farmers in future to devise suitable control programme and also to prevent the spread of paratuberculosis infection in other animals.

**Keywords:** LFA, 17.8, 20.8, kDa, *Mycobacterium avium subsp. paratuberculosis*, ELISA

**Introduction**

Johne's disease or paratuberculosis is bacterial zoonotic disease widespread throughout the world caused by an intracellular pathogen *Mycobacterium avium* subspecies *paratuberculosis* (Map). It is a chronic enteric disease of domestic and wild ruminants including primates (Chiodini, 1984).

Paratuberculosis is an enzootic disease on the B list of the Office des International Epizootes (OIE). The zoonotic potential of Map is not fully understood. It was shown that the Map is more readily isolated from Chron's disease (CD) patients (Waddle *et al.*, 2015). It is estimated that 68.0% of US dairy herds are infected with JD, costing between \$200 million to \$1.5 billion per year to the dairy industry (Sohal *et al.*, 2015) [13]. In India using protoplasmic antigens from 'Bison Type' genotype of Map, Singh *et al.*, (2008) [14] reported seroprevalence of paratuberculosis in buffaloes, 28.67%, cattle, 29.86%, and 23.63%, in Northern India. Cumulative seroprevalence in Uttar Pradesh and Punjab states of Northern India was 32.9% and 23.0%, respectively.

ELISA is a more sensitive and specific test for serum antibodies than any other serological tests and it has been widely used for screening herds. Different ELISAs such as unabsorbed, absorbed, lipoarabinomannan (LAM) and affinity purified antigen (APA) have been developed (Manning and Collins, 2001) [10]. Later Speer *et al.*, (2006) [15] reported ELISA using whole bacilli treated with formaldehyde (WELISA) as well as a surface antigen, obtained by treatment of Map bacilli with formaldehyde and sonication (SELISA). These appeared superior to commercial ELISA used for the diagnosis of JD.

Eda *et al.* (2006) [8] demonstrated an ethanol vortex enzyme-linked immunosorbent assay (EVELISA) based on surface antigens of Map. It had diagnostic specificity and sensitivity rates of 96.9 and 100%, respectively. EVELISA identified 96.6% of the low fecal shedders and 100% of the midlevel and high-level shedders. The JTC ELISA based on using antigens secreted in early to mid-log phase had a higher diagnostic sensitivity low-level fecal shedders of Map (40%). The JTC ELISA worked effectively both on serum and milk samples for detection of cattle with subclinical Map infections (Shin *et al.*, 2008) [11].

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A flow cytometric method (FCM) has also been used to diagnose early as well as chronic Johne's disease by testing serum for antibodies against *Mycobacterium avium subsp. paratuberculosis* (Eda *et al.*, 2005) [9]. The FCM had diagnostic sensitivity and specificity levels greater than 95% and was capable of detecting Johne's disease 6 to 44 months earlier than the fecal test and 17 to 67 months earlier than commercial ELISA.

Bannantine *et al.* (2008) [2, 3] in another study, developed a partial protein array using 92 Map specific recombinant proteins using the sera from experimentally infected cattle and evaluated the humoral immune response on 70th, 194th and 321st-day post infection. The studies revealed that the proteins encoded by Map 0862, Map 1087 and Map 1204 elicited strong humoral immune responses as early on 70th-day post infection. However, the antibody titer against the protein encoded by Map 0862 reduced with the progression of time. Moreover, antibodies against these three proteins were detected in the sera from naturally infected animals.

Gold nanoparticle-based lateral-flow assays have been developed for rapid serodiagnosis of many diseases. Gold nanoparticles coupled with antibody to LipL32 protein commonly found in pathogenic *Leptospira* stain was used in LFA for detection of leptospirosis (Chirathawom *et al.*, 2014) [6]. Therefore, the present study was envisaged to address the critical importance of B cell epitopic regions from two Map specific immunodominant antigens secreted at early stages in paratuberculosis-infected animals. The proteins from locus tag Map 862 and Map 1637 (Map complete genome accession no. 016958) encoding 17.8 kDa and 20.8 kDa proteins have been expressed in *E. coli* earlier in the laboratory. The C terminal region of 483 bp from Map 862 and N terminal region of 546 bp from Map 1637 used in the present study encodes for 17.8 kDa and 20.8 kDa proteins respectively.

