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Rapid diagnosis of paratuberculosis by map recombinant fusion 24.4 kDa fusion protein based lateral flow assay

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Abstract
Paratuberculosis is a chronic progressive granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *Paratuberculosis* (Map). The disease 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 is also not readily adapted for rapid pen-side testing because 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, we use Map recombinant fusion 24.4 kDa protein generated in the laboratory by fusing the epitopic region of 300bp each from locus tag Map 0862 and tag Map 1637 to produce lateral flow assay (LFA). A total of 52 animals were screened by commercial kit followed by Map recombinant fusion 24.4 kDa protein based LFA. LFA showed 83.33% specificity and 70% sensitivity. Thus Map recombinant fusion 24.4 kDa protein based LFA in the present study will prove useful as a reagent in the rapid diagnosis of infected animals in the field condition.

Keywords: 24.4 kDa, *Mycobacterium avium* sub sp. *Paratuberculosis*, LFA

Introduction
Mycobacterium avium subsp. *paratuberculosis* (Map) is a zoonotic intracellular pathogen causing paratuberculosis (Johne's disease), chronic granulomatous enteritis of domestic and wild ruminants and primates (Manning and Collins, 2001) [15]. The organism is acid-fast, slow growing, fastidious bacterium requiring exogenous ferric mycobactin for its growth *in vitro* (Chiodini and Kruiningen, 1984) [4]. Clinically infected animals develop chronic treatment-resistant diarrhea, progressive weight loss, infertility, debilitation and eventually death. This organism has also been implicated/ associated with Crohn’s disease, a chronic multifactorial inflammatory bowel disease in humans (Abubakar *et al*., 2008) [1].

Johne’s disease is widespread throughout the world and causes substantial economic losses to the farmers and the dairy industry. Johne’s disease is considered as one of the most costly infectious diseases of the dairy herds (Hasonova *et al*., 2006) [8]. The annual economic impact on the dairy industry is estimated at $276 per cow per lactation for infected cows on a dairy (Raizman, 2009) [19]. Reportedly, the prevalence of Map infection in US dairy herds ranges from 20 to 40% and costs to the dairy industry are estimated at $220 million annually (Ott *et al*., 1999) [18].

Currently used diagnostic tests for Johne’s disease need to be improved due to deficiencies in their sensitivity and specificity. Problems of specificity are due to the high degree of similarity that exists between Map and environmental mycobacteria, especially the closely related *Mycobacterium avium* subsp. *avium* (Maa). The cell-mediated immune assay such as the intradermal (Johnin) test uses a complex and undefined Map derived protein preparation to stimulate an immune reaction in the host. The test is less specific, prone to be false-positive and associated with non-specific reactions due to cross-reactivity with similar proteins present in other mycobacteria (Jungersen *et al*., 2002) [10].

The ELISA based assay is ideal and sensitive for the screening of sera from infected animals. But the test is not readily adapted for rapid pen-side testing because it requires equipment and skilled technicians. Different ELISAs such as unabsorbed using a lipoarabinomannan based (LAM) antigen, absorbed ELISA based on protoplasmic antigen and affinity purified antigen (APA) have been developed (Manning and Collins, 2001; Rajkumar *et al*., 2001) [15-20].

