Journal of Microbiological Methods 177 (2020) 106024

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Gold nanoparticle based immunochromatographic biosensor for rapid diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection using recombinant protein

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ARTICLE INFO

Keywords:
Gold nanoparticles
Paratuberculosis
Lateral flow assay
MAP
Recombinant protein
44 kDa

ABSTRACT

Highly infectious and obvious withstand ability of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) to environment as well as lack of on-site field diagnostic methods notably hampers the paratuberculosis (PTB) control. The existing intricacy, time-consuming and complicated diagnostic methods of PTB accentuate the development of novel and easy-to-perform on-site test. A gold nanoparticle (GNP) based lateral-flow assay (LFA) using MAP recombinant protein (44 kDa) has been developed for sensitive and specific detection of PTB in field conditions. The diagnostic sensitivity and specificity of the LFA for MAP specific antibodies was found approximately 84.2% and 83.3% in comparison to indirect enzyme-linked immunosorbent assay. Consequently, the newly developed GNP based LFA offers on-site and cost-effective method for the prompt diagnosis of PTB and precludes the time-consuming laboratory screening.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) implicated in chronic and contagious paratuberculosis (PTB) infection in ruminants as well as captive and free-ranging wild animals accounted for 200 million dollar loss in dairy sector of the US (Losinger, 2006). The clinical complications *viz.* granulomatous enteritis, persistent diarrhoea, progressive weight loss and debilitation frequently observed during PTB infection (Manning and Collins, 2001). Because of the global presence of PTB, it is frequent screened during International trade consequence to the mass culling of positive animals (Fecteau, 2018). Prevalence of PTB was maximum in African continent (53%) in comparison to other continents *i.e.* Europe (20%), Australia (6.8%), North America (16.9%), South America (18.3%) and India (23.3%) (Agrawal et al., 2019). On the basis of restriction fragment length polymorphisms (RFLP) analysis, MAP has been characterized into two main host-adapted types: type I or S strains found in sheep and type II or C strains found in cattle (de Juan et al., 2006). Young one's are highly susceptible as the maximum transmission of MAP occurs through milk (Steuer et al., 2020).

The diagnosis of disease is complicated and challenging in preclinical stage (*i.e.* infected animals do not exemplify any symptom of disease at an early stage) (Sergeant, 2003). At present, routine diagnosis of PTB is made at several laboratories by enzyme-linked immunosorbent assay (ELISA), skin testing for delayed type hypersensitivity, bacterial isolation, PCR and reverse transcriptase PCR (RT-PCR) (Reichel et al., 1999; Clark Jr et al., 2006; Shin et al., 2008; Weber et al., 2008; Thakur et al., 2019; Kalis et al., 2003; Cook et al., 2010;Eamens et al., 2000; Brey et al., 2006; Cook and Britt, 2007; Slana et al., 2008; Jaravata et al., 2006; Sonawane and Tripathi, 2019; Herthnek and Bölske, 2006). However, most of these diagnostic methods require the availability of a dedicated laboratory facility,

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https://doi.org/10.1016/j.mimet.2020.106024
Received 8 July 2020; Received in revised form 28 July 2020; Accepted 3 August 2020
Available online 12 August 2020

highly trained laboratory personnel, stable reagents and multistep sample handling or preparation. In particular, MAP isolation requires well-established laboratory facility, which can be difficult and expensive to maintain, besides requiring 5 weeks to 6 months for the appearance of primary colonies on solid media. PCR test is based on actual DNA of organism, which is irrelevant in case of MAP, as sometime; there is no manifestation of disease despite organism present in body.

A rapid and easy-to-perform test, which would allow for on-site diagnosis to be made in the case of a suspected disease would circumvent issues associated with the transportation of samples to the laboratory and would be especially useful for a faster diagnosis in areas where the disease is endemic. When the disease occurs in a remote area which is poorly connected with road and railways, the requirement for a pen-side diagnostic test that is quick and easy to perform, devoid of sophisticated equipment or *per se* laboratory expertise will be highly valuable. For these reasons, a recombinant protein based lateral-flow assay (LFA) for the detection of PTB specific antibodies might be a key to overcome the issues associated with other assays and become an effective tools to diagnose PTB. The detection of PTB specific antibodies by direct application of clinical sample from animals of an infected farm may reduce the chances of diagnostic error arising from nonspecific reactions. The assay may be used to quickly diagnose the disease and could be an effective tool in controlling PTB.

2. Materials and methods

The MAP2963 recombinant protein (44 kDa) expression clone and the hyperimmune serum raised in guinea pig against MAP2963 recombinant protein (44 kDa) were gifted by Dr. P.P. Goswami (Head, Division of Veterinary Biotechnology, IVRI, Izatnagar, Bareilly (U.P.) India.

