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Recombinant fusion 24.4 kDa protein based in house ELISA for serodiagnosis of paratuberculosis

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Abstract
Paratuberculosis is a zoonotic chronic infectious enteric disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis*. ELISA based assay is ideal and sensitive for the screening of sera from infected animals. In this study, we use Map recombinant fusion 24.4 kDa protein developed by fusing the epitopic region of 300bp each from locus tag Map 0862 and locus tag Map 1637 to produce an in-house ELISA. The recombinant fusion 24.4 kDa was purified by single step chromatography using Ni-NTA Agarose. The recombinant fusion 24.4 kDa was reacted with anti- recombinant fusion 24.4 kDa antibodies as well as bovine and sheep sera infected with Map on western blot. A total of 106 animals samples were screened by commercial kit followed by Map recombinant fusion 24.4 kDa protein-based in-house ELISA. In-house ELISA using well-characterized sera (n=106) sample from sheep and bovine yielded a sensitivity of 85% and the specificity of 84.62%. The recombinant fusion 24.4 kDa used in the present study will prove useful as a diagnostic reagent in an in-house ELISA test.

Keywords: 24.4. kDa, *Mycobacterium avium* subsp. *paratuberculosi*s, ELISA

Introduction
Paratuberculosis (Johne's disease) 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) [4].

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 patients (Waddle *et al*., 2015) [19].

The incomplete understanding of the host immune response against this pathogen has hindered the development of an effective vaccine. The dairy industry incurs substantial economic losses due to reduced milk production, premature culling and reduced slaughter value (Stabel *et al*., 2014) [17].

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) [15].

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. Since the sequencing and analysis of the entire Map genome were obtained (Leroy *et al*., 2005) [12], several specific proteins have been detected in the genome of Map and the immunoreactivity of these proteins investigated (Hughes *et al*., 2008) [10]. Bannantine *et al*. (2008) [1] developed a spot protein array for initial antigen screening. EV-ELISA based on surface antigens of Map identified 96.6% of the low fecal shedders and 100% of the midlevel and high-level shedders (Eda *et al*., 2006) [7]. JTC ELISA based on antigens secreted in early to mid-log phase worked effectively both on serum and milk and showed a higher diagnostic sensitivity and detected low-level fecal shedders of Map (40%) (Shin *et al*., 2008) [14]. The studies from experimentally infected cattle on 70th,194th, and 321th-day post-infection 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 antibodies against these three proteins were detected in the sera from naturally infected animals. However, the antibody titer against the protein encoded by Map 0862 reduced with the progression of time (Bannantine *et al*., 2008) [2]. However, individual antigens are able to identify only a subset of paratuberculosis-infected animals. Then, a mix of antigens or a recombinant protein generated after fusing epitopic regions from the two individual proteins could be a good candidate for serological diagnosis.

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In the present study the proteins from locus tag Map 0862 and locus tag Map 1637 from Map complete genome accession no. AE 016958 which encodes for 39.7 kDa and 53.2 kDa respectively were expressed fully or in part in Gene Expression laboratory of the Veterinary Biotechnology Division (Accession No. JQ976670 & JQ976669). The immune response of these proteins was studied in Map-infected cattle sera in ELISA based formats (Goswami *et al.*, 2017) [8]. The further recombinant fusion protein was developed by fusing the epitopic region of 300bp each from locus tag Map 0862 and locus tag Map 1637 encoding for 24.4 kDa protein by primer splice overlap technology was used as the diagnostic reagent in in-house ELISA.

Material and method
Guinea pigs were obtained from the Laboratory Animal Resource Section, IVRI, Izatnagar. Standard prescribed guidelines for care and use of laboratory animals were followed during the experimentation with these animals. The. *E. coli* strain (M15) from Qiagen (USA), kanamycin (25 µg/mL) from Sigma. The plasmid pQE30 contains an ampicillin resistance marker was used as expression vector supplied by Qiagen, HRP-labelled goat anti-guinea pig IgG (Sigma), donkey anti-sheep HRP conjugate (Sigma), rabbit anti-bovine HRP conjugate (Sigma), ELISA reader (Tecan, Austria), 96 wells ELISA plates (Grenier, Germany).

