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Original article

Molecular cloning of the OMP19 gene from *Brucella melitensis* strain H38 and its antigenicity compared to that of commercial OMP19

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Abstract

Brucellosis is a worldwide zoonosis, that can still be classified as endemic despite its ancient origins which causes economic losses and public health problems. Although effectively controlled by vaccination in animals, there is currently no vaccine for use in humans. Outer Membrane Proteins (OMP) that play an active immunogenic and protective role in the Brucellae family. OMP19 is present in all Brucella species as a surface antigen and is a potent immunogen responsible for Brucellosis intracellular infection. For this reason, the study was aimed to be used safely as a potential recombinant vaccine candidate against all Brucella infections, especially in humans and pregnant animals. This study evaluated a *Brucella* lipoprotein antigen, i.e. 19 kilodalton (kDa) outer membrane protein (OMP19), which was amplified and cloned into the pETSUMO vector system. The immunogenic power of the purified recombinant OMP19 antigen against brucellosis was compared with that of OMP19 (Raybiotech Inc, USA) in a mouse model and the obtained rOMP19 antigen was found to be similar to the commercially available recombinant protein.

Key words: rOMP19 antigen, *Brucella melitensis*, recombinant protein

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Introduction

Brucellosis, a zoonotic disease endemic in the Mediterranean basin, has caused global epidemics (especially throughout Europe and America) when control and eradication programs were reduced, especially vaccination and out-of-control animal movements. The World Health Organization has classified brucellosis as neglected zoonosis and this disease represents one of the most severe bacterial zoonose (ranking among the top seven) (WHO 2006). Brucellae are aerobic gram-negative non-motile facultative coccobacilli and form part of the α-proteobacteria family (Garrity and Holt 2001). Brucella replicates in mammalian host cells, the bacteria are able to affect articular and reproduction system and even pass the brain barrier to avoid the host's immune response (Amjadi et al. 2019). *Brucella melitensis* is responsible for ovine brucellosis and causes great economic losses due to abortion and the shedding of contaminated dairy products (Blasco and Molina-Flores 2011). Attenuated *Brucella* vaccines are still used in the World for protection and eradication (Hou et al. 2019). Attenuated *Brucella* vaccines have disadvantages for safety during vaccine manufacture, or the potential for the organism to mutate to a virulent strain, and the spread of the organism to the environment and infection in immunocompromised animals (e.g., pregnant, old, immunosuppressed with drug) or even humans (Schurig et al. 2002). Therefore, recombinant protein-based subunit vaccines that are easy to produce, safe, and mutation-free with quality assurance have been recognized as candidates for the generation of safer and more effective interventions against human brucellosis. For this reason, an increasing number of studies have started to focus on the development of different vaccine models that might facilitate serological diagnosis to ultimately control and eradicate brucellosis (Yang et al. 2013, Gheibi et al. 2018).

It is known that outer membrane protein (OMP) can be found in the cell wall of plant bacteria as well as cell membranes of soil bacteria (e.g. *Ochrobactrum* spp., *Phyllobacterium* spp., *Rhizobium* spp., *Agrobacterium* spp.) and gram-negative bacteria (e.g. *Brucella* spp., *Yersinia enterocolitica* O:9, *Salmonella* spp., and *Escherichia coli* O157:H7) (Gomez-Miguel et al. 1987, Weynants et al. 1996, Velasco et al. 1998). Specifically, OMP 19 forms part of the small outer membrane proteins group and shows antigenic determination in α-proteobacteria and all biovars of *Brucella* spp. (Tibor et al. 1996, Cloeckaert et al. 1999). OMP19, furthermore, displays an amino terminal signal peptide that ends in a tetrapeptide sequence required for lipoprotein modification and processing for synthesis (Hayashi and Wu 1990). Unlike other gram-negative bacterial infections based on lipopolysaccharides (LPS) and lipoproteins (e.g. OMP16, OMP19) are responsible for disease pathogenesis in brucellosis (via Th1 and cytokine stimulation as a result of cell invasion) (Giambartolomei et al. 2004, Kim et al. 2013). Analyzes confirmed that these proteins are external surface antigens (Tibor et al. 1999). Monoclonal antibodies prepared with Omp19 and Omp16 protected mice against the *B. ovis* challenge (Bowden et al. 2000). It has been confirmed that naturally infected sheep with brucellosis have higher levels of antibodies against OMP19 than cattle (Tibor et al. 1996).