Later an ELISA was reported using whole bacilli treated with formaldehyde (WELISA) as well as surface antigens obtained by treatment of Map bacilli with formaldehyde and sonication (SELISA). These appeared superior to commercial ELISA used for the diagnosis of JD (Speer *et al*., 2006) [21].
The development of rapid, simple and specific assay is crucial for detection and surveillance of Map infection in animals. Gold nanoparticles based immunoassays have been developed and applied increasingly as dipstick as well as lateral flow assays in various research fields such as for the detection of the pregnancy hormone, bacterial, viral and parasitic pathogens (Huang, 2006; Karthik *et al*., 2011; Victoria *et al*., 2011; Bermudez *et al*., 2012) [9, 11, 22, 3]. Lateral flow assay has several advantages over traditional serological tests, such as the simplicity of the procedure, low cost, and ease of use in the field.
With the completion of the *Mycobacterium avium*sub sp. *paratuberculosis* genome, it was possible to identify sequences that were present in *Mycobacterium avium* subsp. *paratuberculosis* but not found in the other mycobacteria (Liu, 2005; Leroy *et al*., 2005) [14, 12]. The immune response of proteins from locus tag Map 0862 (1083 bp) and tag Map 1637 (1440 bp) from Map complete genome accession no. AE 016958 which encodes for 39.7 kDa and 53.2 kDa respectively were was studied in Map-infected cattle sera in ELISA based formats (Goswami *et al*., 2017; Leroy *et al*., 2009) [7, 9]. The further recombinant fusion protein was also generated in the laboratory by fusing the epitopic region of 300bp each from locus tag Map 0862 and tag Map 1637 encodes for 24.4 kDa protein by primer splice overlap technology. The aim of the present study was to analyze the diagnostic potential of Map recombinant fusion 24.4 kDa protein in a Lateral flow immunoassays based format for rapid diagnosis of the infected animals in field condition.

Material and Method

Colloidal gold 20 nm was purchased from Sigma-Aldrich Corp. (USA), Protein G from Sigma-Aldrich Corp. (USA), NC membranes containing strips were purchased from advanced micro devices Pvt. Ltd., Ambala Cantt, India), Nanotrac Wave (Microtrac) to measure the size of protein G coated gold nanoparticles, PBS (pH 7.4), Expressed, purified and characterized Map recombinant fusion 24.4kDa protein from our lab, complete Freund's adjuvant (FCA) and Freund's incomplete adjuvant (IFA) (DIFCO), antisera raised in guinea pig Serum samples, BSA from Sigma-Aldrich Corp. (USA).
A total of 52 samples including 20 bovine paratuberculosis-positives and 20 sheep paratuberculosis-positive serum to determine the sensitivity of the assay. Healthy 10 sheep and 2 bovine samples were used to determine the specificity of the assay. These all samples were already tested in our lab by using a commercial ELISA kit.

Raising of hyperimmune serum against Map recombinant fusion 24.4 kDa protein

Two 8 weeks old guinea pigs were immunized to raise the antibody against Map recombinant fusion 24.4 kDa protein. Before injecting recombinant protein blood was withdrawn from each guinea pigs and serum was separated & stored at-20 °C 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 pigs were bled and antisera were separated and stored in aliquots at -20 °C for further use.

Salt agglomeration test for determining the minimal protective amount

Briefly, 50, 75 and 100 µg recombinant protein G/ml was added to 250 µl of pH 7.2 adjusted gold to each tube and incubated for 30 min at 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. The 10% excess concentration of protein in the tube before the last tubes which retain the red color is chosen for further experimental procedures.

Conjugation of recombinant protein G to colloidal gold (pH adjustment method)

The 20 nm size colloid gold nanoparticles (GNPs) were pelleted at 7500 rpm for 30 minutes and again resuspended in equal volume of 0.01M PBS (pH7.2). Map recombinant fusion 24.4 kDa protein @ 60 µg/ml of GNPs was added and incubated for 30 min at RT. The conjugated GNPs were further incubated with 2% BSA with intermittent shaking for 30 min at RT. Again, gold nanoparticle was pelleted at 7500 rpm for 30 minutes and after centrifugation, conjugated GNPs were resuspended in 0.01M PBS (pH7.2) that contained 0.02% BSA and 0.025% Tween 20. The conjugates ready to use were then stored at 4 ºC for further use.