Expression and purification of the recombinant protein and raising of antisera
Escherichia coli M 15 cells harboring the clone ppg 660 encoding 24.4 kDa protein were grown in LB medium containing ampicillin (75 µg / mL) and kanamycin (25 µg /mL), and induced with 1.0 mM IPTG for 4–6 h. The purification of recombinant 24.4 kDa fusion protein under denaturing conditions was carried out by one-step Ni–NTA (nickel–nitrilotriacetate) agarose (Qiagen) affinity chromatography. The protein was analyzed by SDS-PAGE. The protein Concentration of the protein was determined by nanodrop and aliquots were stored at –20 °C, until used. The hyperimmune sera against the recombinant fusion 24.4 kDa protein were raised in guinea pigs. 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.

Immunoreactivity of recombinant fusion 24.4 kDa protein analysis
Reactivity of the recombinant fusion 24.4 kDa protein with hyperimmune sera was determined by dot blot analysis and clinical sera by western blotting, for this, the proteins from the gels were transferred to nitrocellulose membranes (0.45 µM) using semi-dry electroblotting (Atto, Tokyo, Japan) at 150 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 ºC with positive sheep and bovine serum (1:50 dilution) separately. Following further washings, the blots were

incubated with a 1:500 dilution of donkey anti-sheep HRP conjugate (Sigma) and rabbit anti-bovine HRP conjugate (Sigma) respectively. 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 and bovine were taken as negative control.

Standardization of recombinant fusion 24.4 kDa protein based in-house ELISA
In-house ELISA was standardized using the checker-board titration method. Different concentrations of the recombinant fusion 24.4 kDa protein100 µg, 150 200 and 300 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 4ºC. The wells were washed thrice with PBS-Tween 20 (PBS-T) and blocked with 5% skim milk powder in PBS-T for 2hr at 37ºC 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 37ºC 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 37ºC. 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_2O_2 in substrate buffer. After sufficient color development, the reaction was stopped by the addition of 50 µl 2.5N H_2SO_4 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.

Testing of the field sera with in–house ELISA
The clinical sera from the paratuberculosis infected and uninfected sheep were tested according to the protocol mentioned earlier. The plate was coated with 150 ng recombinant antigen and incubated at 37 ºC 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 ºC 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 ºC 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.

Statistical analysis
The Kappa statistic was calculated using the standard formula (Cohen, 1960) [5]. The kappa value was interpreted as described previously (Viera and Garrett, 2005) [18]. 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. 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) [16], and then gradually replaced by a Th2 humoral immune response(Coussens 2001) [6]. As the humoral response is not able to fight against intracellular infection, the symptoms in the clinical phase becomes 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) [3], moreover the bacterium is characterized by having a slow growth rate in vitro (Sechi et al., 2005) [13] and is capable to carry on a persistent infection with a slow course that makes it difficult to detect the infection with early diagnosis and microbiological cultural methods, respectively. Most of the mechanisms underlying the development of disease caused by Map have been explained following those based on diseases triggered by *Mycobacterium tuberculosis* (MTB) and *Mycobacterium avium* subsp. *avium* (Kuehnel et al., 2001) [11]. Mycobacteria infect mainly macrophage cells (Hestvik et al., 2005) [9], for this reason, they evolved to develop defense mechanisms to face the hostile environment they encounter within the phagosomal compartment.

The fecal culture, the current gold standard test, detects Map during subclinical and clinical stages of infection, but it is time-consuming requiring more than 12 weeks of incubation and also labor intensive. PCR is sensitive and rapid, but applicable in bacterial shedders. Further, both fecal culture and PCR could not differentiate between the pass-through Map and Map colonization.

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 ELISA using Map 0862, Map 0865, Map 0852 and Map 2737 suggested for its use in antibody detection from infected animals (Bannantine et al., 2008) [2].

Therefore, in the present study, a Map recombinant fusion protein that was already developed 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 serodiagnostic potential in an in- house ELISA.