Therefore, we have planned a study on recombinant Omp19 protein with these findings. This study, aimed to recombinantly produce OMP19 (i.e. the immunodominant protein of *B. melitensis* H38 (Hur et al. 2011)) in an *E. coli* vector system. The immunogenic effect of the potential recombinant vaccine candidacy for human and pregnant animals against brucellosis would evaluate by comparing it with commercial biosimilar OMP19 in a mouse model.

Materials and Methods

Bacterial strains and cultivation conditions

In the current study, bacterial DNA of the OMP19 gene was sourced from *B. melitensis* strain H38 (ATCC, 23456), which had been obtained from the Department of Microbiology, Selcuk University, Faculty of Veterinary Medicine (SUVF) culture collection. As ingredients, One Shot® Mach1™-T1R chemically competent *E. coli*, One Shot® chemically competent *E. coli* BL21(DE3), pET SUMO TA cloning reagents (linearized-pET SUMO vector, 10X PCR buffer, dNTP mix, 10X ligation buffer, T4 DNA ligase, SUMO forward sequencing primer, T7 reverse sequencing primer and control PCR template) included content in the Champion pET SUMO Protein Expression System (Invitrogen, USA) was used for protein production. *B. melitensis* H38 was cultured in Brucella broth (Sigma-Aldrich, USA) for five days at 37°C (shaking at 160 rpm), whereas Luria-Bertani (LB) medium was used for *E. coli* cultivations. SOC and LB media containing 50 μg kanamycin/ml were used for the selection of transformed cells.

PCR amplification and cloning of OMP19

The OMP 19 open reading frame from *B. melitensis* H38 was amplified by PCR, using the primers OMP19-F (5'-ATGGGAATTTCAAAAGCAAGT-3') and OMP19-R (5'-TCAGCGCGACAGCGTCA-3') which were designed based on information obtained from the

National Center for Biotechnology Information (NCBI) (accession number KP101384). PCR amplification was achieved using 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 90 s and extension at 72°C for 90 s. The resulting amplified product of 534 bp (which would synthesise a 19 kDa outer membrane protein) was eluted from agarose gel using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, U.S.) and ligated (because the PET SUMO vector is linearized) to the pET-SUMO vector with T4 DNA ligase before being transformed (with a heat shock method) into One Shot® Mach1™-T1R competent *E. coli* cells. The linear pET SUMO vector has single 3′ deoxythymidine (T) residues at its ends, allowing the PCR products by Taq polymerase to bind conformably to the vector with a single deoxyadenosine (A) added to their 3' ends. After transformation, resistant *E. coli* colonies were selected that could grow on LB agar containing kanamycin (50 µg/ml). Positive inserts were screened via direct colony PCR using the designed vector-specific primers (i.e. pET SUMO forward (5′-AGATTCTTG TACGACGGTATTAG-3′) and T7 Reverse primer (5′-TAGTTATTGCTCAGCGGTGG-3′)). PCR amplification was performed according to the Champion pET SUMO Protein Expression System (Invitrogen, USA) kit instruction. Positive PCR products were sequenced. Nucleotide sequences were analysed using the NCBI database's Basic Local Alignment Search Tool (BLAST®). According to the results of PCR and sequence, the *E. coli* colony carrying the pET-SUMO--OMP19 clone (the OMP19 ORF in the most accurate one) was passaged in 5 ml of LB broth for plasmid production and incubated for 12 h at 37°C. The pET--SUMO-OMP19 plasmid clone in *E. coli* grown in broth was isolated with the ZymoPURE Plasmid Miniprep Kit (Zymo Research, U.S.). Once again, with a heat shock method pET-SUMO-OMP19 clone replicates were transformed into *E. coli* BL21(DE3) for protein expression. Similar to the first transformation, colonies with antibiotic resistance in LB medium were confirmed by PCR with pET SUMO forward and T7 reverse universal primers.