Confirmation of conjugation by agarose gel electrophoresis and Zeta analyzer

Modification of the gold surface with proteins generally results in a change in the surface charge, which can be seen by an altered migration pattern (direction or migration distance) in agarose gel electrophoresis. 1% the agarose gel was prepared and allowed to cool, 15 µl of control and recombinant protein G conjugated nanoparticles were pipped in each well. The gel was run at 50 volts for 45 min. Because surface coating of protein increases the size of the nanoparticles thereby slowing down their electrophoretic speed when compared to unmodified gold nanoparticles. Size of control and recombinant protein G conjugated nanoparticles was also measured by Zeta analyzer.

Development of Lateral- flow assay

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 protein G and at the other end by an absorption pad. A sample application pad, in turn, flanks the reagent pad. Map recombinant fusion 24.4 kDa protein (1mg/ml) was deposited as a 1-mm narrow line onto the nitrocellulose strip as test line. Protein G mg/ml) 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 ready to use 5-mm-wide test strips. These ready to use 5-mm-wide test strips were sealed in plastic bag and stored at 4 ºC.

Procedure for testing the lateral flow device

For standardization of the lateral flow assay, hyperimmune serum raised in guinea pig was used as a positive and uninoculated guinea pig sera as a negative control. The hyperimmune sera were diluted with PBS at 0,1:100,1:200 and 1:500 dilution then tested with the strip. The commercial ELISA kit tested serum samples were used up to dilutions of 0, 1:10,1:20,1:50 for testing the efficacy of LFA. Previously prepared 5µl of the recombinant-protein G-gold-nanoparticles conjugate was pipetted to the conjugate pad and 20µl of diluted hyperimmune sera was dispensed in to sample pad, kept it for few seconds for reaction. After 5 seconds, 100 µl of PBS-T was flown to the strip. The assembly was placed horizontally for 3-5 min to observe the result. Similarly, clinical sera were also tested. In positive samples, most of the antibody-protein G-gold complexes are retained by Map recombinant fusion 24.4kDa protein in the NC test line producing a colored band. The excess of Map recombinant fusion 24.4kDa protein gold conjugate with free recognizing epitopes is not retained in the test line and continues to migrate until reacting with protein G present at the control line of the NC membrane, thus producing a second colored band. In negative samples antibody-protein G-gold complexes not retained in the test line 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.

Statistical analysis

The Kappa statistic was calculated using the standard formula (Cohen, 1960) [6]. The kappa value was interpreted as described previously (Viera and Garrett, 2005) [23]. Confidence intervals for sensitivity and specificity were calculated using a free online tool at the website: https://www.medcalc.org/calc/diagnostic_test.php.

Result and Discussion

Mycobacterium avium subsp. *paratuberculosis* (Map) is the etiological agent of bovine paratuberculosis or Johne's disease. However, despite several years of development, currently available diagnostic tests still lack performance to be used in control programs. 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 the rapid diagnosis of contagious agalactia in goats (Arun *et al*., 2014) [2]. Recently, it has been effectively used for bacterial disease diagnosis in both veterinary and medical field (Meng *et al*., 2014; Chirathaworn *et al*., 2014; Niu *et al*., 2014) [16, 5, 17].

Therefore, in the present study, a Map recombinant fusion protein that was already generated in the laboratory by fusing the epitopic region of 300bp each from locus tag Map 0862 and tag Map 1637 encodes for 24.4 kDa protein by primer splice overlap technology was used for analyzing its diagnostic potential in Lateral flow immunoassays format.

Minimal protection amount of protein G was found 50 µg/ml (Fig.1).

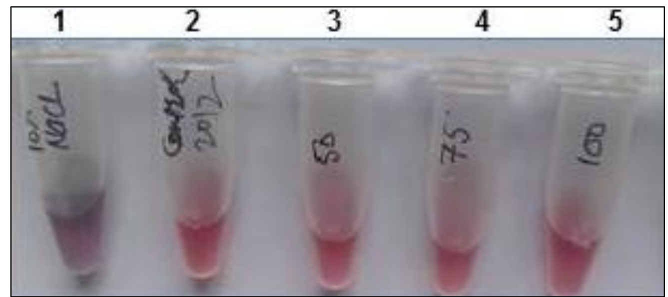

Fig 1: Salt agglomeration test for determining the minimal protective amount of protein G the concentration of protein G added per ml of gold nanoparticles in tube no. (1) 0, (2) control, (3) 50, (4) 75, (5) 100 µg. Tube 1 showed salt agglomeration while tube 2-5 revealed no salt agglomeration

Zeta analyzer showed protein G coated GNPs were of 46.4 nm in diameter as compared to 24.4nm of control GNPs (Fig. 2) as well as conjugated GNPs were slowly migrated in the agarose gel as compared to control GNPS (Fig. 3).