The grown *E. coli* cultures harboring plasmid ppg-660 clone induced with 1 mM IPTG generated a prominent band of histidine-tagged recombinant fusion 24.4-kDa protein on SDS-PAGE (Fig.1, lane 2). No such band was observed in IPTG induced *E. coli* M15 cells harboring plasmid ppg clone

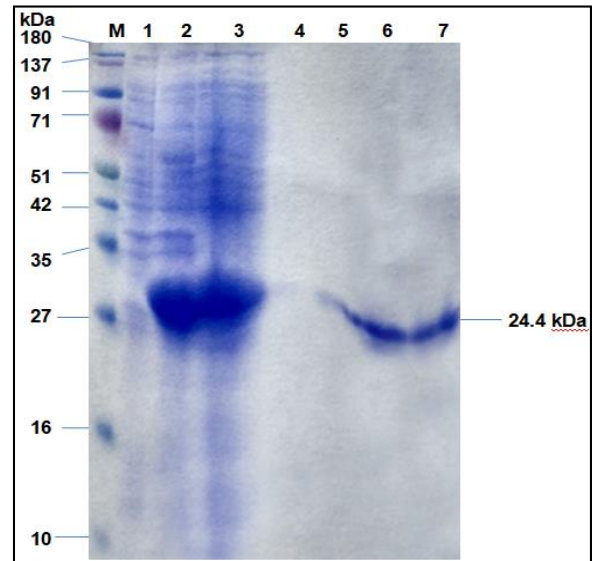660 encoding recombinant fusion 24.4-kDa protein (Fig. 1, lane 1).

Fig 1: SDS-PAGE analysis of recombinant fusion 24.4 kDa protein
1. Uninduced M-15 cells harboring ppg 660 clone
2. Induced M-15 cells harboring ppg 660 clone
3. Flow through
4. Wash
5. Elute 1 protein
6. Elute 2 protein
7. Dialysed protein

The purified recombinant fusion 24.4-kDa protein was eluted in buffer E (pH 4.5) gave a monomeric band of recombinant fusion 24.4-kDa protein on12% SDS-PAGE (Fig. 1, lane 5,6 respectively). The pooled fractions of the purified recombinant proteins were dialyzed by sequential reduction of the concentration urea & finally against PBS for proper refolding of the proteins (Fig.1, lane 7). The yield of the purified protein was found to be about 12 mg/l of cultures as determined by Nanodrop method

The pooled polyclonal sera raised against purified recombinant fusion 24.4-kDa protein in guinea pigs could bind to 4 hour IPTG induced *E. coli* (M15) cells harboring ppg 660 was purified protein on dot blot and generated a sharp brown color (Fig.2b). No reaction was observed in uninduced *E. coli* (M15) cells harboring recombinant plasmid ppg 660 (Fig.2a).

Fig 2: Dot bot analysis recombinant fusion 24.4 kDa protein
a. Immunoreactivity with 0-day serum of Guinea Pig.
b. Immunoreactivity with 35 days hyperimmune serum of Guinea Pig.

The purified recombinant fusion 24.4-kDa protein also recognized by the serum from a sheep and bovine naturally infected with paratuberculosis showed brown color spot on

