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Research Article

The Omp31r Recombinant Protein of *Brucella melitensis* and Immunological Evaluation for its Possible Use for the Diagnosis in Bovine Brucellosis

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Abstract

In humans, Brucellosis is a potentially mortal disease, caused by intracellular Gram-negative bacteria, which belongs to the *Brucella* group and is worldwide distributed. In humans, the infection is mediated through the consumption of milk or fresh cheese no pasteurized and raw meat. Therefore, sanitary control in farm animals is greatly important, thus the serologic diagnosis is key to detect antibodies against *Brucella´s* lipopolysaccharide, by ELISA tests or agglutination tests. The aim of the work is testing a recombinant protein, to use it as a potential protein for serological diagnosis of brucellosis in bovine. Bioinformatics analysis results in the OMP31 gene identification from *Brucella melitensis* M15, forward cloned from genomic DNA. The OMP31 protein was expressed in a BL21 (DE3) system, using pET28a vector, and isolated by polyhistidine-NI affinity chromatography. Polyclonal antibodies against OMP31 were produced in rabbit and a western blot against *Brucella abortus* RB51, *Brucella melitensis* M15 and *Brucella abortus* 1119 were performed. The antibodies produced did recognize *Brucella abortus* RB51 and *Brucella melitensis* M15, but didn´t recognize the vaccine strain *Brucella abortus* 1119. A standardized ELISAi was performed, using healthy bovine´s serums and *Brucella abortus* positive bovine´s serums. The specificity and sensibility were estimated contrasting an ELISAi using OMP31r obtaining 90.6% and 77.17% and *Brucella abortus* 1119, 100%. The recognition of OMP31r by antibodies from serums of *B. abortus* infected bovines is quite promising, opening the possibility to produce both, rapids test kit and massive diagnosis to enable the control of bovine brucellosis.

Keywords: *Brucella sp; Omp31r; Brucellosis; Recombinant Protein; Indirect ELISA*

Intruction

Brucella melitensis and *Brucella abortus* are gram-negative, facultative, intracellular bacteria classified within the genus *Brucella,* involved in ovine and bovine infections respectably [1-3]. It is transmitted to humans through contact with infected livestock or by consumption of farm animal products. In humans, it causes weakness and debilitating febrile illness, knowns as an undulant fever by chronic infections, or aborts and infertility in bovines and other animals, resulting in severe economics lost and public health problems [4]. By

now, there is any reliable vaccine for humans, therefore, the sanitary controls in farm animals are the best option to prevent brucellosis by the consumption of infected animals. Many diagnosis methods have been designed, like microbiology, serology and DNA detection [5-7]. Among these methods, serology is taking as the main option for its sensibility, and economic advantage [8-11].

The detection of specifics antibodies against bacteria's liposaccharides by ELISA test is one of the main elements in the diagnosis of brucellosis in humans and other animals, as well as standard tube agglutination test (STAT), and the rose bengal plate agglutination test (RBPT). In Venezuela, the RBPT is used as a serologic detection method and STAT as a confirmation method test for brucellosis´s diagnosis [12-14]. The RBPT is non-expensive but low specific, therefore, the development of a faster and reliable method for brucellosis´s detection is quite important.

Although S-LPS can induce a very powerful antibody response, making the serological method quite sensitive, the antibodies have the critical drawback of cross-reactivity among Gram-negative bacteria such as *Yersinia enterocolitica O:9, Escherichia coli* 0157, *Salmonella typhimurium*, and *Vibrio cholera* among others. The cross-reactivity was attributed to the high structural similarity of the O-polysaccharide (OPS) epitope of the S-LPS molecule [15-18].

The identification of immunogenic proteins in pathogens by immunoproteomic, which combines proteomic coupled to western-blotting, has been quite useful. In fact, some outer and/or cytoplasmatic components of *Brucella* has served as potentials markers in assays for brucellosis diagnosis [19,20]. Some examples of these proteins are Lumazina synthetase, a protein from the secretion system type IV VirB5, the outer-layer protein Omp28 and the immunogenic periplasmatic protein Bp26 [21-25].

