Development of in-House Recombinant OMP31 (rOMP31) Protein Based Latex Bed Test for the Diagnosis of Ovine and Caprine Brucellosis

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Abstract: ***Brucella melitensis is a major cause of brucellosis in human and small ruminants. For prevention and control of brucellosis accurate, inexpensive, simple, rapid and specific diagnosis is prerequisite in livestock as well as in humans. Many tests are available including gold standard isolation methods. However, there is a paucity of early, effective, sensitive door step diagnosis tests for detection of brucellosis in both livestock and humans. For the purpose, outer membrane proteins of B. melitensis are considered as important Non-lipo-polysacchride immunodominant candidate antigen. The present study describes the development and evaluation of a B. melitensis specific recombinant Omp31 based latex agglutination test (LAT) for the detection of antibodies to B. melitensis in goats. The rOmp31 gene was cloned in pET-32 and protein was expressed in E. coli BL21 cells and purified by Ni-NTA chromatography. Carboxylated modified polystyrene latex particles were coated with purified rOmp31 and evaluated for detection of antibodies against B. melitensis in goat sera. The validation of developed recombinant Omp31 antigen with other serological tests and sera containing B. abortus antibodies revealed that it was more sensitive and accurate as compared to the Rose Bengal Plate test (RBPT) with specificity for B. melitensis. Moreover, the test is rapid, simple, inexpensive, and suitable for detection of specific antibodies in B. melitensis infection under field conditions. LAT test more sensitive and specific for diagnosis as compare to RBPT. Outer membrane protein (OMP), polymerase chain reaction (PCR), recombinant outer membrane protein (rOmp), Latex aggutilation test (LAT), Enzyme linked immunosorbent assay (ELISA), Rose Bengal Plate test (RBPT)***

1. Introduction

Goat is one of the important species of livestock with estimated about 1 billion populations all over the world. The population of goat has shown an increase by more than 20% in the last decade. Out of this goat population, about 95% of goats are located in the developing countries of Asia and Africa, where they are considered one of the most important sources of protein for human beings (Tosser-Klopp *et al.,* 2014; Rossetti *et al.,* 2017). In India goat population contributes a lot to the agrarian economy, particularly in regions where crop and dairy-farming are not economical, thus play an important role in the livelihood of landless, small and marginal farmers (DADF, 2017). Therefore, there is the requirement of new serological diagnostic methods for the detection of antibodies to some alternative antigens such as outer membrane proteins (OMPs) and cytoplasmic proteins instead of LPS (Pathak et al., 2017). To reduce the false positive serological reactions in serodiagnosis of Brucella spp., outer membrane proteins (OMPs) were considered more suitable antigens in comparison to LPS (Garin-Bastuji et al., 2006). The outer membrane proteins (OMPs) of Brucella sp. were initially identified in the early 1980s (Dubray, 1985) and have been extensively characterized as potential immunogenic and protective antigens (Cloeckaert et al., 1996; Cassataro et al., 2004). OMPs of Brucella spp. are classified as per their apparent molecular weights into three groups such as group 1 (94 or 88 kDa), group 2 (36–38 kDa), and group 3 (25–27 and 31–34 kDa). Among these, group 1 was identified as minor whereas group 2 and 3 OMPs were identified as major OMPs (Cloeckaert et al., 2002). The two major OMPs are further subdivided like group 2 (Omp2a and Omp2b) and group 3 (Omp25 and Omp31) (Gupta et al., 2012). Omp31 is expressed in all Brucella species, except Brucella abortus, which has a 25-kb chromosomal deletion comprising omp31 and other genes (Cherwonogrodzky et al., 1988).