Therefore In this study, the recombinant proteins from *E. coli* harboring pQE862 C+ and pQE1637 N+ clones respectively encoding 17.8 kDa and 20.8 kDa proteins were purified with the aim of developing ELISA based test as well as lateral flow assay for detection of paratuberculosis in infected animals.

### Material and Methods

Guinea pigs were obtained from the Laboratory Animal Resource Section, IVRI, Izatnagar.

The *E. coli* strain (M15, pREP4) from Qiagen (USA), Plasmid pQE30 contains an ampicillin resistance marker was used as expression vector supplied by Qiagen, HRP-labelled goat anti-guinea pig IgG (Sigma), Coomassie brilliant blue G 250 (Sigma, USA), complete Freund's adjuvant (FCA) and Freund's incomplete adjuvant (IFA), ampicillin and kanamycin (Sigma, USA), vertical slab mini apparatus (Atto, Japan), rabbit anti-sheep HRP conjugate (Sigma), 96 wells ELISA plates (Grenier, Germany).

### Expression and purification of the recombinant his 17.8 and his 20.8 kDa proteins

The colonies from two Map specific clones ( Map 483 C+ and Map 862 N+ were chosen from the master plates and inoculated in 4 ml of LB broth ampicillin (100 µg/ml) and kanamycin (25 µg/ml) and incubated overnight at 37 °C with constant shaking at 2000 rpm for 12- 16 h. Two hundred milliliters of fresh LB broth containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) was then inoculated with 1000 µl of the overnight culture and further incubated at 37 °C with constant shaking until mid-log phase to reach an OD600 of

0.6. One milliliter of the culture was collected from each flask, centrifuge and the pellets were kept at -20 °C as an uninduced control. To the rest of the cultures, IPTG was added at a final concentration of 1mM and kept at 37 °C, with constant shaking at 200 rpm. One milliliter of the culture was collected from each flask and the pellets were kept at -20 °C to check the expression. The cultures from both the flasks were collected and centrifuged at 6,000 rpm for 20 minutes. The pellets were kept at -20 °C till further use. Expression of the His-tagged proteins was analyzed by SDS-PAGE analysis. The poly-histidine (6x-his) tagged 17.8 kDa and 20.8 kDa proteins were purified under denaturing conditions by nickel chelating affinity column chromatography as per Qiagen protocol. Eluates obtained were checked on SDS-PAGE along with the protein flow through & wash through. Fractions showing single band on SDS-PAGE were pooled and stored at -20 °C.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The induced & uninduced pellets collected from grown *E. coli* cultures harboring plasmids pQE 862 C+ and pQE 1637 N+ respectively and *E. coli* M15 cells were resuspended in 50 µl of SDS-PAGE sample buffer (2X). The volume was made to 100 µl by addition of distilled water to each sample. The pellets were boiled for 10 min in a water bath in order to lyse and denature the bacterial proteins. Samples were then spun at 12,000rpm to pellet the cellular debris, following which 20 µl of the supernatant was loaded into wells, SDS-PAGE was carried out on a vertical slab mini apparatus (Atto, Japan) as described by Laemmli (1970). 12% separating and 5% stacking polyacrylamide gel containing 0.1% SDS was used. Electrophoresis was carried out in 1X Tris-glycine electrode buffer (pH 8.3) at 100V for 2-3 hr till the bromophenol blue migrated to the bottom of the gel. The gels were stained for 2hr with Coomassie brilliant blue G 250 (Sigma, USA) and destained in a solution containing methanol and acetic acid. The fractions containing the uninduced, induced and purified his 17.8 and 20.8 proteins and M 15 cells were resolved. The size of the proteins was determined against the protein molecular weight marker.