Production and purification of recombinant OMP19 protein

E. coli BL21(DE3) cells containing the pET-SUMO--OMP19 clone were incubated in LB medium (in a bottle twice the volume) at 37°C for 2 h at 200 rpm until an absorbance of 0.5 (OD at 600 nm) was reached. Thereafter, cells were induced with 1 M Isopropyl β-d-1-thiogalactopyranoside (IPTG) and allowed to grow for a further 12 h at 20°C. The supernatant was passed through 50 kDa-10 kDa filters to concentrate the 19 kDa protein. In addition, the His-tag labelled insoluble recombinant protein was eluted (with high purity) using the Ni-NTA Spin Kit (Qiagen, Germany) lysis protocol which allowed extracting proteins remaining in the cell pellet. The supernatant and purified OMP19 were analysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and this protein was named SUVF-OMP19.

Protein concentration, SDS-PAGE and immunoblot assays

The concentration of supernatant and pellet proteins were measured using the Bradford method (Bradford 1976). Thereafter, purified proteins were analysed with SDS-PAGE before being transferred to nitrocellulose membranes in a semi-dry transblot system (Trans-Blot Turbo Transfer System, BioRad). High titer brucellosis--infected sheep serum, i.e. the primary antibody, was diluted in 5% skimmed milk powder in a blocking solution with a final concentration of 1:200. The membrane was subsequently incubated overnight in this primary antibody solution at 4 °C (shaking at 50 rpm), before being washed with TBS-T for 5 min (repeated three times). Anti-Sheep donkey IgG (whole molecule) peroxidase conjugated antibody (Sigma-Aldrich, USA), i.e. the secondary antibody, was diluted in 5% skimmed milk powder in a blocking solution with a final concentration of 1:2000. The membrane was incubated in the secondary antibody solution for 1 h, after which the latter was collected and the membrane was washed in TBS/T buffer (repeated three times). Colorimetric detection was performed using a Chemiluminescent peroxidase substrate (Sigma-Aldrich, USA).

Compared immunogenic power of recombinant OMP19 in a mouse model

After obtaining approval from the Ethical Committee, Selcuk University Experimental Medicine Application and Research Center (Konya, Turkey) (decision #2017-17, dated 25.04.2017), immunogenicity mice trials were performed according to World Organisation for Animal Health (OIE) chapter 3.1.3 Brucellosis (infection with *Brucella abortus*, *B. melitensis* and *B. suis*). Mice aged 5-7 weeks and weighing 18-23 grams made up the experimental groups and control group (with six female mice in each group) (OIE 2022). Vaccine doses containing 20 µg SUVF-OMP19 purified protein (generated in the current study) and 20 µg commercial OMP19 protein (Raybiotech Inc, USA) were separately mixed with incomplete Freund's adjuvant (IFA) (F5506, Sigma-Aldrich, USA) (Lai et al. 2012) and were administered intraperitoneally to mouse. The negative control group was intraperitoneally