Some outer layer protein (OMP) has been evaluated by immunoproteomic, and it was found in positive *B. abortus* bovine´s serums and humans accidentally infected [26], but not in its LPS-lacking mutant strain, *B. abortus* RB51 bovine´s serums [27-29]. These could be an interesting way to minimize cross-reaction in brucellosis detection [30]. Therefore, these OMP´s proteins seem to be a good option to develop a brucellosis´s multi-diagnosis method, not only for livestock but also in humans. Them we decide to analyze Omp31 by bioinformatic, cloned the gene that expresses the protein and evaluate its potential as a serologic marker or for the diagnosis of brucellosis in bovines.

Materials and Methods

Bioinformatic analysis

Amino acids sequences of OMP31 from were aligned by multialin http://multalin.toulouse.inra.fr/multalin/), thus, CAJ11595.1, EFH35395.1, W17222.1 from *Brucella abortus* and GQ184729.1 from *Brucella melitensis* were selected. The identity percentage of the amino acids was obtained using UGENE V1.27.0 software. The oligos were designed using oligoexplorer V1.4 software, and the access number of the OMP31 gene sequence (*Brucella melitensis*) is GQ184729.1.

Bacteria´s strain and grown conditions

Brucella melitensis 16M virulent type was used as a reference strain. An *Escherichia coli* BL21(DE3) was used to cloned the genes, and incubated at 37°C in Luria-Bertani (LB) or supplemented agar using Kanamycin at 30 mg/ml as an antibiotic.

Expression and purification of recombinant OMP31

The gene of Omp31 from *Brucella melitensis* was cloned in pET28a (Novagen, USA) and transformed in *E. coli* BL21. The expression of the protein was induced in LB medium/Kanamycin (30 mg/ml) with Isopropyl beta-D-thiogalactopyranoside (IPTG) 1mM. The cells were incubated for 4 hours, pelleted (12,000xg 15 min, 4°C), and stored at -20°C until used. The cells were resuspended in a solution of Tris 50

mM, EDTA 5 mM, and 1% Triton X-100 (pH 8.0), followed by 3 cycles of sonication for 1 min at 4°C. The Inclusion bodies were recollected by centrifugation at 15,000g x 15 min at 4°C, followed by two washes with the resuspension solution without Triton X-100. The pelleted Inclusion bodies were resuspended in a solution (Tris 50 mM, 5 mM EDTA, urea 8M; pH 8.0) at room temperature with agitation overnight. Followed by centrifugation (15,000x g, 15 min, 4°C), the protein was purified by chromatography using ProBond resin (Invitrogen, USA). The recombinant OMP31r was verified by western blotting, using a polyclonal antibody anti-OMP31 produced in rabbits in our laboratory. The purity was evaluated by SDS-PAGE 12%.

SDS-PAGE and western blot

The OMP31r was boiled for 10 min at 95*°C* and diluted in sampler buffer 2X (SDS 4%, Glycerol 20%, 2-mercaptoethanol 10%, bromophenol blue 0,004%, Tris-HCL 0,125 M; pH 6.8), and the sample was run in SDS-PGE electrophoresis. At the end of the electrophoresis, the protein was transferred to a nitro-cellulose Immobilon-P membrane (Millipore, EE. UU.). the transference was performed using the buffer (Tris 25 mM, glycine 192 mM, methanol 20%), using a transfer equipment. The Blot was blocked with TBS-(skim milk 5%) during 1h at 4*°C*, and the washed tree times with PBS-(Tween20 0,05%). The western-blot was incubated with positive serum (immunized rabbit) anti-OMP31r, dilution 1:1000, during 2h at 4*°C*. The membrane was washed with PBS-(Tween20 0,05%), followed by an incubation with antibodies from goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP dilution 1:5000, Sigma Aldrich, EE. UU.) during 1h at room temperature, the detection was performed using diaminobenzidine- H_2O_2.

Bovine`s serum was also used for wester-blot in a dilution 1:100. The positive serum was recollected from a bovine infected with *Brucella sp.,* and negative serum from a bovine not infected.

ELISA

The right conditions for the ELISA, such as titer of either primary and secondary antibodies and concentration of the antigen, were standard (data not showed).