### Raising of hyperimmune serum against his17.8 and his 20.8 kDa proteins

Six 6- 8weeks old guinea pigs were immunized to raise an antibody against his17.8 and his 20.8 kDa proteins respectively. Before injecting recombinant proteins blood was withdrawn from each guinea pigs and serum was separated & stored at -200C for future use. Each guinea pigs were injected subcutaneously with 250 µg of the immunogen each in 0.5 ml PBS (pH 7.4) along with equal volumes of complete Freund's adjuvant (FCA) making oil in water emulsion. The guinea pigs were subsequently injected with three boosters, first with 200 µg & two subsequent boosters with 100 µg protein each mixed with Freund's incomplete adjuvant (IFA) at 21st, 28th, 35th-day intervals. One week after the fourth injection, the guinea pig was bled and antisera were separated and stored in aliquots at -20 °C for further use.

### Western Blot analysis of his 17.8 and his20.8 kDa proteins

Reactivity of the recombinant proteins was determined by western blot analysis. For western blotting, the proteins from the gels were transferred to nitrocellulose membranes (0.45 µM) using semi-dry electroblotting (Atto, Tokyo, Japan) at

100 volts for 2 hours. The blots were blocked with 2% skimmed milk powder in PBS-T buffer (PBS containing 0.1% Tween-20) for 2 hr at room temperature. After washing with PBS-T buffer three times, the membranes were incubated for 2 hr at 37 °C with sheep serum. The serore activity of his 17.8 and his 20.8 protein with the clinically positive sheep serum (1:50 dilution) was recognized using a 1:500 dilution of rabbit anti-sheep HRP conjugate (Sigma). Antigens were visualized on the blots by incubation with 0.02% diaminobenzidine suspended in PBS containing 0.03% hydrogen peroxide. The sera from a healthy sheep were taken as negative control.

#### **Standardization of his 17.8 and his 20.8 kDa protein-based indirect ELISA**

Indirect ELISA was standardized using the checker-board titration method. Different concentrations of the recombinant proteins (his 17.8 & his 20.8) 10 µg -100 µg /ml in carbonate-bicarbonate buffer (pH 9.6) were made and 50 µl of each concentration was coated into 96 wells ELISA plates (Grenier, Germany). The plates were incubated overnight at 4°C. The wells were washed thrice with PBS-Tween 20 (PBS-T) and blocked with 5% skim milk powder in PBS-T for 2hr at 37°C to remove excess un-absorbed antigen. Then, 1:5000 serial dilution of serum in 100 µl volume of PBS-T were added in duplicate and incubated at 37°C for one hour. The plates were washed thrice with PBS-T for 3min at each wash. Conjugate anti-guinea pig IgG HRPO at a dilution of 1: 2,000 in 100 µl volume was added to each well and the plates were incubated for 1hr at 37°C. The plates were then washed three times with PBS-T and color was developed with 100 µl of 10 mg/ml OPD with 10 µl of 30% H<sub>2</sub>O<sub>2</sub> in substrate buffer. After sufficient color development, the reaction was stopped by the addition of 50 µl 2.5N H<sub>2</sub>SO<sub>4</sub> and the plates were read at 490nm in an ELISA reader (Tecan, Austria).

During the process of standardization the controls viz., antigen control, serum control, and conjugate control were also used. For paratuberculosis positive & negative sera 1:250 & 1:500 dilutions were added to each well in blocking buffer.

#### **Purification of IgG from hyperimmune sera**

IgG from hyperimmune sera raised in guinea pigs against his 17.8 & his 20.8 kDa proteins were purified using Protein A column purification kit (Sigma) as per the manufacturer instructions. IgG concentration was measured by nanodrop and was 3.72mg/ml and 3.75mg/ml for against his 17.8 & his 20.8 kDa proteins respectively. Confirmation of the purified IgG was resolved and analyzed on SDS-PAGE.

#### **Preparation of colloidal gold for conjugation (pH adjustment)**

The 30nm colloid gold nanoparticle was pelleted at 8000 rpm for 10 minutes and again re-suspended in an equal volume of 1 mM 3-[N-Morpholino]-2-hydroxypropane sulfonic acid (MOPS) buffer, (pH7) for conjugation with recombinant antigens (17.8 kDa & 20.8 kDa). Gold particles were then stored at 4°C for further use.