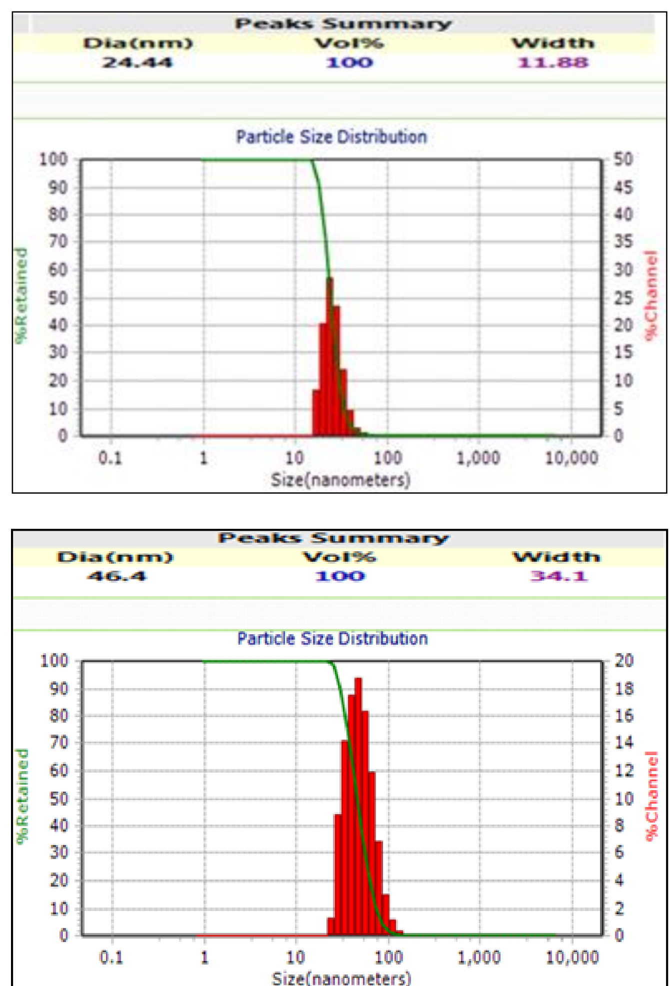

Fig 2: Zeta analysis of control (Left) and protein G coated GNPs (Right)

Fig 3: Agarose gel electrophoresis analysis of migration pattern of protein G conjugated gold nanoparticles

1) Control gold nanoparticles whereas
2-5) Protein G conjugated gold nanoparticles

Different dilutions of hyperimmune and clinical sera were used with the PBS buffer and tested with this assay. GNPs conjugate binds to the antibody forming a gold nanoparticle protein G-antibody complex that binds to antigen immobilized on test line and forms a red colored line. An absence of the test line indicated a negative result. The excess GNP conjugates continue to move by capillary action and encountered a control line composed of protein G, a red line always appear at the control line as gold conjugate binds to protein G, regardless of the presence of specific antibodies in the sample. Hyperimmune sera diluted up to 200 times and clinical sera diluted up to 20 dilutions showed a positive result. The result in this assay in case of control, hyperimmune samples as well as healthy and clinical samples were showed (Fig. 4 and Fig. 5 respectively).