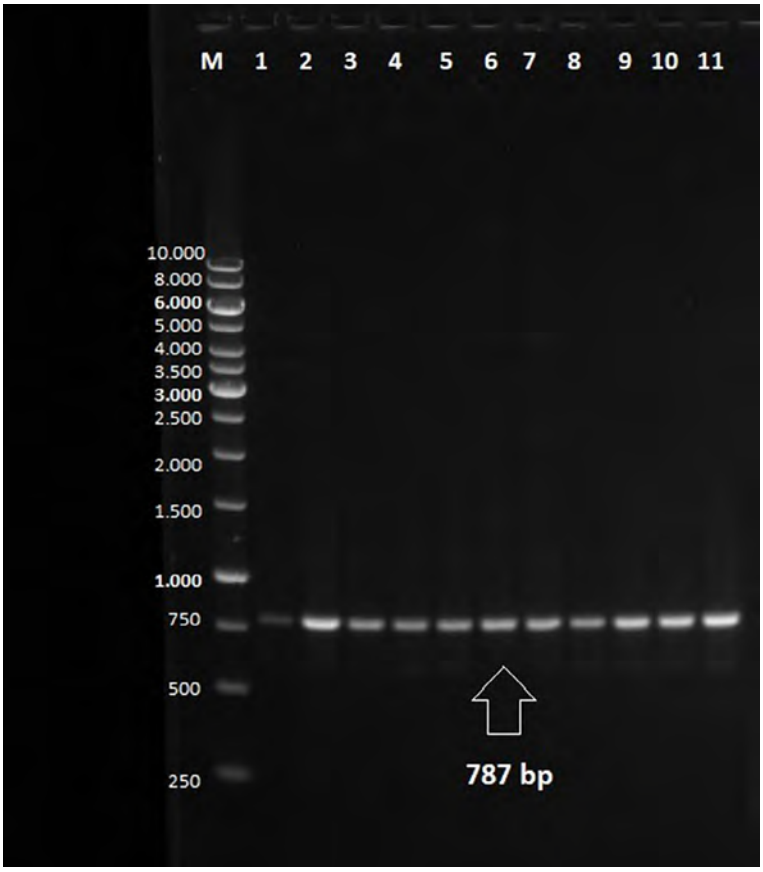

Fig. 1. PCR products in agarose gel electrophoresis of 11 transformed *E.coli* OneMatch cells using pET-SUMO vector-specific primers with product size 787 bp (253 bp vector backbone and 534 bp OMP19 insert) (line M), 1 kb DNA ladder (Thermo Scientific, USA).

injected with PBS. Mice in the two recombinant protein vaccine groups and the negative control group were administered the vaccines twice, at 21 days intervals, for a total of 42 days.

ELISA procedure of Whole-Cell *B. melitensis* H38 and Recombinant OMP19

B. melitensis H38 whole cell lysate used as antigens for homemade ELISA. *B. melitensis* H38 was passaged on blood agar. A single colony from the isolated bacteria was passaged into 25 ml of Brucella broth. The enriched culture was transferred into 100 ml Brucella broth. The culture was centrifuged at 9000 rpm for 5 minutes and the bacterial pellets were collected in sterile bottles. The obtained *B. melitensis* H38 bacteria pellet was frozen at -80 degrees and thawed 10 times. Sonication was performed at 25 msn x 10 pulse during each thawing process (Uslu and Erganis 2021). According to the Bradford assay procedure, the amount of protein in lysed whole-cell *B. melitensis* H38 antigen was measured (Bradford 1976). Because the ELISA evaluation is sensitive, coating with recombinant protein was performed only with commercial recombinant OMP19 protein, 100 µg /ml (Raybiotech Inc, USA). After diluting (0.5 µg in each well) both whole-cell *B. melitensis* H38 and recombinant OMP19 antigen, with 0.01 M phosphate-buffered saline (PBS), pH 7.2, was distributed in flat-bottom 96-well ELISA plates (Kartell S.p.a, Italy) and incubated at + 4°C overnight. After incubation, the wells were emptied and washed (five times) with phosphate buffer saline-Tween20 (PBST) before being blocked with 5% skimmed milk powder. Thereafter, plates were charged with mouse sera at a dilution of 1:50 and were incubated at 37 °C for 1 h. After washing with PBST (five times), the plates were incubated with rabbit anti-mouse horseradish peroxidase (HRPO) conjugate (A9044, Sigma-Aldrich, USA) at 37°C for 1 h. After subsequent washing with PBST, the wells of immunoassay plates were filled with substrate solution containing ortho-phenyl diamine (OPD) (p8287, Sigma-Aldrich, USA). Then, it was incubated in dark place for 30 minutes, and the results were evaluated with the ELISA reader at 450 nm.

Statistical analysis

Variables were determined as mean±standard deviation, median (max-min) percentage, and frequency values. Also, the homogeneity of variances, one of the