#### **Determination of minimal protecting amount of his 17.8 and 20.8 kDa proteins**

The minimal protecting amount is the minimal amount of protein required to protect the GNP's against salt agglomeration and pH changes during the experimental procedure. Briefly 25, 50 75 and 100 µg recombinant protein/ml of pH adjusted gold was added to each tube and

incubated for 10 min in room temperature. Then 100 µl of 10% NaCl in distilled water was added to each tube. The tubes that contain enough protein to stabilize the gold colloids maintain the red color even in the presence of electrolytes while those tubes which contain less protein, flocculation takes procedure instability of gold colloids and the color changes from red to dark purple/black. The 10% excess concentration of protein in the tube before the last tubes which retain the red color is chosen for further experimental procedures.

#### **The lateral- flow assay format**

The lateral-flow assay made consists of a detection strip made of nitrocellulose that is flanked at one end by a reagent pad that contains the dried colloidal gold-labeled recombinant protein his 17.8 and his 20.8 kDa protein respectively for each strip and at the other end by an absorption pad. A sample application pad, in turn, flanks the reagent pad. The recombinant antigens were deposited as a 1-mm narrow line onto the nitrocellulose strip as test line. Anti his 17.8 and anti his 20.8 kDa proteins antibody was deposited in a second line parallel to the test line to function as a reagent control. The composite was backed by support and was cut into 5-mm-wide test strips.

#### **Procedure for testing the purified hyperimmune sera with the lateral flow device**

The lateral flow assay device was first tested with the purified IgG from hyperimmune sera. The purified IgG was diluted with PBS at 0,100,200,500 and 1000 dilution then tested with the strip. Previously prepared 4µl of the recombinant-antigen-gold-nanoparticles conjugate was pipetted to the conjugate pad and 8µl of diluted purified IgG was dispensed in to sample pad, kept it for few seconds for reaction. After some time, 20 µl of PBS-T was flown to the strip. After reaching the binding complexes to the test line they must bind to his 17.8 and his 20.8 kDa proteins.

#### **Testing of the field sera with ELISA**

The clinical sera from the paratuberculosis infected and uninfected sheep were tested according to the protocol mentioned earlier. The plate was coated with 200ng recombinant antigen and incubated at 37 °C on an ELISA plate shaker for 1 hr. The plate was washed three times with washing buffer (PBS- T). 50 µl of diluted 1:2000 in blocking buffer rabbit anti-sheep HRPO conjugate to all the wells and the plate was incubated at 37 °C on an ELISA plate shaker for 1 hr. The plate was washed three times with washing buffer (PBS- T). 50 µl of freshly prepared chromogen substrate solution (OPD) to all the wells and the plate was incubated at 37 °C for 10 to 20 minutes in the dark till color develops. 50 µl of stopping solution was added to all wells and the plate was tapped gently. The absorbance of the colored solution of all wells was recorded at 492nm in an ELISA reader.

#### **Interpretation of ELISA results**

For the purpose of interpretation of results comparison of P & N values were used, where P stands for OD of unknown samples and N stand for OD of the negative sample. The value of N was calculated by taking the ODs of five known paratuberculosis negative serum samples. The samples showing the P/N ratio of  $\geq 2$  were considered as positive for paratuberculosis infection.

The test serum showing OD<sub>492</sub> values double or more than

double the mean of negative serum control was considered as positive for Map 483 and Map 1637 antibodies respectively.