western blot assay (Fig. 3 left side and Fig. 3 right sides respectively)
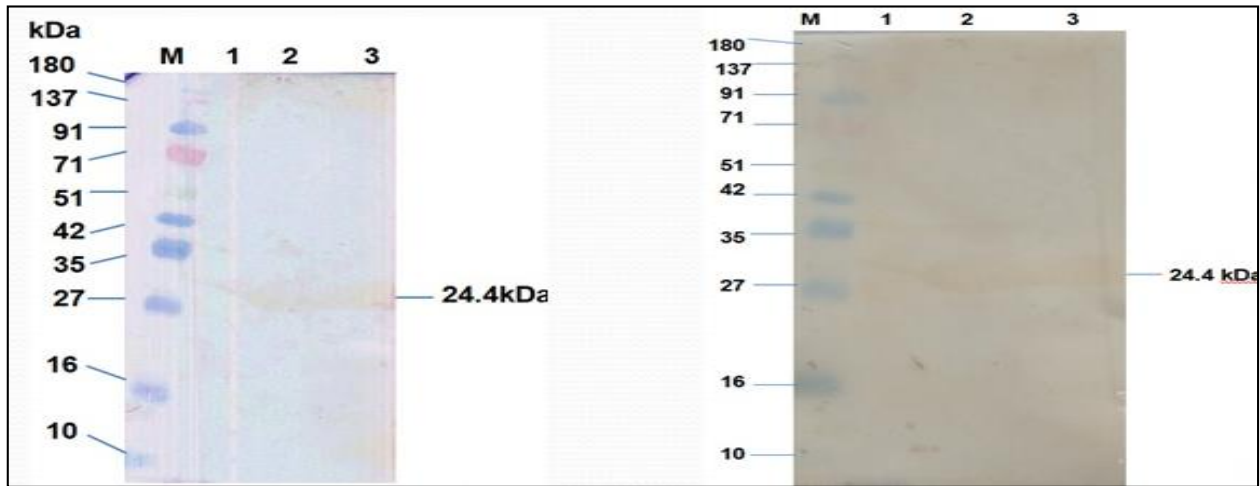
Fig 3: Western blot analysis of recombinant fusion 24.4 kDa protein with positive sheep (left side) and bovine (right side) serum
1. Uninduced M-15 cells harboring ppg 660 clone
2. Induced M-15 cells harboring ppg 660 clone
3. 3(left) Reaction of recombinant fusion 24.4 kDa protein with positive sheep serum
4. 3(right) Reaction of recombinant fusion 24.4 kDa protein with positive bovine serum

A mix of 106 (bovine and sheep) commercial kit tested sera sample having P/N value, >2.0 than that of sera obtained from the healthy animal were analyzed to determine sensitivity and specificity of in-house ELISA.

Screening of 106 serum samples (bovine and sheep) by Map recombinant fusion 24.4 kDa protein in-house ELISA revealed 34 and 72 negative and positive reactors, respectively (Table 1). The result demonstrated that the specificity was The serum dilutions up to (1:1200) were used

by in-house ELISA from sheep infected with paratuberculosis with his recombinant fusion 24.4 kDa revealed higher P/N value, ≥2.0 than that of sera obtained from healthy sheep and cattle. The specificity was 84.62% (95% CI 65.13- 95.64%), while the sensitivity was 85% (95% CI 75.26- 92.00%), relative to infection status as indicated by the commercial kit test. In-house ELISA based on Map recombinant fusion 24.4 kDa protein showed kappa value agreement with commercial ELISA kit (k = 0.72) (Viera and Garrett, 2005) [18].

Table 1: Sensitivity and specificity of an in-house ELISA

Test	Disease				
	Present n Absent n Total				
Positive	True Positive	a = 68	False Positive	c = 4	a + c = 72
Negative	False Negative	b = 12	True Negative	d = 22	b + d = 34
Total		a + b = 80		c +d = 26	
Statistic		Formula	Value	95% CI	
Sensitivity		a / a+b	85.00%	75.26 to 92.00%	
Specificity		d / c+d	84.62%	65.13 to 95.64%	
Kappa agreement = 0.72					

Therefore, the Map recombinant fusion 24.4 kDa protein is a good candidate for an in-house ELISA based serological test that is easy to perform and cheap to produce.

Conclusion
The use of gene splice techniques in the production of recombinant fusion protein also completely eliminated the preparation and use of protein mixtures. The design of the recombinant fusion 24.4 kDa protein and its ease of expression and purification have the potential to make this a highly effective approach in the development of reagents for the diagnosis of bovine paratuberculosis. Comparing with commercial ELISA kit as the standard method, this in- house ELISA test showed good specificity and sensitivity and can be used for routine diagnosis of paratuberculosis in animals.

Ethical approval
Compliance with ethical standards

Conflict of interest
The authors declare no conflict of interests.

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