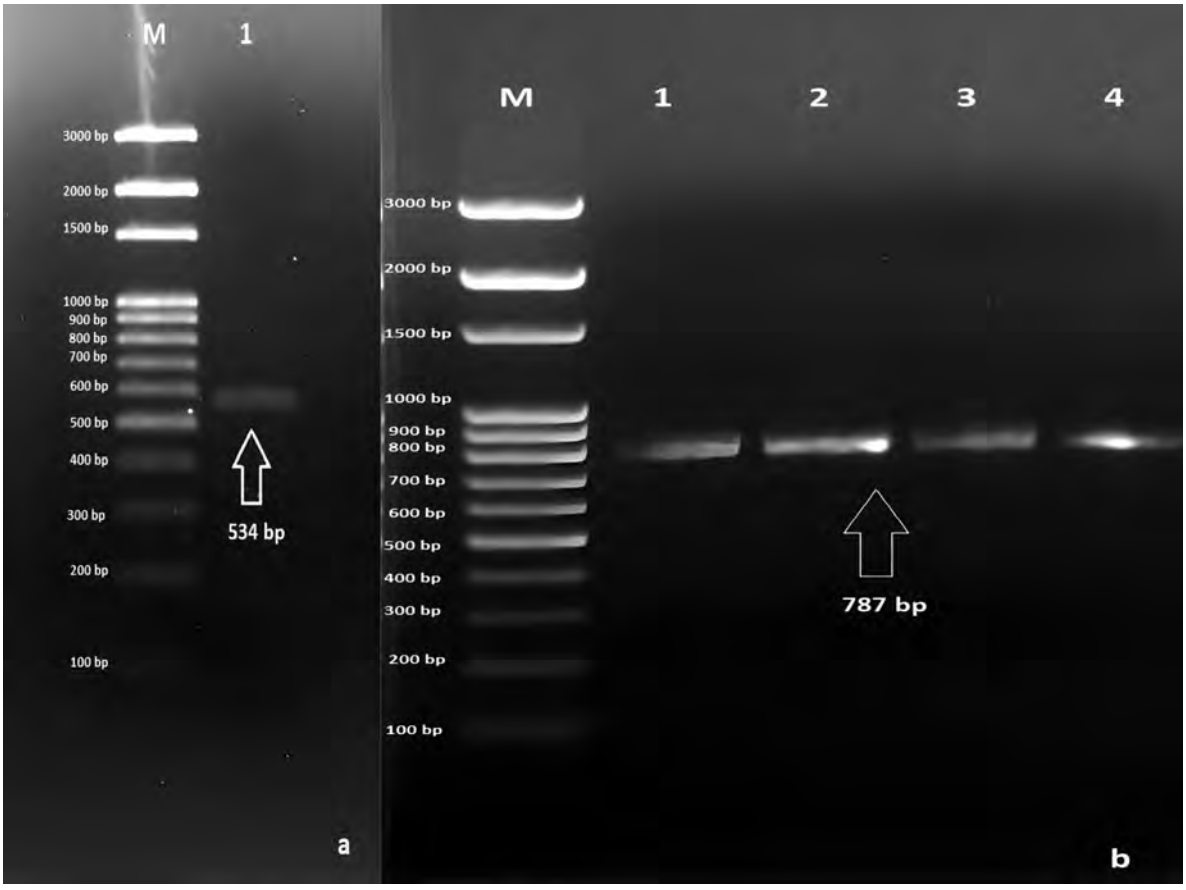

Fig. 2. PCR products in agarose gel electrophoresis a. *Brucella melitensis* H38 using gene-specific primers for OMP19 amplification (line M), 100 bp DNA ladder (Solis biodyne, Estonia); line 1- OMP19 (534 bp) b. Four transformed *E.coli* BL21(DE3) cells using pET-SUMO vector-specific primers with product size 787 bp (253 bp vector backbone and 534 bp OMP19 insert) (line M), 100 bp DNA ladder (Solis biodyne, Estonia); transformed cell 1 (line 1), transformed cell 2 (line 2), transformed cell 3 (line 3), transformed cell 4 (line 4).

prerequisites of parametric tests, was checked using the "Levene" test. The normality assumption was examined by the "Shapiro-Wilk" test. "Student's t-Test" was used for parametric data, and the "Mann Whitney-U test" was used for non-parametric data to evaluate the differences between the two groups. Additionally, the multiple comparison tests ANOVA and Bonferroni were used to compare three or more groups, and in case of non-availability of these tests, Kruskal Wallis and Bonferroni-Dunn tests were used for multiple comparisons. The antibody titer for the sera potential of ELISA groups was evaluated by ROC analysis. Thus, the AUC value, sensitivity, selectivity values were calculated. SPSS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) software was used to evaluate the data. p-values of <0.05 and <0.01 were considered statistically significant.