2.1. Production and purification of MAP recombinant protein (44 kDa)

The MAP recombinant protein (44 kDa) expression clone inoculated in 4 ml of Luria Bertani (LB) broth having ampicillin (100 μg/ml) and kanamycin (25 μg/ml) followed by overnight (O/N) incubation at 37 °C. The 1 ml of this O/N culture was subsequently inoculated in 200 ml of LB broth containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml) and further O/N incubated at 37 °C in shaking incubator. One ml of the culture collected from each flask was used as uninduced control. Isopropylthiogalactoside (IPTG) was added to the remaining culture at a final concentration of 1 mM and incubated at 37 °C for 6 h. The culture(s) were pelleted by centrifugation at 6000 rpm for 20 min and kept at −20 °C till further use. For the purification of the His-tagged protein, the pellets were collected and lysed with 5 volumes of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea: pH 8.0) by sonication at 12 μM amplitude for 4 cycles of 15 s each. Subsequently, the debris was removed by centrifugation at 12,000 rpm for 30 min and the supernatant was transferred to a clean tube. Then 1/4th of Ni-NTA slurry was added on the column (Qiagen) which was equilibrated with lysis buffer and kept on shaker for a period of 1 h for thorough mixing. Then lysate-resin mixture was loaded on the 10 ml column equilibrated with binding buffer (pH 8.0). The column was washed thrice with 2 ml of wash buffer (pH 6.3) to remove proteins and other cellular debris leaving His tagged recombinant proteins (44 kDa) conjugated to Ni-NTA slurry. The column was further equilibrated with 2 ml of elution buffers of pH 5.9 and pH 4.5 and the flow through from the column was collected in different tubes. The renaturation of the recombinant protein was performed by dialysis at 4 °C against PBS (pH 7.4) using a dialysis tube with a cut off molecular weight of 15 kDa (thermo scientific) in order to remove urea and bring the protein in its native form. Dialysis of this eluted denatured recombinant protein (in 8 M urea) was carried out against decreasing molar concentration of urea as 6 M, 4 M, 2 M and PBS buffer (pH 7.4) at 4 °C. After renaturation recombinant protein was quantified by Bradford assay (Bradford, 1976) and analysed on SDS-PAGE to determine the purity.

2.2. Purification and concentration of IgG from hyperimmune serum raised against MAP recombinant protein (44 kDa)

The IgG from hyper immune sera was purified using Protein A column by affinity purification method (Himedia antibody purification kit). After purification, the purified IgG against MAP recombinant protein (44 kDa) was concentrated by Amicon ultra 0.5 centrifugal filter device having a cut off filter of 10 kDa molecular weight followed by analysis on SDS-PAGE gel. Immunoreactivity of MAP 2963 antigen and its purified hyper immune sera was also tested with dot blot which in turn reflected specificity. The quantification of purified hyper immune sera against MAP 2963 antigen was also done by Bradford (Bradford, 1976) assay.

2.3. Synthesis and characterization of gold nanoparticles

The gold nanoparticles (GNPs) used in this study were synthesized by sodium citrate method as per the standard protocol (Turkevich et al., 1951; Verma et al., 2014). The concentrated 50 mM solution of auric chloride was prepared by dissolving 1% tetrachloroauric acid in 50 ml distilled water. From this concentrated solution, 200 μl was taken and make final volume of 50 ml in MQ water to prepare 0.2 mM auric chloride solution. Prepare 1.5% solution of sodium citrate *i.e.* 30 mg in 2 ml MQ water as a reducing agent. Keep the gold solution containing flask on hot plate for 10–15 min along with magnetic stirrer and gradually add the sodium citrate solution. The solution was boiled for another 10 min and stirred vigorously for the next 15 min. Once the colloidal gold suspension reached the room temperature, the final volume was adjusted to 50 ml using HPLC graded water and was stored at 4 °C for further use. The adsorption spectrum of the synthesized gold nanoparticles was recorded at 450 nm to 600 nm by UV–visible spectrophotometer (SpectraMax, M5, molecular devices, USA). Zeta study using zeta sizer nano-ZS (Malvern instrument, UK) was conducted to determine particle size and their distribution. The varying concentrates solution (0.1 to 0.2 mM) of auric chloride consequence to varied size of GNPs. The effect of size of GNPs on the performance of LFA was also taken into an account.

2.4. Conjugation of secondary antibody with GNP

The conjugation of GNP and secondary antibody is a pH dependent process and relies on its physical properties. For conjugation, the secondary antibody (anti-guinea pig /anti-bovine IgG) adjusted to a pH 7.5 by mixing up with 50 mM Potassium dihydrogen phosphate (KH_2PO_4, loba chemie, India) and final concentration of 50 μg/ml was prepared. The 100 μl of this secondary antibody solution was made up to final volume of 1000 μl with synthesized GNPs solution and further incubated at the room temperature for 30 min. After incubation, 1% polyethylene glycol (PEG 20000, MP Biomedicals, France) and 10% bovine serum albumin (BSA, Amresco, USA) solution were added at the rate of 50 μl and 100 μl per ml of GNP suspension respectively. After being stirred for another 15 min, the coupled gold solution was centrifuged at 13000 RPM for 30 min at 4 °C temperature (Eppendorf centrifuge 5804R, Germany) and the resulting supernatant was discarded in order to remove unbound secondary antibody. The pellet formed was then sonicated in an ultrasonic bath (Ultrasonicator, Imaco) and 1 ml of preserving solution was added in order to resuspend the pellet. The suspension was centrifuged again and the final pellet was sonicated and suspended in 50 μl of preserving solution by vortexing. This secondary antibody derivatized GNPs conjugate was stored in a refrigerator at 4 °C.

2.5. Spotting of test and control lines on nitrocellulose membrane

The expressed MAP recombinant protein (44 kDa) and protein A (Merck, India) were spotted on the nitrocellulose membrane as the test and control lines respectively. Approximately 2 μl/cm of protein was applied on the nitrocellulose membrane using an Easy printer (MDI, LPM03, India). The membrane was dried at room temperature and stored at 4 °C.

2.6. Construction of lateral-flow device (LFD)

The sample pad, conjugate pad and the absorption pad (MDI, India) were assembled over nitrocellulose membrane laminate (MDI, India) with 1 mm overlap. The assembled laminates were cut into strips of 4 mm width. Then, the individual strips were carefully inserted into the plastic cassette having windows.