#### Procedure for testing the field sera with the lateral flow assay

To test for anti-Map IgG antibodies in serum, 4  $\mu$ l GNPs conjugate applied on conjugation pad and 8  $\mu$ l the sera applied on sample pad. The sample fluid consists of phosphate-buffered saline containing 0.66 mg of bovine serum albumin per ml and 3% Tween during the assay, the antibodies present in the sample migrate with the buffer through the device and bind to the his 17.8 and his 20.8 gold conjugate respectively forming antibody-recombinant protein-gold complexes, which continued migrating through the NC membrane. In positive samples, most of the antibody-recombinant protein-gold complexes are retained by recombinant antigen in the NC test line producing a colored band. The excess his17.8 and his 20.8 gold conjugate with free recognizing epitopes are not retained in the test line and continue to migrate until reacting with anti his 17.8 and anti his 20.8 protein antibodies, which is present in the control line of the NC membrane, thus producing a second colored band. In negative samples recombinant his17.8 and his 20.8 gold conjugate not retained in the test line because the antibody-recombinant protein-gold complexes are not formed, thus producing a single colored band (control line). The assay was scored positive when distinct staining of the test line is observed. When no staining was observed the test scored negative.

#### Result and Discussion

*Mycobacterium avium* subsp. *paratuberculosis* (Map) is acid-fast slow growing the etiological agent of bovine paratuberculosis or Johne's disease. The disease is

characterized by several phases that include, besides the initial phase of infection, a subclinical asymptomatic stage dominated by a Th1 type immune response, which usually is not able to eliminate the infection due to bacterial mechanisms of evasion (Sohal *et al.*, 2008) [14], and then gradually replaced by a Th2 humoral immune response (Coussens 2001) [7]. Since the humoral response is not able to fight against intracellular infection, the symptoms in the clinical phase become evident with the appearance of granulomatous lesions in loco, intestinal disorders, and weight loss, culminating in the death of the infected animals (Beard *et al.*, 1999) [4]. The ELISA based assay is ideal and sensitive for a screening of sera from infected animals. But the test is not readily adapted for rapid pen-side testing because it requires equipment and technical expertise.

LFA technique is recognized as a promising diagnostic for rapid on-site testing due to its simple procedure, rapid operation, quick results low cost, and non-requirements for special skills or expensive equipment. A gold nanoparticle-based lateral flow assay was developed using sonicated antigens for rapid diagnosis of contagious agalactia in goats (Arun *et al.*, 2014) [1].

The grown *E. coli* cultures harboring plasmid pQE 862 C+ and pQE 1637 N+ respectively induced with 1 mM IPTG generated a prominent band of histidine-tagged protein (his 17.8 and his 20.8) on SDS-PAGE (Fig. 1a & 1b, lane 3 respectively). No such band was observed in IPTG induced *E. coli* M15 cells and uninduced *E. coli* M15 cells harboring recombinant plasmid pQE 862 C+ and pQE 1637 N+ respectively (Fig. 1a & 1b, lane 1 and 2 respectively).

The purified his17.8 and his 20.8 protein gave a monomeric band of his 17.8 and his 20.8 proteins respectively on 12% SDS-PAGE (Fig. 1a & 1b, lane 4 respectively).