The plates (Polysorp Nunc, Denmark) were coated with the recombinant protein OMP31r diluted in PBS (0.2 µg/well), followed by blocking with BSA and washed using PBS- (Tween20 0,05%). The serums were diluted in PBS-tween20-(BSA 1%) and placed in each well. Bounds antibodies from bovine’s serums were detected using anti-bovine peroxidase conjugated (Sigma, St. Louis, EUA). The reaction was performed adding 3,3’,5,5’-Tetrametilbencidina (TMB, 2 g/l) in buffer 0,1 M citrate phosphate (0,03% H_2O_2). The cut off value was set using negative serums (not infected serums). The cutoff, which is the value that discriminates between positive and negative samples, was calculated based [31], to determine healthy control serums.

$$X_s = X + 3\delta$$

X_s = Cut off point
X = Average of healthy controls
δ = standard deviation

The samples that exhibit values above the cut off value at 450nm OD, has been considered as positive, and those below the cut off negative.

Specificity and sensibility

The specificity and sensibility of the *Brucella*´s tests of infected and healthy bovine´s serums were calculated using the methods as described [32].

Results and Discussion

The alignment analysis of amino acid sequences between the OMP31 from *B. melitensis* and those OMP31b from *B. abortus* displayed a 70% of identity (Figure 1 and table 1).

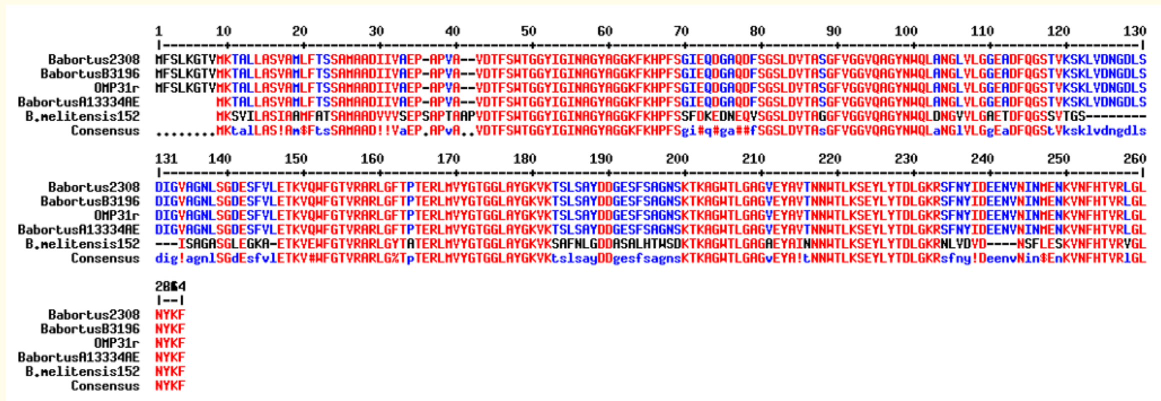

Figure 1: *Alignment analysis in order. OMP31b protein from Brucella abortus (Babortus2308, BabortusB3196), OMP31r (recombinant protein), BabortusA13334AE, and OMP31 protein from B. melitensis (B. melitensis 152) respectively, using Multialin program. B. abortus 2308 (GenBank Access number: CAJ11595.1), B. abortus B3196 (GenBank Access number: EFH35395.1), B. abortus A13334AE (GenBank Access number: A13334AE), B. melitensis 152 (GenBank Access number: GQ184729.1).*

	***B. abortus* 2308**	***B. abortus* B3196**	***B. abortus* A13334AE**	***B. melitensis* 152**	***OMP31r***
B. abortus 2308	100%	100%	100%	70%	100%
B. abortus B3196	100%	100%	100%	70%	100%
B. abortus A13334AE	100%	100%	100%	70%	100%
B. melitensis 152	70%	70%	70%	100%	70%
OMP31r	100%	100%	100%	70%	100%

Table 1: *Identity in percent among OMP31proteins of Brucella sp.*

Bioinformatic studies, show an elevated percent of identity (70%) among OMP-likes proteins from different *Brucella* species, such as *B. abortus* and *B. melitensis.* The high percent of identity among OMP-like proteins from these two species implies the existence of common immunogenic epitopes. When an evaluation of serums from bovines infected with brucellosis was displaced, the antibodies produced against OMP31-like (from *B. abortus 1119*) also recognized OMP31r (from *B. melitensis*). The cross-reactivity between both proteins was demonstrated by the results of ELISA tests (Figure 3). These results suggest potential advantages in the diagnosis of brucellosis in serum's evaluation of different infected animals.