2.7. Test principle and procedure

The LFA is an immunochromatographic assay using MAP recombinant protein (44 kDa) and gold conjugated secondary antibody (anti-guinea pig or anti-bovine IgG) to detect MAP specific antibodies. MAP specific antibodies present in the sample binds to the secondary antibody conjugated GNP and form anti-guinea pig/antibovine (secondary) gold conjugates-MAP specific antibody complex and then this complex captured by the recombinant protein (44 kDa) resulting in a red coloured band in the test line. The remaining unbound secondary antibody conjugated GNPs move further through pores of nitrocellulose membrane and binds with the protein A resulting in red colour band on control line. The appearances of two bands are indicator of a positive test result. Single colour band in the control line indicates for a negative test result as the protein A on control line will bind the gold conjugate with both positive and negative samples and ensures correct test performance. Appearance of only test line or no line will indicate an invalid test result. (Fig. 1).

2.8. Standardization and validation of LFA

The running buffer (PBS-T, pH 7.5) as negative control and purified IgG against recombinant protein (44 kDa) as positive control were used in the standardization of LFA. The minimum concentration of MAP 2963 specific antibody, which made the colour of test line to appear along with a strong red band at control line, was evaluated by serial 2 folds dilution of hyperimmune serum (2.03 mg/ml) up to 1:2048 dilutions in order to determine the lower detection limit of visual immunochromatographic strip test. The developed LFA was validated by accessing 31 non- hemolysed serum samples collected from bovine of dairy farms in Bareilly having the history of progressive emaciation, profuse diarrohea and sub-mandibular oedema. The LFA was also compared with ELISA in terms of sensitivity, specificity and positive predictive value (PPV).

2.9. Statistical analysis

Lateral flow assay results were compared with ELISA using kappa statistics. The data were analysed in GraphPad Prism 8 to test the agreement between these two assays as described by Landis and Koch (1977).

3. Result

3.1. Characterization of bare and conjugated gold nanoparticles (GNPs) by UV- visible spectrophotometry and zeta sizer

`The absorption spectrum of synthesized bare 0.2 mM GNPs was measured from 450 nm to 600 nm and maximum absorbance (λ_{max}) found at 520 nm was 0.599 (Fig. 2A). Similarly, the GNPs conjugated secondary antibody (anti-guinea pig/anti-bovine) absorption spectra were also measured from 450 nm to 600 nm and compared with bare GNPs absorption spectra, indicated the shifting of λmax from 520 nm to 522 nm (Fig. 2B and C). With an increase in the particle size of GNPs, the shifts in absorption peak towards higher wavelength was observed due to Surface Plasmon Resonance. Variable particle size of the GNPs leads to the colour variation which can be visualized through naked eyes as the absorption spectrum of colloidal GNPs fall in the visible region. Zeta sizing was done to detect particle size and its distribution in bare and conjugated GNPs. It was observed that most of the bare and conjugated GNPs are in the range of 10–30 nm and 25–45 nm in size having an average diameter of 18 nm and 36 nm respectively. It was also seen that few gold nanoparticles after conjugation clumped together indicates a high peak in 100–110 nm range which might be due to change in surface potential (Fig. 3A and B).

3.2. TEM images of bare and conjugated gold nanoparticles

The size, shape and distribution of the bare and conjugated GNPs have been determined by transmissible electron microscopy (TEM). The TEM was done on Phillips CM10 at AIIMS, New Delhi. The average diameter of GNPs solution was found in the range of 10–30 nm with a few particles of higher and lower size distribution. The TEM images showed that gold colloid is in monodispersion state, this is because of negatively charged layer of citrates ions, which prevents the aggregation of the particles due to the effect of repulsion. Moreover, the TEM images show that most of the GNPs are spherical in shape which is required for conjugation of antibody. The effect of conjugation on the size of GNPs was also shown in this study. The size of bare GNPs and conjugated GNPs under TEM were revealed and it was found that most of the size of bare GNPs and conjugated GNPs particles were in the region of 10–30 nm and 35–46 nm respectively (Fig. 4A and B).

3.3. Quantification and spotting of purified recombinant protein

SDS-PAGE gel of recombinant protein (44 kDa) and purified IgG was done (Figs. 5 and 6). Then this recombinant protein (44 kDa) and concentration of MAP 2963 specific antibody in hyperimmune serum sample were 1.23 mg/ml and 2.03 mg/ml respectively. Later on this expressed recombinant protein (1.23 mg/ml) and protein A (1 mg/ml) were spotted on the nitrocellulose membrane as test and control lines respectively. The membrane was dried at room temperature and then sample pad, conjugate pad and adsorbent pad assembled over it.

3.4. Testing of chromatographic strip and standardization of strip

To standardize the working efficiency of LFA strip 4 μl of antibody gold conjugate (anti-guinea pig/anti-bovine) was applied over conjugate pad. Then running buffer (PBS-T, pH 7.5) was poured on sample pad drop by drop to carry the whole immune-complex of gold-antibody conjugate through nitrocellulose membrane results in development of red colour line on control line (Fig. 7A). In the next step 3 μl of purified IgG against recombinant protein (44 kDa) raised in guinea pig along with 4 μl of antibody gold conjugate (anti-guinea pig) was applied over conjugate pad, results in development of red colour line both on test and control line (Fig. 7B). Here the device was also checked successfully for testing clinically affected field bovine sera using anti-bovine IgG gold conjugate and is shown in Fig. 8A and B which were parallel tested with indirect ELISA and results were found in consonance. The positive test gave the coloured line or band at test line because of the relationship between analytes (recombinant protein (44 kDa) and purified IgG against recombinant protein (44 kDa)/clinical sera) and also on control line (anti-guinea pig/anti-bovine IgG and protein A) while in negative test colour seen in control line only.