Results

Construction and sequencing of pET-SUMO-OMP19 cloning vector

The OMP19 gene was amplified from the chromosomal DNA of *B. melitensis* strain H38 (producing the expected product of 534 bp) and subsequent ligation and transformation (of the OMP19 PCR product into the vector) produced 11 colonies of One Shot® Mach1™-T1R competent *E. coli* (Fig. 1). Among these 11 colonies, the colony with the clone vector was selected according to the PCR result and sequence analysis. Analysis of nucleotide sequence of Omp19 was deposited in the Genbank with accession number [GenBank: ON813304]. The selected colony was incubated, and sufficient pET-SUMO-OMP19 clone DNA was obtained for transformation with the Plasmid Miniprep Kit. Four transformed colonies were obtained after transferring pET-SUMO-OMP19 to BL21 (Fig. 2).

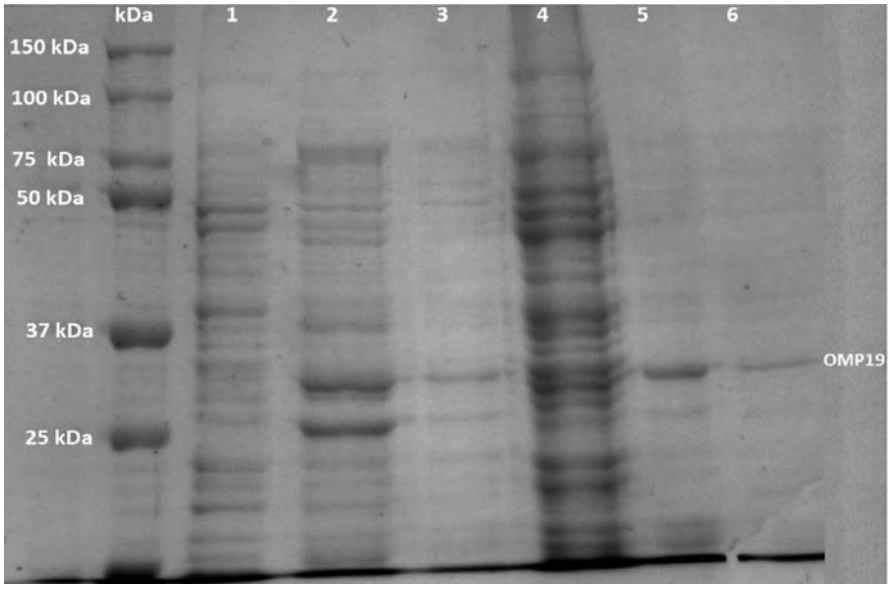

Fig. 3. SDS–PAGE analysis of recombinant OMP19 (expressed in *Escherichia coli*). 1- uninduced cell lysate; 2- cell lysate protein (isolation with Ni-NTA); 3- protein in the supernatant; 4- cell lysate column adhesion control; 5- cell lysate and supernatant (post cassette filter protein isolation with Ni-NTA); 6- OMP19 (Raybiotech Inc, USA).

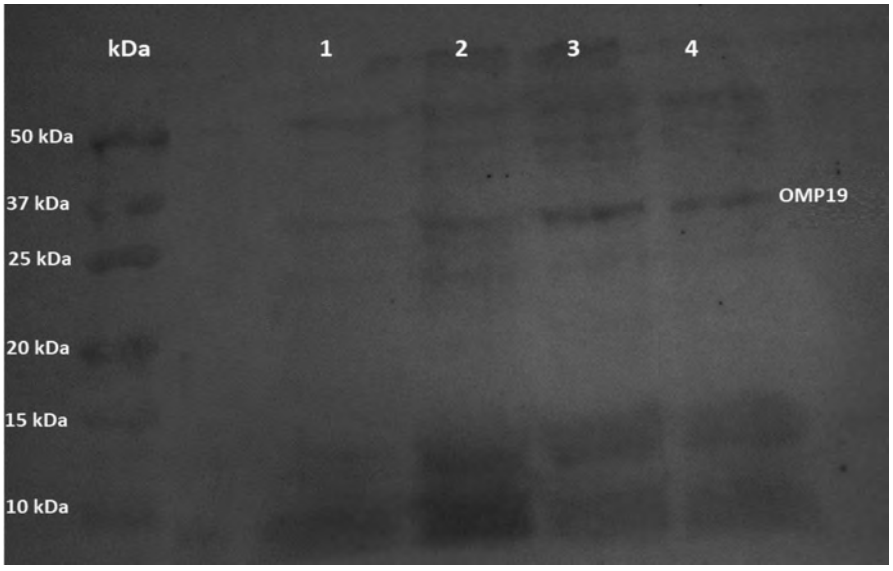

Fig. 4. Western-Blotting with naturally Brucellosis-infected sheep sera reacting with Omp 19, 1- supernatant protein; 2- cell lysate (protein isolation with Ni-NTA); 3- cell lysate and supernatant (post-cassette filter protein isolation with Ni-NTA); 4- OMP19 (Raybiotech Inc, USA).