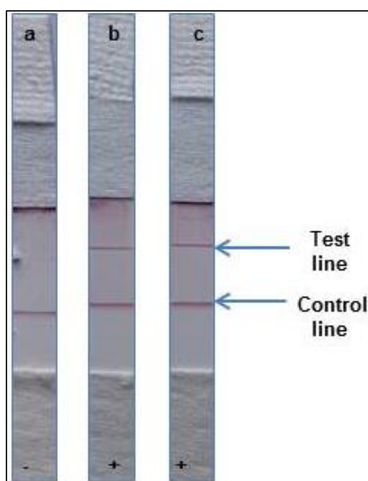

Fig 4: Lateral flow assay with recombinant fusion 24.4 kDa protein on test line and protein G on the control line respectively

a) Control guinea pig serum showed a red colored line only on the control zone, whereas.
b-c) Hyperimmune guinea pig serum showed red colored lines on both test and control zones.

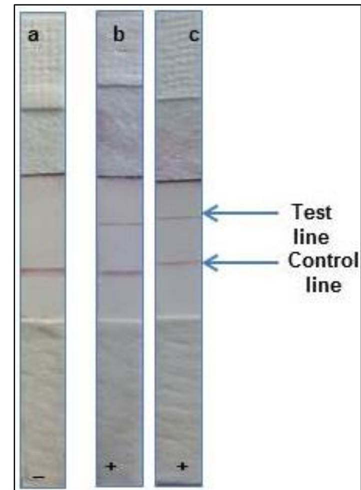

Fig 5: Lateral flow assay with recombinant fusion 24.4 kDa protein on test line and protein G on control line respectively

a) Negative sheep serum showed a red colored line only on the control zone, whereas.
b) Positive sheep serum showed red colored lines on both test and control zones and.
c) Positive bovine serum showed red colored lines on both test and control zones.

A mix of 52 commercial kit tested sera sample having P/N value, >2.0 than that of sera obtained from the healthy animal was analyzed to determine sensitivity and specificity of LFA. At the dilution of 1:20, the concentration of Map-specific antibodies was comparatively higher than other mycobacterial antibodies, causing competitive inhibition between the specific and non-specific antibodies for Map antigen. This resulted in a more intense pink line onto the nitrocellulose, compared to the poor visible line at higher dilutions. Therefore, the detection limit of the assay was found to be 1:20 which is specific for *Mycobacterium avium* sub sp. *paratuberculosis*.
Screening of 52 serum samples (bovine and sheep) by Map recombinant fusion 24.4 kDa protein LFA revealed 22 and 30 negative and positive reactors, respectively (Table 1). The result demonstrated that the specificity was 83.33% (95% CI 51.59-97.91%), while the sensitivity was 70% (95% CI 53.47-83.44%), relative to infection status as indicated by the commercial kit test. The LFA-based test based on Map recombinant fusion 24.4 kDa protein showed a kappa value agreement with commercial ELISA kit (k = 0.5) (Viera and Garrett, 2005) [23]. Therefore, the Map recombinant fusion 24.4 kDa protein is a good candidate for an LFA based serological test that is easy to perform and cheap to produce.

Table 1: Sensitivity and specificity of LFA

Test	**Disease**				**Total**
	Presentn		**Absentn**		
Positive	True Positive	a = 28	False Positive	c = 2	a + c = 30
Negative	False Negative	b = 12	True Negative	d = 10	b + d = 22
Total		a + b = 40		c + d = 12	
Statistic		Formula	Value	95% CI	
Sensitivity		a/ a + b	70.00%	53.47 to 83.44%	
Specificity		d/ c + d	83.33%	51.59 to 97.91%	
Kappa agreement = 0.5					

Conclusion

The present study is a leap towards the development of rapid, sensitive and simple LFA test for the detection of paratuberculosis infections in animals. Map specific recombinant fusion 24.4 kDa protein based LFA was found to be efficacious for rapid diagnosis of paratuberculosis. Although the test is less efficient in comparison to ELISA. However, it would be proved useful for screening of Map-infected animals in the field level at a low cost. Overall, the developed test will be useful to farmers in the future to devise a 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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