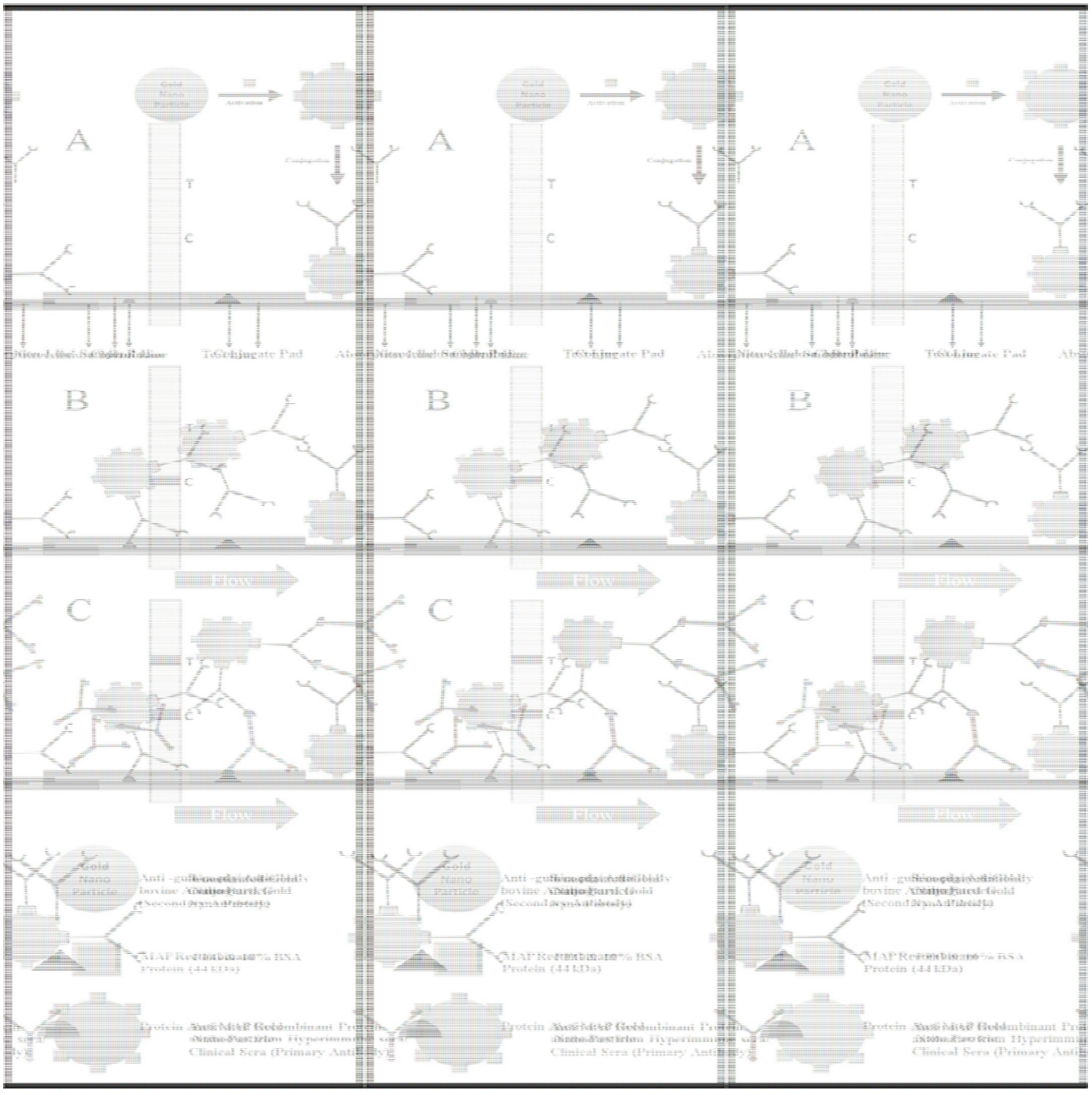

Fig. 1. Schematic diagram of lateral flow assay strip test (LFA) developed for *Mycobacterium avium* subspecies paratuberculosis (MAP) specific antibodies using MAP recombinant protein (44 kDa). **A**: Strip having anti-guinea pig/anti-bovine antibody (secondary) gold conjugates in the conjugate pad along with MAP recombinant protein (44 kDa) on the test line (T) and protein A on control line (C) in the nitrocellulose membrane. **B:** Strip without MAP specific antibodies on sample pad leads to binding of anti-guinea pig/anti-bovine antibody (secondary) gold conjugates with the protein A on control line (C) to form red colour visible band. **C:** Strip with MAP specific antibodies on sample pad binds with anti-guinea pig/anti-bovine antibody (secondary) gold conjugates on conjugate pad to form anti-guinea pig/anti-bovine antibody (secondary) gold conjugates- MAP specific antibodies complex and then this complex captured by the recombinant protein (44 kDa) on the test line (T), producing a red visible band while unbound anti-guinea pig/anti-bovine antibody (secondary) gold conjugates move through the nitrocellulose membrane and captured by protein A on the control line (C) to form another red colour visible band. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Diagnostic performance of LFA

The size of GNPs in the range of 10–40 nm was found to be appropriate for optimal performance of LFA. The GNPs of less than 10 nm size consequence to the formation of diffuse line on the both test and control line. Moreover, the GNPs larger than 40 nm flow slowly through the nitrocellulose membrane. Consequently, colour intensity of line was dim. The diagnostic performance of LFA in term of sensitivity was maximum at size of 18-20 nm.