Similar results were observed by Connolly [26]; detecting the OMP31 from *B. abortus biovar* 224 evaluating positive bovine´s serums, suggesting that these OMP-like proteins could be found in others *B. abortus* wildtype.

The gene of OMP31 used for this study was detected using PCR from *B. melitensis* genomic DNA (723 bp fragment) and validated by sequencing [33]. The gene was forward cloned in pQE30 expression vector and induce to expression in TG1 *Escherichia coli* strain (Figure 2). The OMP31 recombinant (OMP31r) was purified by IMAC chromatography reaching 70 ug/ml of purified protein.

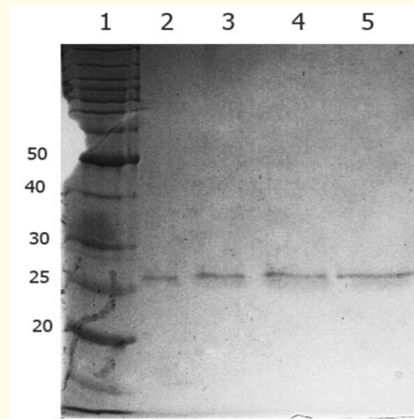

Figure 2: *SDS-PAGE. Lane1, weight marker; lanes 2 to 5, recombinant protein OMP31r. The apparent molecular weight of 27KDa.*

The purified protein was tested by performing an ELISA to evaluate if the recombinant OMP31r is capable to act as antigen, thus being recognized by the antibodies against *Brucella.* We observed recognition in serums of positive animals for brucellosis but no significant differences between positive and negative serums (Data not showed). A similar result, but in ovine and caprine was observed in a study made by Navarro in 2012 [34]. These farm animals have usually been exposed to bacteria and pathogens, developing a widely diverse repertoire of antibodies, and some of these would recognize *E. coli* proteins from the expression system. When some *E. coli* peptides co-elute in the purification of the expressed protein; cross-reaction results between positive and negative serum could occur, producing false positives or overrated results.

In order to eliminate these nonspecific signals, a pre-absorption of serum antibodies using the powder extract from *Escherichia coli* method was used [35], keeping only the antibodies that recognize our recombinant protein.

The Omp31r is well recognized in the western blot by positive bovine´s serum, and not for negative serum In figure 3A the recognition of co-eluted *E. coli* proteins is a problem (Figure 3B), that is affecting either blotting and ELISA results, but fixed after the pre-absorption method, producing a clean result of the Omp31r specific recognition (Figure 3C). The pre-absorption of the non-specific OMP31r antibodies in the bovine´s serums allowed us to continue performing the immunological evaluation of this protein.

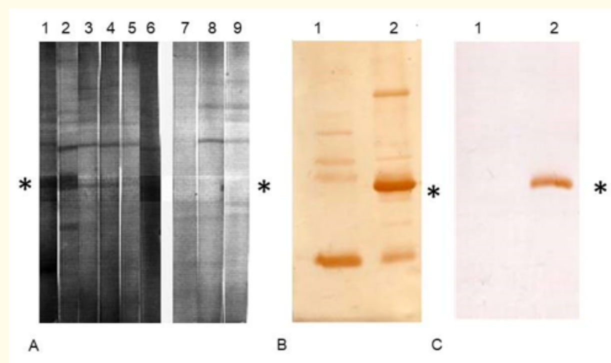

Figure 3: *Western blot with the recombinant protein of Brucella melitensis (OMP31r). Asterisk (*) is used to point the Omp31r location protein in the blot. A) Cross-reactivity against OMP31r and E. coli proteins, strip 1-6 is using a positive serum for Brucella sp. and strips 7 - 9 is using negative serum for Brucella sp. B) the recombinant protein of Brucella melitensis (OMP31r) before and C) after the pre-absorption of positive Brucella sp. serum´s antibodies using the powder extract from E. coli. Line 1. E. coli extrat Line 2. Purified recombinant protein Omp31r.*

The OMP31r and a phenolated extract of *B. abortus biovar* 1119 (positive control) were evaluated in ELISA tests (Figure 4). The cut-off value for positive antigen in the ELISA test was calculated using the average of n = 32 healthy serums DO (negative serums).