SDS-PAGE and Western Blot analyses

Protein (90.7 µg/µL) from BL21 cell lysates and supernatants (containing the clone pET-SUMO-OMP19 induced by 1M IPTG) that had been isolated with the Ni-NTA kit and concentrated by the cassette filter, banded in the same region as the 5 µg/µL commercial OMP19 during SDS-PAGE analysis. Using filter concentration, 5 µg/µL protein had been obtained from the supernatant, whereas 18.9 µg/µL protein had been obtained from induced cell lysate. No OMP19 production was detected in uninduced *E. coli* cells (Fig. 3).

Western blot analysis of purified recombinant OMP19 (1:200 dilution) was performed with polyclonal serum from *Brucella*-infected sheep (Fig. 4). Due to the presence of the His-tag protein (11 kDa), the synthesized protein was displayed as 30 kDa.

OMP19 humoral immune responses

Mice were immunised with synthesised recombinant OMP19 protein (SUVF-OMP19) in IFA. Alternatively, mice were treated using IFA with commercial OMP19 protein (positive control) or IFA with PBS (negative control). Results indicated that the SUVF-OMP19 synthesised protein produced the

Table 1. Antigenic power comparison of two different recombinant OMP19 vaccines in a mouse model.

Group/Variable (mean ± SD) n=6	Negative control	Omp19 vaccine	SUVF-Omp 19 vaccine	p value
Whole Cell ELISA	0.15±0.01	0.16±0.08	0.3±0.02	0.001
Protein ELISA	0.22±0.04	1±0.05	0.86±0.07 [a]	0.001

a: Significantly Different from Negative Control
b: Significantly Different from Omp 19 Vaccine
c: Significantly Different from SUVF-Omp 19 Vaccine