The lower detection limit of developed LFA assay was 1.98 μg/ml (*i.e.* 1: 1024 dilution of hyperimmune serum). The diagnostic sensitivity and specificity of LFA and ELISA were assessed with 31 clinical serum

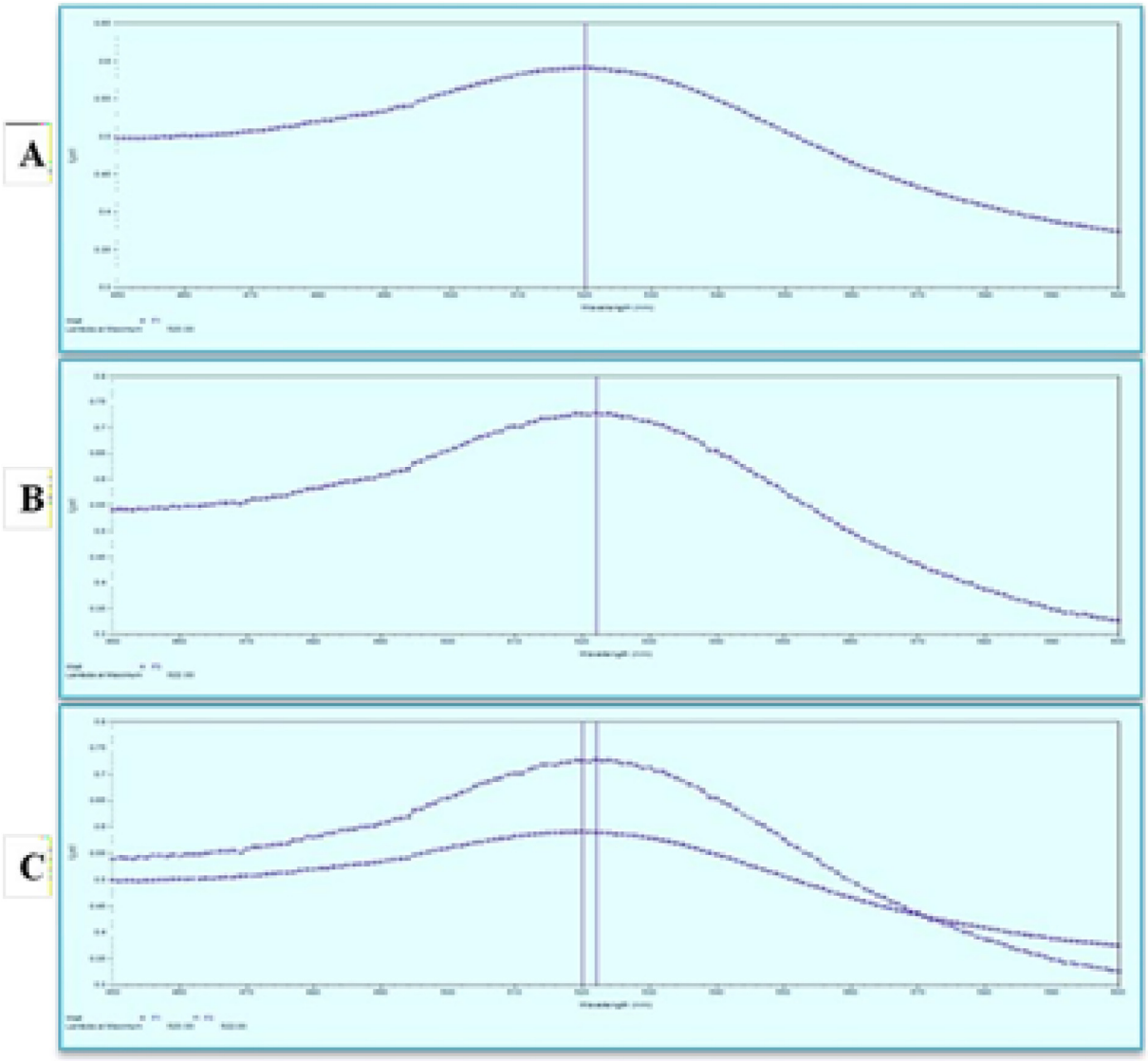

Fig. 2. Characterization of GNP's by UV/VIS Spectrophotometer. **A:** Characterization of 20 mM bare GNP's. **B:** Characterization of anti-guinea pig/anti-bovine IgG conjugated GNP's. **C:** Comparison of 20 mM bare and anti-guinea pig/anti-bovine IgG conjugated GNP's.