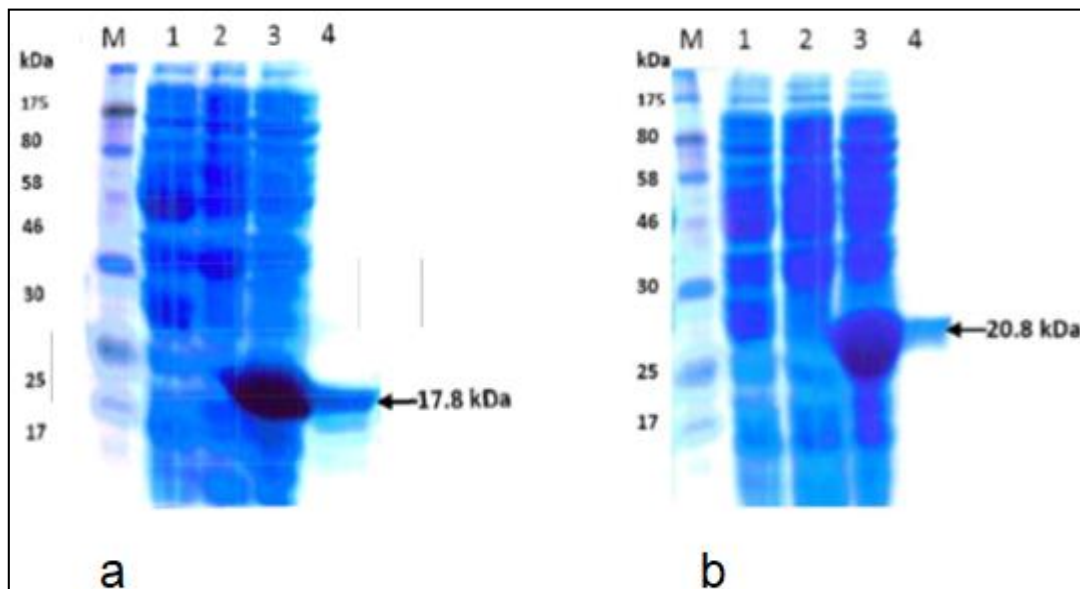
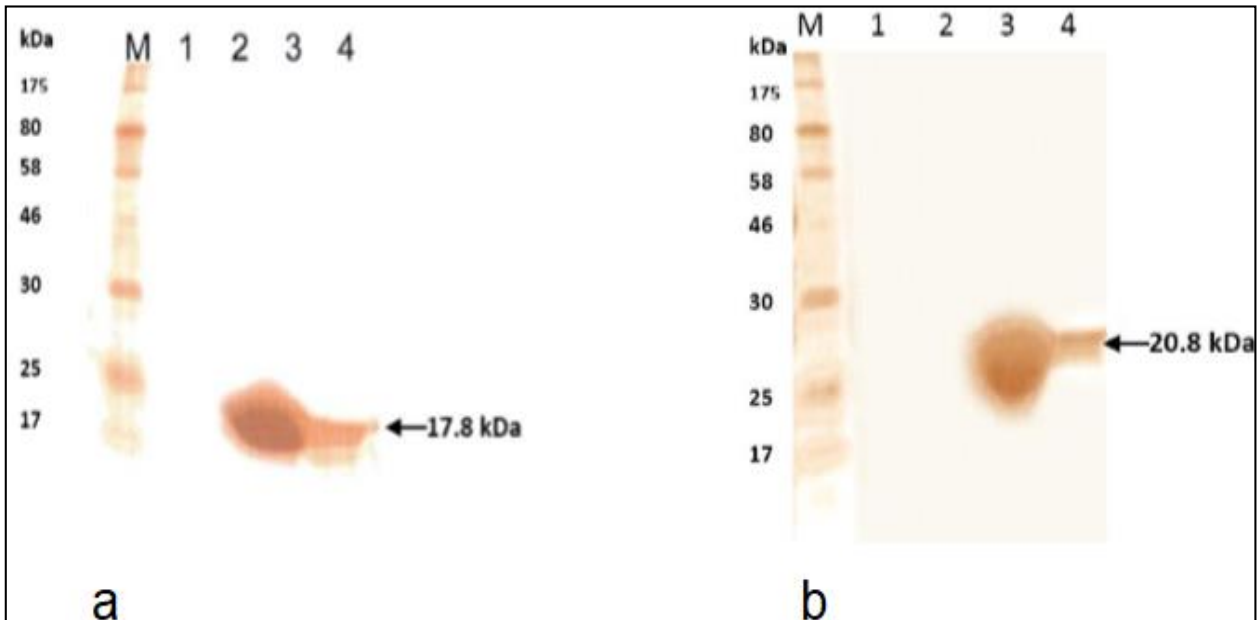


Fig 1: SDS PAGE analysis of expressed and purified his 17.8 and 20.8 kDa proteins.

The yield of the purified protein was found to be about 10 mg/l of cultures as determined by Nanodrop method.