2. Review

The limitations of serological methods is that the bacterial suspension used to diagnose infections caused by smooth species *viz*., *B. abortus, B. melitensis* and *B. suis* are not suitable for diagnosing infections caused by rough species like *B. ovis* and *B. canis* and *vice-versa*. Apart from that the techniques, which are based on detection of antibodies against LPS antigen have the chances of false positive results due to extensive cross reactions with other Gram negative bacteria, impairing the unambiguous serological diagnosis of disease (Corbel, 1985; Wright and Nielsen, 1990; Reynauld *et al.,* 1993; Kittleberger *et al.,* 1995; Weynants *et al.,* 1996). There are several advantages of use of the recombinant proteins instead of protein extracted from *Brucella* spp. for diagnosis of brucellosis has several advantages such as free from other antigens of *Brucella* spp. that might interfere in diagnosis, high yield, avoidance of handling of zoonotic *Brucella* organism (Seco-Mediavilla *et al.,* 2003). Cassataro *et al.* (2004) purified the recombinant OMP31 protein of *B. melitensis* and tested on human and animal sera suffering from brucellosis by iELISA and suggested the limited value for the diagnosis of *B. melitensis* in human and animals. There are various studies for cloning and expression of different individual *omp* genes of *Brucella* spp. and their use in

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immunoenzymatic assays for serological diagnosis of brucellosis in man as well as animals. Various outer membrane proteins (OMP36, OMP25, OMP19, OMP16, OMP10, P17, P15 and P39) have been cloned and expressed in competent cells and used as candidate antigen for diagnosis of brucellosis, out of these, the cytoplasmic protein P15-P39 (in sheep and goat) and P17-P39 (in cattle) gave the most useful results (Letesson *et al.,* 1997). Pathak *et al.,* 2017 suggested the use of the rOmp2a as a candidate antigen for diagnosis of brucellosis in human beings with a sensitivity of 93.75% and specificity of 95.83%. The omp31 gene of *B. melitensis* 16 M has been considered as suitable gene for cloning and expression and showed the protection in murine model and natural host against a *B. ovis* challenge (Vizcaino *et al.,* 1996; Estein *et al.,* 2003, 2004).