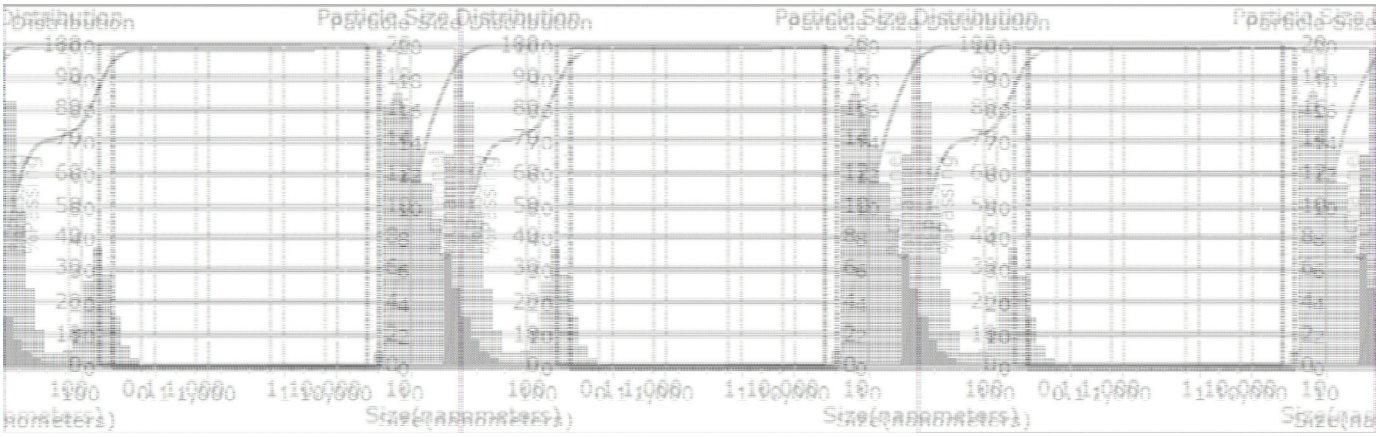

Fig. 3. Determination of particles size and distribution by zeta sizing **A:** A 20 mM bare gold nanoparticles. **B:** A 20 mM anti- guinea pig/ anti- bovine IgG conjugated gold nanoparticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

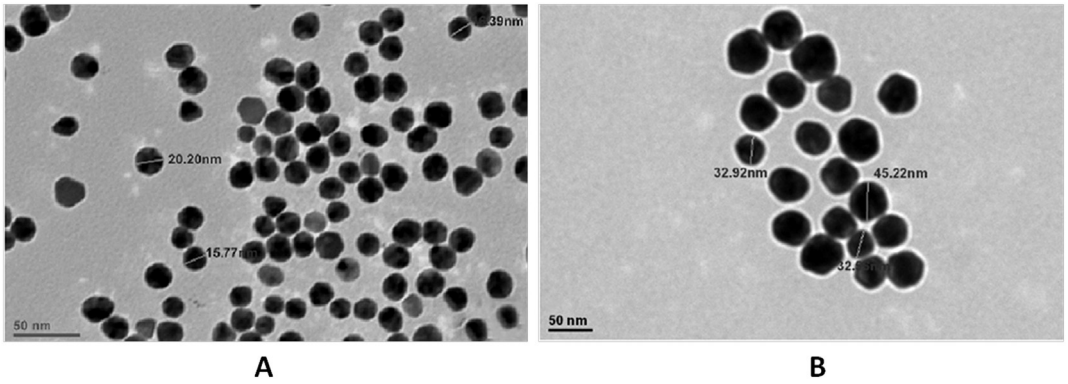

Fig. 4. TEM images to determine particles size and distribution. A: Size of 20 mM bare gold nanoparticles under TEM. B: Size of anti-guinea pig/anti-bovine IgG conjugated gold nanoparticle under TEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

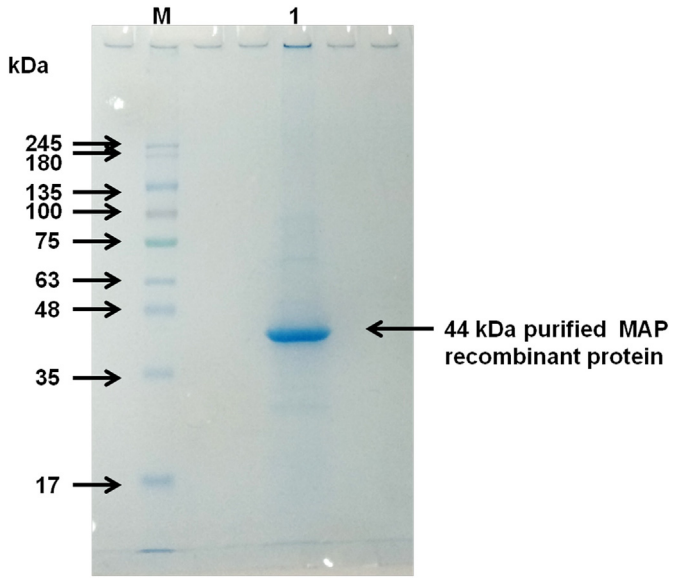

Fig. 5. SDS PAGE of purified 44 kDa MAP recombinant protein. Lane M: Marker prestained protein ladder (abcam 10 to 245 kDa): Lane 1 Purified 44 kDa MAP recombinant protein.

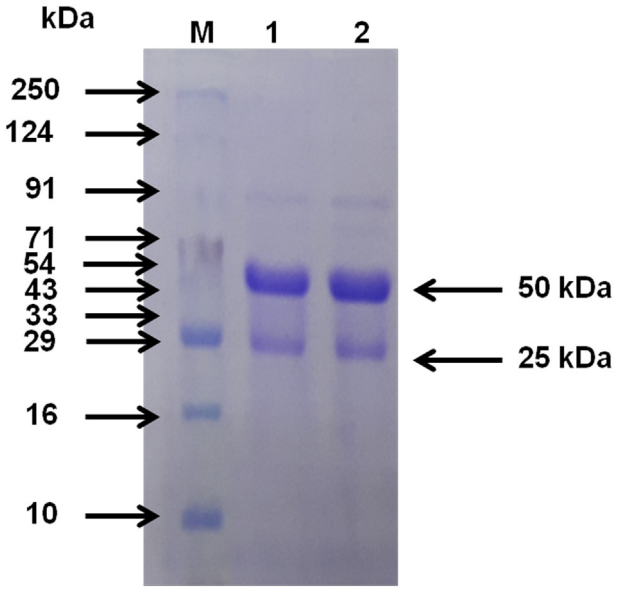

Fig. 6. SDS-PAGE analysis of purified IgG against MAP recombinant protein (44 kDa). Lane M: MARKER Molecular weight markers (PUREGENE 10 TO 250 kDa). Lane 1 and 2: Reduced/heated purified IgG against MAP.

samples. The comparative performance of LFA and ELISA are summarized in Table 1. The overall sensitivity, specificity and PPV of the LFA were 84.2%, 83.3% and 88.89% respectively in comparison to results obtained by ELISA (Table 1). Out of these 31 clinical samples, 19 (61.29%) were found positive for *M. paratuberculosis* in ELISA while 18 (58.06%) were found positive in LFA assay (Table 2). The results generated by the LFA were compared with optical density values derived from ELISA. A mean optical density value of ≥ 0.1 in the ELISA was taken as indicative of a positive reaction. Based on the above findings, the recombinant protein (44 kDa) based lateral flow assay should be used to test field samples in the future.