Purified his 17.8 and his 20.8 kDa protein on western blot generated a sharp brown color band (Fig. 2a & 2b, lane 3 and 4

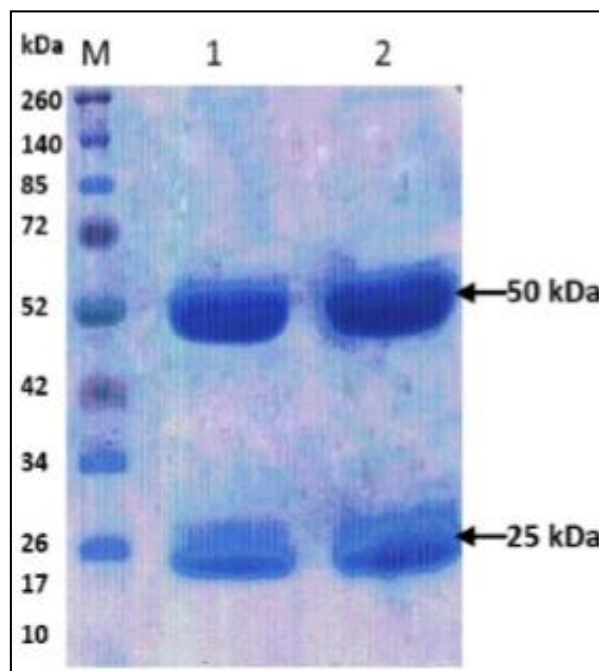
respectively). No reaction was observed in induced *E. coli* (M15) cells and uninduced *E. coli* (M15) cells harboring recombinant plasmid pQE 862 C+ and pQE 1637 N+ (Fig. 2a & 2b, lane 1 and lane 2 respectively).



**Fig 2:** Western blot analysis of expressed and purified his 17.8 and 20.8 kDa proteins.

Antibody IgG from hyperimmune sera against his17.8 and his 20.8 proteins were purified by protein A-based kit. SDS-

PAGE analysis revealed the presence of 50 kDa and 25 kDa band of IgG (Fig. 3, lane 1 and 2).

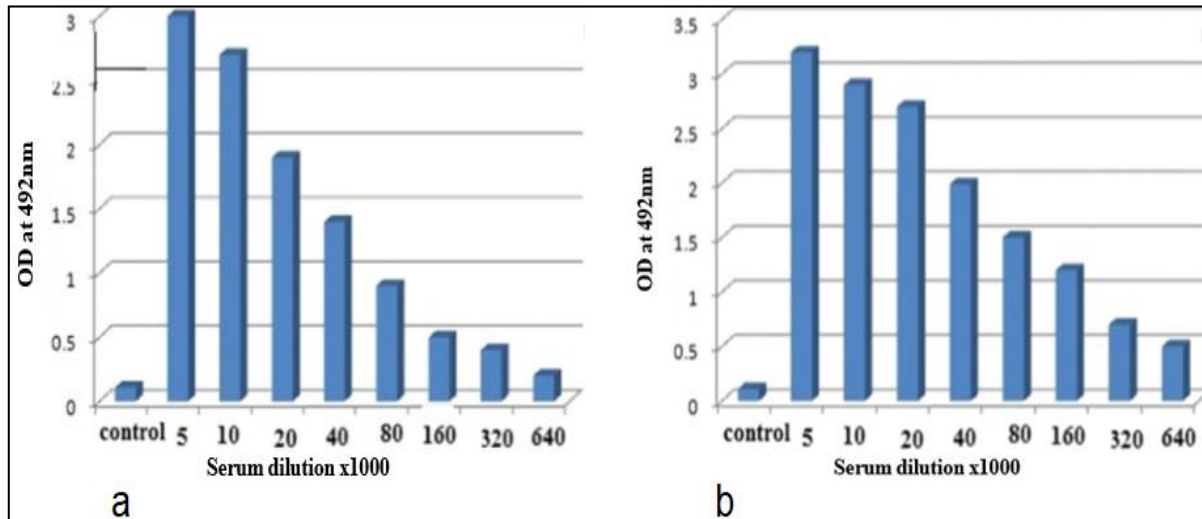


**Fig 3:** SDS Page analysis of purified antibody IgG from hyperimmune sera against his17.8 and his 20.8 kDa proteins.

The checkerboard titration was used to determine the optimum concentration of proteins (50, 100, 200 & 500 ng) per well and serum dilution for use in indirect ELISA. Antigen concentration of 100ng per well was found optimum which was detected the antibodies at higher dilutions. Further using 100ng antigen per well high titers of antibodies up to 6,40,000 dilution were observed in hyperimmune sera raised

in guinea pigs against his17.8 and his 20.8 proteins (Fig. 4a& 4b respectively), whereas no significant antibody titers were observed in control groups.