3. Material and method

The heat killed bacteria of *B. melitensis* vaccine strain Rev 1 was obtained from the Brucellosis department of Razi Vaccine and Serum Research Institute (Hesarak, Karaj, Iran). Escherichia coli strain Top10 (Stratagene, USA) was used for initial cloning, sequencing and maintenance of different DNA fragments. A prokaryotic expression vector pET32b(+) (Novagene, USA) was used for recombinant protein production. The recombinant plasmid was transformed into E. coli, BL21 (DE3) pLysS (Stratagene, USA) as host strain. LB broth or LB agar was supplemented when required with 100 µg/mL ampicillin. All chemicals were purchased from Merck Company (Germany). Sera were collected from five patients who were diagnosed with a history of brucellosis in Dr. Taheri Medical Diagnostic Laboratory, Mashhad, Iran. Sera from five healthy volunteers were considered to act as negative control. The pooled sera of the subjects as positive and negative controls were prepared individually. The killed *B. melitensis* Rev 1 was used for DNA isolation by phenol chloroform method (Sambrook and Russell, 2001). The purity of the obtained DNA was verified by NanoDrop ND-100 spectrophotometer. Full length open reading frame (ORF) of the Omp31 gene was amplified by PCR with Pfu DNA polymerase from the extracted DNA by the forward primer that has EcoR I restriction site (5'-ATAGAATTCGATGAA GTCCGTAATTTTGGCGTCCAT-3') and the backward primer with restriction site for Xho I (5'-TATTGGAGCTCGAGGAACTT GTAGTTCAGACCGACGC-3'). These primers were selected according to the reported Omp31 nucleotide sequence deposited in the NCBI GenBank database and were synthesized by GenFanAvaran Co., Tehran, Iran. PCR reaction was performed in a 25 µl volume containing 250 ng of template DNA, 0.5 µM of each primer, 2.5 mM MgCl2, 200 µM (each) deoxynucleoside triphosphates, 1× PCR buffer and 1.5 unit of pfu DNA polymerase. The following condition was used for the amplification: hot start at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 3 min, annealing at 65°C for 45 s and extension at 72°C for 1 min. Then a final extension step of 10 min at 72°C was performed. The PCR products were analyzed by electrophoresis on 1% agarose gel in TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The predicted 723-bp PCR product was purified from the agarose gel by AccuPrep™ Plasmid Mini Extraction kit (Bioneer, Korea) according to the manufacturer's instruction. The purified digested PCR product was cloned into the pET32b(+) plasmid, in which the recombinant protein includes a six-histidine tag (His-tag) at the C-terminal end for easier purification. Standard techniques for these steps such as plasmid DNA preparation, ligation, competent cell preparation and transformation were followed, as described previously (Sambrook and Russell, 2001). The resulting construct, pET32b + Omp31, was transformed into competent TOP10 E. coli cells (Invitrogen, Carlsbad, CA) using transformation kit (Fermentas, Lituania). Ampicillin-resistant colonies were grown until OD 600 = 1.0 in LB medium containing 50 µg/ml of Ampicillin, at 37°C with agitation. Expression and purification of rOmp31 For expression of Omp31, the purified pET32b+ Omp31 construct was transformed in E. coli BL21 (DE3) cells (Novagen, USA) by transformation kit (Fermentas, Lithuania) and selected on LB ampicillin plates. Protein synthesis was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) in a culture of bacteria with an OD 600 of 0.6. Bacteria were incubated for 3 h at 37°C then harvested by centrifugation (3000 g, 20 min, 4°C) and stored at -80°C. The pellet from a 100 ml bacterial culture was resuspended in lysis buffer (Tris 50 mM, EDTA 5.0 mM, urea 8.0 M, pH = 8.0) and subjected to three freeze-thaw cycles using liquid nitrogen. Cell lysate was subjected to centrifugation at 9000 g for 15 min at 4°C to separate the supernatant containing soluble materials from the pellet. Both the supernatant and the pellet were evaluated by SDS-PAGE to analyse the expression of rOmp31. Expressed protein was purified by chromatography through Ni-agarose (Invitrogen), from the insoluble phase of lysate using Guanidine hydrochloride 6 M to dissolve the pellet, according to the manufacturer's protocol. Briefly, two ml of Ni-NTA resin was packed into a syringe, washed and equilibrated in 10 column volumes of deionized water, followed by 10 column volumes of binding buffer (pH = 7.8, K3(PO4) 50 mM, NaCl 400 mM, KCl 100 mM, 10 mM Imidazole, 10% Glycerol, 0.5% Triton X-100). The filtered supernatant of lysate insoluble phase through a 0.45 µm membrane was loaded onto a Ni-NTA column and then washed with 10 column volumes washing buffer containing 20 mM Imidazole. Target protein was eluted using an Imidazole gradient (100-500 mM) in the binding buffer. rOmp31 protein coated on latex beds by the help of binding buffer then using for diagnosis of brucellosis.

4. Result

Out of 46 samples tested, 9 (19.56%) were positive and 37 (80.43%) were negative by rOmp31 antigen based Latex agglutination test (LAT). In comparison to RBPT, the relative sensitivity and relative specificity of rOMP31-latex bed test were found 92.85% and 93.59%. Thus, the test gave comparable results with the standard agglutination assay. It is important to note that 05 samples that were negative by RBPT were detected as positive by the rOMP31-LAT, suggesting that low-titre samples that are missed by the agglutination test were detected by the sensitive LAT. However, 01 sample that

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was positive by RBPT was detected as negative by the rOMP31- LAT. Positive predictive value is the proportion diseased among subjects with a positive test result. Negative predictive value is the proportion non diseased among subjects with a negative test result. Positive predictive value of rOMP31-LAT was found 72.22%, while negative predictive value was 98.64%, rOMP31-LAT showed 93.40% in accuracy of prediction against RBPT.

Concordance was higher between RBPT and rOMP31-LAT test with a concordance of (93.48%). Agreement between two tests can be calculated using Kappa statistics. Kappa statistics between RBPT and rOMP31-LAT showed substantial agreement as their Kappa value was 0.774.