3.6. Statistical analysis of LFA with ELISA

Among the 31 samples, 18 were found positive by LFA whereas 19 were tested as positive by ELISA. A total of 12 were tested as negative in ELISA whereas 13 were negative by LFA (Table 2). The agreement of LFA with the ELISA is represented in Table 2. A substantial agreement between the two assays (LFA and ELISA) were observed with kappa value of 0.665 (at $P < 0.05$).

4. Discussion

`An indispensable part of any disease control strategy includes the deployment of diagnostic assays to rapidly confirm the initial clinical determination of infection. This is of particular importance because of the highly infectious nature of MAP, its fecal shedding during the prolong subclinical period or prior to any obvious clinical signs and remarkable withstand ability to environment. PTB differential diagnosis from parasitic infestations is a herculean task. Although bacteriological culture and its biochemical testing (Eamens et al., 2000), ELISA (Clark Jr et al., 2006; Shin et al., 2008; Weber et al., 2008), an antigenic tripeptide (derived from 'S' strain of MAP) based diagnosis (Bannantine et al., 2020) and RT-PCR (Herthnek and Bölske, 2006) are in use to detect MAP. However, the aforesaid intricacy and time-consuming methods require expertise personnel along with a well-equipped laboratory. Transportation of clinical samples from the suspected animals to the laboratory is also necessary and it is a major constraint in making rapid and accurate decisions for disease control. Researchers are examining alternative assay systems that allow more rapid confirmatory clinical diagnosis without need of a laboratory setting and may be performed at the pen side instead.

The LFA is a valid technology as it fulfills the criteria of any rapid assay. The technique permits rapid diagnosis, allowing time for the

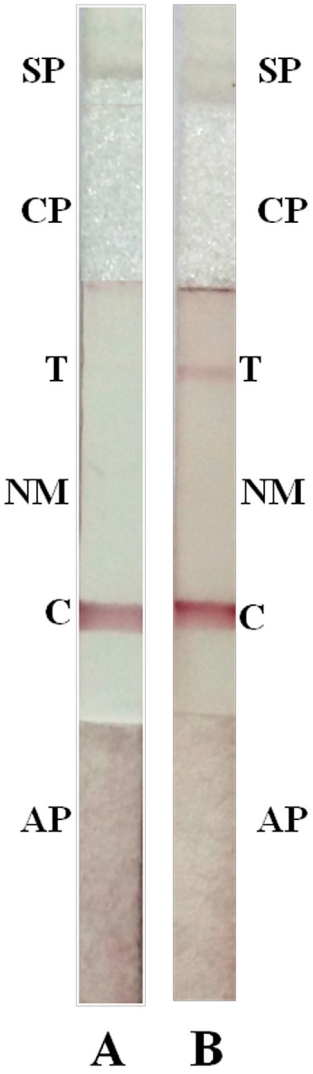

Fig. 7. Testing and standardization of chromatographic strip with anti-guinea pig antibody gold conjugate. **A**: Without / Mock purified IgG against recombinant protein (44 kDa) raise in guinea pig. **B:** With purified IgG against recombinant protein (44 kDa) raise in guinea pig. (SP-Sample Pad; CP-Conjugate Pad; *T*-Test line; NM-Nitrocellulose Membrane; C-Control line; AP-Absorption Pad). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

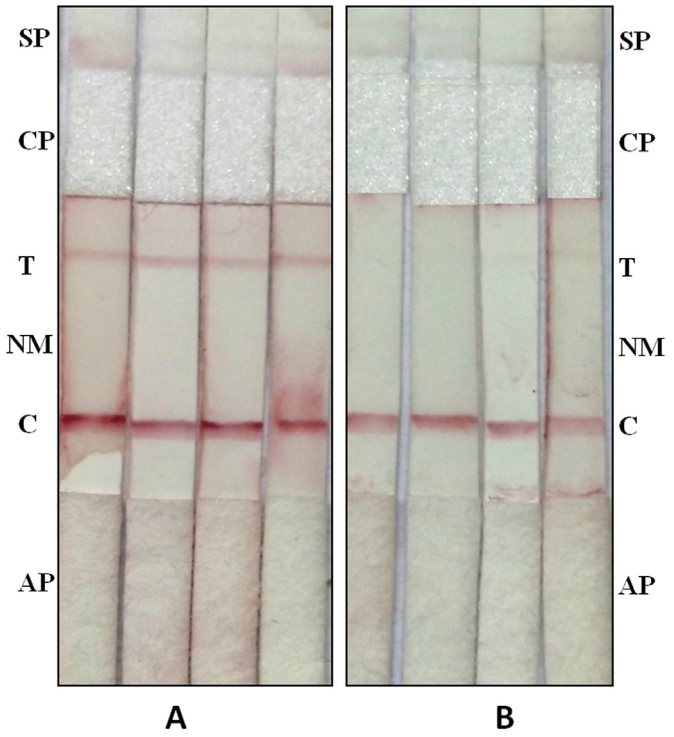

Fig. 8. Testing of chromatographic strip with anti-bovine antibody gold conjugate. **A:** With clinically affected field bovine sera positive against paratuberculosis. **B:** With field bovine sera negative against paratuberculosis. (SP-Sample Pad; CP-Conjugate Pad; T-Test line; NM-Nitrocellulose Membrane; C-Control line; AP-Absorption Pad. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Specificity and sensitivity measurement using predictive value of LFA and ELISA assays to diagnose MAP infection.