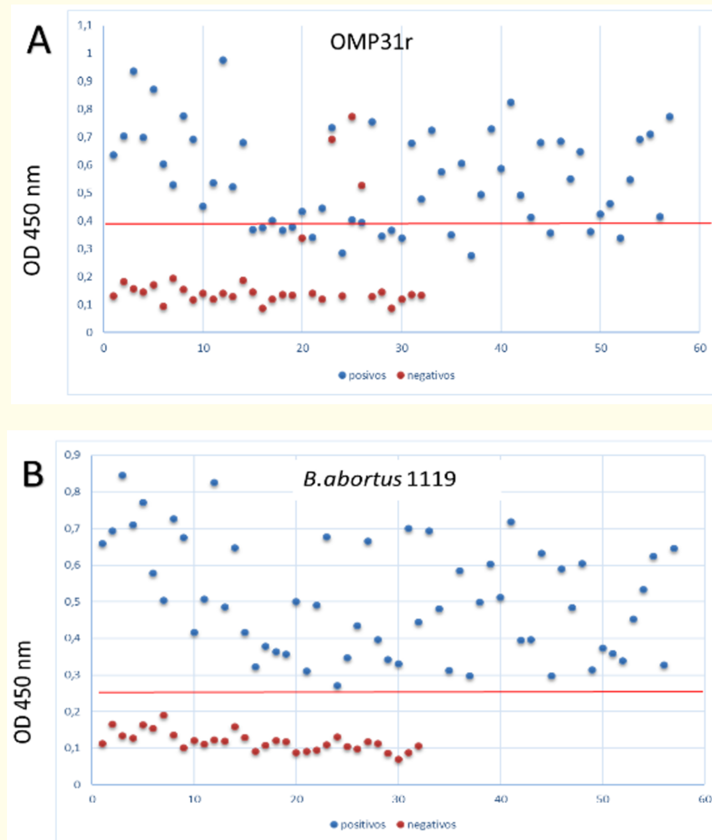

Figure 4: *ELISA reactivity serologic test. A) The evaluation recombinant antigen OMP31r (20 ng/well), and B) the evaluation of the antigen B. abortus biovar 1119 (2 ng/well) extract were used as antigen. 82 bovine's serums were classified into two groups: In blue, the brucellosis positive serums, and in red, brucellosis negative serums. The red line indicates the cut-off value.*

For OMP31r as an antigen, the value average of negative serums was DO = 0.1919, thus the cutoff value was D.O = 0,3838 and for *Brucella* strain 1119 used as antigen, the value average of negative serums was optic density (OD) = 0.1183, thus the cut-off value was D.O. = 0.2366.

The positive bovine´s serums (n = 57), shown an OD between 0.285 and 0.975. the anti OMP31 antibodies were detected in 43 of the 57 positive serums, which means, 75.43% of recognition against OMP31r. when *Brucella* strain 1119 was used as an antigen, 100% of the positive serums (n = 57) were detected.

Facing these results, only 9 positive serums were not detected. Therefore, the sensibility, specificity, and precision of the OMP31r with

Calculation	Antigen OMP31
Sensitivity (%)[a]	77.17%
Specificity (%)[b]	90.62%
Precision (%)[c]	82.02%

Table 2: *Comparison of sensitivities, specificities, and precision of indirect ELISAs using the recombinant antigen OMP31r.*

[a] *Sensibility (%) = (true positives/(true positive + false negative)) X 100.*

[b] *Specificity (%) = (true negatives/(true negatives + false positive)) X 100.*

[c] *Precision (%) = (true positives + true negatives/(true positives + true negatives + false negative + true negatives) X 100.*

the positive serums tested were 77,17 %, 90.6 %, and 82.02% respectively (Table 2).

Although these promising results, 3 of 32 negative serums had reactivity against OMP31r; this inconsistency could be as a result of the reactivity with proteins from *E. coli,* that were co-isolated with the recombinant protein OMP31r. A similar result has been reported with a membrane protein Bp26 [36-38], used as antigen in the brucellosis diagnosis in sheep and goats Omp28 [39-40].