Further optimization of antigen in ELISA revealed that 100 ng of his17.8 and his 20.8 proteins detected the antibody consistently and resolved between positivity and negativity.

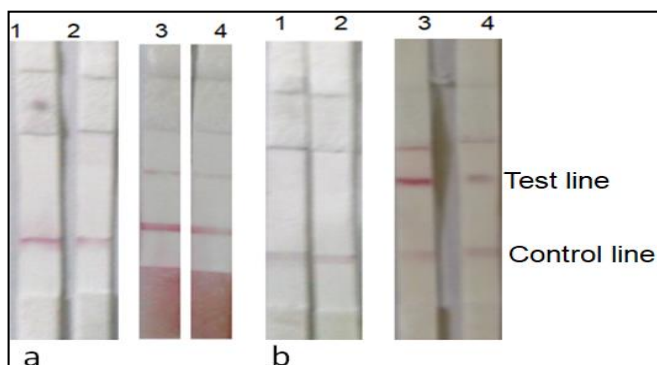


**Fig 4:** ELISA showing antibody titer of anti his 17.8 and anti his 20.8 kDa protein sera.

The serum dilutions up to (1:1200) were used by ELISA from sheep infected with paratuberculosis with his17.8 and his 20.8 kDa proteins revealed higher P/N value,  $\geq 2.0$  than that of sera obtained from healthy sheep.

In this study, a lateral flow assay platform was developed for rapid serodiagnosis of paratuberculosis in which recombinant antigens spotted on test line and recombinant IgG on the control line. Recombinant antigen coated gold nanoparticles as the detection reagent. For a positive serum sample, conjugate binds to the antibody forming a gold nanoparticle-recombinant- Antigen-antibody complex that binds to antigen immobilized on test line and forms a red color. The absence of a test line indicated a negative result. The excess GNP conjugates continue to move by capillary action and encountered a control line composed of recombinant IgG, a red line always appear at the control line as gold conjugate binds to recombinant IgG, regardless of the presence of specific antibodies in the sample.

Different dilution of hyperimmune sera was used with PBS buffer and tested with this assay. The result in this assay in case of control and hyperimmune samples were shown (Fig.5a, lane 1 & Fig.5 a, lane 3 respectively of his 17.8 protein) and (Fig. 5b, lane1 & Fig.5b, lane 3 of his 20.8 protein). Hyperimmune sera diluted upto 500 times shown a positive result. The undiluted clinical sera of affected sheep were tested with lateral flow assay. The in this assay in case of control and paratuberculosis infected samples (Fig.5a, lane 2 & Fig. 5a, lane 4 respectively of his 17.8 protein) and (Fig. 5b, lane 2 & Fig. 5b, lane 4 respectively of his 20.8 protein).



**Fig 5:** LFA using his17.8 and his 20.8 kDa proteins with hyperimmune and clinical sheep sera.

Commercial ELISA kit tested 5 positives and 5 negative samples are taken for comparison. Using his 17.8 kDa and his 20.8 kDa proteins, all the positive sera reacted with both ELISA & LFA while negative sera were not reacted in both assays.

**Conclusion**

In the present study Map specific his 17.8 and his 20.8 kDa proteins were found to be efficacious both in ELISA and LFA for diagnosis of paratuberculosis. Although the test is less efficient in comparison to ELISA. It would be proved useful for screening of Map-infected animals in the field level at low cost. Though a Large number of samples are required for proper validation yet Overall, the developed test will be useful to farmers in future to devise suitable control programme and also to prevent the spread of paratuberculosis infection in other animals.

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**Ethical approval**

Compliance with ethical standards

**Conflict of interest**

The authors declare no conflict of interests.

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