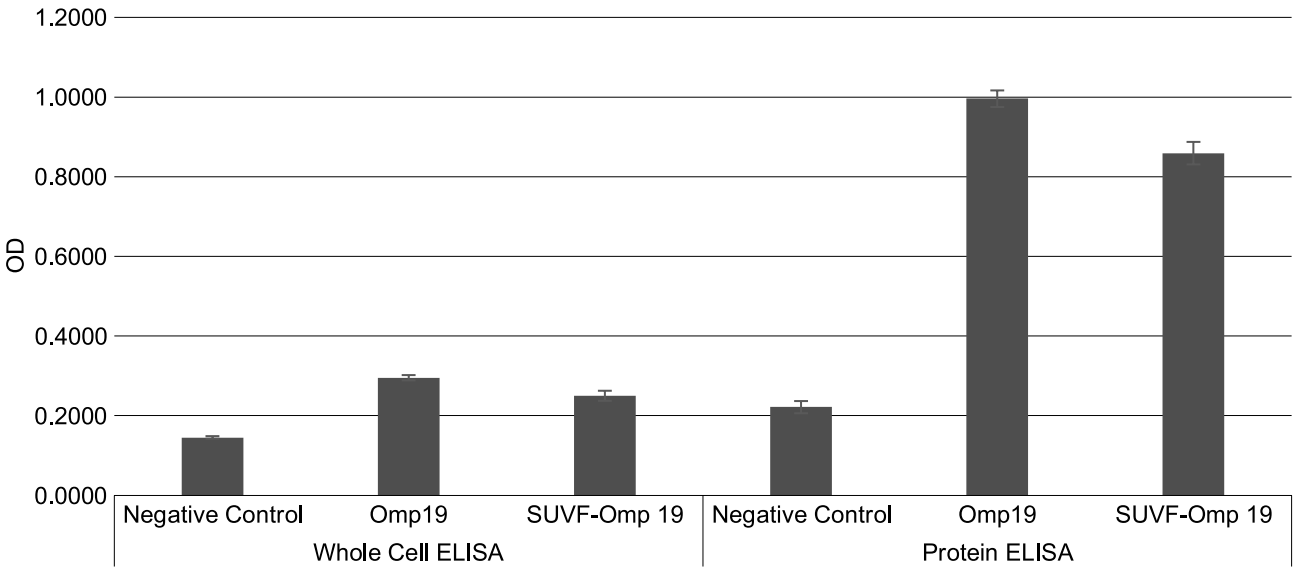

Fig. 5. Comparative results of the antigenic potency of synthesised recombinant OMP19 protein (SUVF-OMP19) in ELISA when coated with whole-cell or OMP19 protein.

same antibody strength as its commercial counterpart (Table 1).

In the ELISA coated with OMP19 protein, when the two groups were compared, the recombinant SUVF-OMP19 vaccine group gave as strong reactions as the positive control, but the whole-cell ELISA reactions remained lower (Fig. 5).

Discussion

Classically attenuated or inactivated bacterial vaccines are being replaced by subunit vaccines that are prepared from immunodominant antigens (Hou et al. 2019, Yin et al. 2020). Due to its lipoprotein structure, OMP19 from *Brucella* spp. has been shown to have immunodominant, protective, and antigenic properties (Zai et al. 2021). As such, the OMP19 antigen has been tested in model animal trials that stimulate different mucosal immune systems (Pasquevich et al. 2011). In such *Brucella* vaccine studies, the rapid rise and fall of antibody levels and continued immunity via the cellular immune system is desirable. Recombinant synthesis and purification of this lipoprotein, which plays an essential role in the pathogenesis of brucellosis, might thus contribute to the development of differential diagnostic kits and new vaccine strategies.

In the current study, the bands obtained for (insoluble) recombinant OMP19 during SDS-PAGE and Western blot analyses were larger than the expected 19 kDa. Wingfield (2015), suggested that this may be as a result of post-translational modifications and changes in the protein's folding and 3D structure (due to His-tag fusion). This fusion protein of approximately 2.5 kDa does not affect protein function during synthesis (Booth et al. 2018), although some researchers have reported that it may assume different positions and lengths during recombinant protein synthesis (Araújo et al. 2000, Mohanty and Wiener 2004). Although the synthesised recombinant protein of the current study had an increased average kDa weight, it also showed high binding strength in the ELISA coated with OMP19 protein – confirming that it was antigenically similar.

Unlike the target animal species, it is challenging to produce brucellosis antibodies in mouse models (Grilló et al. 2012). Yet, the two, recombinant OMP19s protein synthesised and used in this study produced a similar and robust level of antibody response. Consi-

dering the ethical principles, the protein whose effectiveness has been proven by other studies, the challenge trials specified in the OIE were not performed in this study (Pasquevich et al. 2009, Uslu and Erganis 2021).

ELISA analysis of the recombinant OMP19 vaccine groups (with *Brucella* whole-cell antigens) detected (weak) binding in all antigenic regions through a single protein epitope. In a previous study focusing on *Brucella* OMP28, crucial differences were noted in whole-cell ELISA and ELISA with recombinant proteins (Chaudhuri et al. 2010).

It has been shown that rapid diagnostic test kits may be developed with synthesised recombinant proteins relevant to the veterinary field (Zhang et al. 2012). Vaccination against brucellosis in humans remains essential because of the infection risks to the human population, such as veterinary medicine and farmer dealing with animal husbandry. However, currently, no live vaccine can be effectively and safely used in humans (Perkins et al. 2010), although safe and effective candidates may be produced with recombinant proteins. Therefore, recombinant vaccines that have been successful in model animals (such as that of OMP19) should subsequently be assessed in human trials. Furthermore, with the development of mRNA vaccine technology, new vaccine models could be prepared by targeting the proteins responsible for the pathogenesis of brucellosis.

Conclusion

The current study reports the production of OMP19 which, similar to previous studies, was proven to be immunogenic and protective. It is thought that the antigenic power of this protein (due to the antibody response created in the mouse model) will provide a step toward creating vaccines for humans. It might also aid in the development of monoclonal antibodies and diagnostic kits for *Brucella* vaccine markers.

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