5. Discussion

A total of 92 sera samples of goats were analyzed by rose Bengal plate test, rOmp31 based i-ELISA and commercially available ELISA for diagnosis of brucellosis. Concordances between tests are calculated to know the agreement between the two tests. This helps to find the correct test combination in identifying the disease. Concordance between rOmp31-LAT and RBPT. Kappa statistics is widely used to study the agreement between the studies. Kappa statistics between RBPT and rOmp31-LAT showed substantial agreement as their Kappa value was 0.774. The high sensitivity comparable to this rOmp31 ELISA in comparison with RBPT reiterates the fact that this system can be used for the routine serodiagnosis of caprine brucellosis in the seroepidemiological studies. The high specificity may be attributed to the presence of species specific recombinant protein whereas low specificity might be due to the detection of antibodies against *Brucella melitensis*, while RBPT detects antibodies against *Brucella abortus* and *Brucella melitensis* both (Tiwari *et al.,* 2013). RBPT is a spot agglutination technique and can give both false as well as true positives (Singh A *et al.,* 2013, 2014, 2018). Another possible reason of low sensitivity of rOmp31-LAT in comparison to RBPT may be explained by a low adherence of the protein antigen (rOmp31) to polystyrene. The discrepancy observed between rOmp31 LAT and RBPT may be due to the following facts: that RBPT mostly detects the antibodies against the S-LPS; and sera with specific IgM but without significant levels of IgG are positive in RBPT and negative in ELISA using protein antigen (Gupta *et al.,* 2007). The sensitivity and specificity of the test varies with pH of the antigen, ambient temperature and temperature of antigen and test serum (Corbel, 1973). Apart from this, the cross-reactive Brucella antibodies (*Brucella abortus* and other *Brucella* spp.) may also influence sensitivity and specificity of the test. Since, the proposed antibodies detection system against rOmp31 antigen is in a plate LAT format, so it allows batch processing resulting into processing of large number of sera samples within a short period of time with ease. Therefore, it is less time-consuming and the results can be analysed quantitatively using an LAT. This development of *in house* rOmp31 based LAT diagnostic test using highly specific recombinant proteins will not only allow cost-effective diagnosis but will also provide reliable field-based methods, in addition circumventing the necessity to handle highly pathogenic *Brucella melitensis* for antigen preparation. The commercial kits available for the diagnosis of brucellosis in goats are not popular in the routine diagnosis of brucellosis in the developing countries like India due to their high cost and the lack of easy availability.

6. Conclusion

Diagnosis of brucellosis in goats can be carried out by various techniques; isolation, serology by RBPT, ELISA and PCR. Among these, molecular tests like PCR is considered reliable than the other diagnostic assays but cannot be used in epidemiological studies to generate large amount of data due to high cost and less practicability. Hence, serological assays are still considered as choice for detecting the natural antibodies in animals. Development of recombinant proteins for diagnosis of brucellosis has solved the drawback of cross-reactivity issues in the diagnosis of brucellosis. Keeping in mind the importance of brucellosis as an emerging infection and the prevalence in India, the present study was carried out to clone the *omp31* gene of *Brucella melitensis* in pET32b(+) prokaryotic system to produce the recombinant Omp31 protein with high yield making the *in house*, cost effective an indirect plate ELISA for serodiagnosis of brucellosis in goats. This also takes into account the safety aspects associated with handling highly zoonotic *Brucella melitensis* in the laboratory for preparation of LPS antigen. Both Kappa value and Concordance percentage of rOmp31-LAT with RBPT were almost perfect and had maximum agreement, respectively. Relative sensitivity and relative specificity of rOmp31-LAT was 94.11% and 97.33%, respectively when compared with commercial ELISA. Thus, the developed *in house* test may be used by the local veterinary diagnostic laboratories for diagnosis of brucellosis in goats. This *in house* developed test will help in the early detection and management of this disease particularly in Uttar Pradesh state.

7. Future Scope

Further evaluation is required to scale up production of this recombinant protein and evaluation of this test on large number of clinical samples from different geographical areas and with various clinical presentations for detection of infection to determine the usefulness of this protein in the serodiagnosis of caprine brucellosis.

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