The western blot, using anti-Omp31r serums from an immunized rabbit, revealed a specific reactivity with the purified Omp31r produced in *E. coli* cells, as well as showing the functional expression in the prokaryotic system. The *B. abortus biovar* 1119 was also observed (Figure 5A).

The no reactivity against OMP31r in the blotting, using a vaccinated *Brucella abortus* RB51 bovine´s serums (Figure 5B), is quite important since it enables to discriminate between vaccinated bovines from *Brucella* infected bovines, reducing false-positive results in the diagnosis. Therefore, the OMP31r as antigen seems to be a good strategy to develop diagnosis test for brucellosis *B. abortus* in bovines.

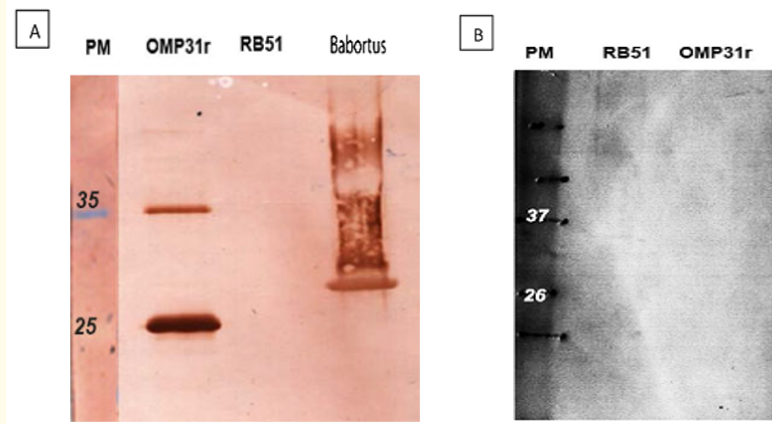

Figure 5: *A) Western blot, (using anti-Omp31r serums from an immunized rabbit): line1: molecular weight marker; line 2: OMP31r; line 3: Brucella abortus RB151 (vaccination strain); line 4: B. abortus biovar 1119. B) Western blot (using vaccinated Brucella abortus RB51 bovine´s serums): line1: molecular weight marker; line 2: Brucella abortus RB51 (vaccination strain); line 3: OMP31r.*

The use of Omp31 proteins has not been reported in the serologic diagnostic of brucellosis in bovines, but if in goats and sheep, since Omp31 is expressed in all *Brucella* species, except *Brucella abortus* which is responsible for brucellosis in bovines. The deprivation of this protein in *B. abortus* is the result of a 25 Kb chromosomal deletion comprising Omp31 among other genes [41,42]. Although, our study revealed that Omp31 from *B. melitensis* shares immunogenic epitopes with its homologue Omp31b from *B. abortus* (Figure 5A). Similar results were found comparing Omp31 from *B. melitensis* with its homologue from *B. ovis* [43].

These results suggest that, this recombinant protein can be used as a multi-diagnosis brucellosis method, not only in both *B. melitensis*-infected goats and *B. melitensis ovis* infected sheep, but also in *B. abortus*-infected bovines.

Conclusion

We observed an elevated sensibility (77.17%) and specificity (90.6%) in ELISAi tests with the bovine´s serums using the Omp31r as antigen against, suggests that this protein exposes immunogenic epitopes capable to be used in the diagnosis of brucellosis in *B. abortus*-infected animals, thus, the recombinant protein Omp31r seems to be very promising as antigen in the detection of antibodies against *Brucella abortus* in infected bovines. This strategy also allowed us to discriminate between not vaccinated and vaccinates RB51 bovines, having a high potential for the diagnosis of brucellosis in bovines. The use of a recombinant protein has many advantages such as safer, cheaper, and easy to be produced, in comparison to cultivates, maintain, and harvest pathogenic *Brucella abortus* bacteria's, inherent danger of operator infection or work environment contamination.

There is no doubt that more studies using a higher quantity of positive and negative brucellosis infected bovines and other gram-negative pathogen bacteria should be used, in order to take a definitive position about it. Although, the potential of the Omp31r in diagnosis, in combination with other recombinant proteins from *B. melitensis and B. abortus,* could be helpful in the diagnosis process and control of the disease.

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