Lateral flow assay	ELISA			%
	Positive	Negative	Total	
Positive	16	2	18	88.89(PPV)
Negative	3	10	13	76.9 (NPV)
Total	19	12	31	
%	84.2	83.3	90.3	
	Sensitivity	Specificity	Coincidence rate	

early implementation of control measures to reduce the possibility of spread of PTB. The LFA has been developed widely to support clinical diagnosis of different diseases (Smits et al., 2008; Oem et al., 2009), and now we have added PTB to the list.

At the start of this study, the number of MAP specific recombinant protein had been reported (Banasure et al., 2001; Bannantine et al., 2003; Basagoudanavar et al., 2006) and we had to opt for the most reliable recombinant protein for the development of the test. To meet this need, number of recombinant protein were evaluated for the ability to react with antibody specific for PTB was evaluated by ELISA (Salgado et al., 2007). Following preliminary investigations on prototype LFA devices, one recombinant protein was selected for further investigation, with the results presented. The MAP 2963 protein is unique in nature and specific to *Mycobacterium avium* subsp. *paratuberculosis* (Paustian et al., 2004). Consequently, the cross-reactivity complications with other species of mycobacterium would not be observed.

The appropriate size-range of GNPs for optimal performance of LFA was 10 nm to 40 nm. Above and below this size range of GNPs, compromised performance of LFA was observed. The minimum variations in the size of GNPs improved the performance of LFA which supports previous findings that minimum variations in particle size refine the performance of diagnostic test and vaccine (Verma et al., 2014; Varshney et al., 2020). The developed LFA was compared with ELISA as the ELISA is the most specific and sensitive test for the detection of MAP specific serum antibodies in cattle. The ELISA is also suitable for MAP infection surveillance purpose or to detect the MAP infection free population (OIE, 2018).

Although the LFA did not react with all the samples, the sensitivity (84.2%) and specificity (83.3%) was similar to those of ELISA for the detection of PTB antibody. Previously, a recombinase polymerase amplification based LFA was developed against MAP showing sensitivity and specificity rate of 88.24% and 78.57% respectively in comparison to ELISA while 100% sensitivity and 97.63% specificity rate as compared to qPCR assay (Zhao et al., 2018). Similar kind of study was also performed earlier against *M. bovis,* in which LFA results indicated that the rate of sensitivity and specificity was 48.8% and 54.7% respectively, along with kappa value of 0.035 as compared to PCR tested samples (Bermúdez et al., 2012). Therefore, results demonstrated that a one- step LFA for the detection of PTB antibody in specimens from

Table 2
Comparison of ELISA and LFA assay results for MAP serum samples.

Dairy farms	No. of samples	ELISA			LFA		
		Positive	Negative	Positive %	Positive	Negative	Positive %
A	11	6	5	54.5	5	6	45.5
B	9	7	2	77.8	7	2	77.8
C	6	4	2	66.7	4	2	66.7
D	5	2	3	40.0	2	3	40.0
Total	31	19	12	61.29	18	13	58.06

suspected animals has successfully been developed. Test results can be available within 5–8 min of collection of a sample from an animal. However, LFA imparts qualitative or semi-quantitative results and inappropriate sample volume may account for compromised precision of test. The diagnostic performance of the LFA can be explored further with the involvement of more numbers of clinical samples. The practicality of the device is awaited from its deployment in cases of disease when confirmation of paratuberculosis is all that may be required to allow appropriate control measures to be implemented.

5. Conclusion

In this study, the lateral flow assay against *M. paratuberculosis* was developed and diagnosis suitability of the assay was determined by using ELISA tested *M. paratuberculosis* positive and negative serum samples ($n = 31$). The results showed that the detection efficiency of lateral flow assay strip test against *M. paratuberculosis* was utmost similar as that of ELISA, an intricacy method. The overall sensitivity, specificity and PPV of the LFA were 84.2%, 83.3% and 88.89% respectively in comparison to ELISA for *M. paratuberculosis*. Therefore MAP recombinant protein (44 kDa) based LFA would be an effective, easy to use, rapid, cost effective and on-site diagnostic test that can detect MAP specific antibodies in the stage of infection. Since the LFA is simple and easy to use, it can be adopted in underdeveloped countries where diagnostic facilities are limited. It can serve as an alternative means of sample testing or can be an addition to existing laboratory-based test procedures. It helps in the implementation of appropriate control measures and reduces the amount of unnecessary animal culling.

Authors' contributions

AA designed the study, carried out the experiment, analysed the data and wrote the manuscript. RV, AG, PK, MHK, RS, SK, and SKP assisted in research work, data curation and edited the manuscript. PS designed this study, analyzed the data and helped in research work.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgment

We are thankful to Dr. P. P. Goswami (Head, Division of Veterinary Biotechnology, IVRI, Izatnagar, Bareilly (U.P.) India for providing MAP Recombinant protein (44 kDa) and its hyperimmune sera raised in guinea pig and providing ELISA tested clinically infected MAP positive and negative serum samples. We are also thankful to IVRI for providing fund to this work.

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