Detection of *Brucella abortus* by immunofluorescence assay using anti outer membrane protein of 19 kDa antibody

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online)

Adv Clin Exp Med. 2018;27(5):643–648

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Funding sources

This investigation was supported by grant No. 96003341 from Iran National Science Foundation (INSF).

Conflict of interest

None declared

Received on December 25, 2016

Reviewed on January 18, 2017

Accepted on February 6, 2018

Abstract

Background. Brucellosis in humans is one of the most prevalent zoonotic diseases around the world with more than 500,000 new cases per year. It is a weakening disease that requires long-term antibiotic treatment, often resulting in permanent and disabling consequences. Outer membrane proteins (OMPs) of *Brucella*, which are non-lipopolysaccharide (LPS) antigens, have been used for the diagnostic kits of brucellosis and vaccine design.

Objectives. The aim of this study was to identify *Brucella abortus* with an immunofluorescence (IF) test using an antibody against recombinant outer membrane protein (OMP) of 19 kDa of this bacterium.

Material and methods. The OMP19 gene of *Brucella* spp. was synthesized, cloned and expressed in *Escherichia coli* cells. The OMP19 protein was purified by metal chelate affinity chromatography and subsequently used for the immunization of rabbits to produce a polyclonal antibody. Then, this antibody was conjugated to fluorescein isothiocyanate (FITC) and used for the detection of *Brucella* by an IF test. Also, the sensitivity and specificity of this antibody for the diagnosis of clinical isolates was calculated.

Results. Outer membrane protein 19 was expressed well and reacted with a commercial antiserum against His-tag in an immunoblot assay. Polyclonal antibodies obtained from the serum of rabbits immunized with the purified protein showed strong reactivity in the enzyme-linked immunosorbent assay (ELISA). Moreover, the polyclonal antibody conjugated to FITC was able to properly identify *Brucella abortus*. Sensitivity and specificity of this IF test in comparison with a polymerase chain reaction (PCR) assay was 84.2% and 50%, respectively.

Conclusions. This high-titer antibody could potentially be valuable for the specific diagnostic test of brucellosis.

Key words: antibody, immunofluorescence assay, OMP19, *Brucella abortus*

DOI

10.17219/acem/85081

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Introduction

Brucella spp. are intracellular Gram-negative bacteria that cause human disease and significant economic losses worldwide due to livestock infection.¹ Lipopolysaccharide (LPS) of *Brucella* spp. elicits long-term serological reactions in vaccinated and infected animals.^{1–4} In serologic tests, LPS of the bacterial cell membrane has been mainly used to identify particular antibodies. For these reasons, it is difficult to differentiate between infected and vaccinated animals using LPS-based serological tests.^{5–8} Moreover, serological examinations on the basis of the detection of LPS, due to a cross-reaction with other Gram-negative microorganisms, for example *Salmonella* spp., *Escherichia coli* and *Yersinia enterocolitica* O:9, cause false-positive reactions.^{9–12} Outer membrane proteins (OMPs) of *Brucella*, which are non-LPS antigens, have been used for the diagnostic kits of brucellosis and vaccine design.^{13–18} The *Brucella* cell wall consists of a peptidoglycan (PG) layer firmly connected with the external layer consisting of at least 75 proteins with several OMPs.¹⁹ These include the major OMPs of group 2 (porin, 34–40 kDa), group 3 (25–30 kDa) that was described initially by Dubray and Bezard²⁰ and Verstrete et al.,²¹ the lipoprotein covalently bound to PG,^{22,23} and the minor OMPs of group 1 (88–94 kDa). The OMPs of *Brucella* spp. have been widely considered as potentially defensive and immunogenic antigens.^{24,25} Monoclonal antibodies (mAbs) against group 1, 2 and 3 OMPs, as well as mAbs to minor surface-exposed OMPs with molecular masses (MMs) of 10, 16, 19, and 31–34 kDa (the latter is a major OMP in *B. melitensis* strains but is less abundant in *B. abortus*), have been produced.²⁶ Physicochemical and practical examination has recently confirmed that OMP10, OMP16 and OMP19 are lipoproteins that are uncovered at the cell surface.²⁷ It has been proven that these lipoproteins are present in several *Brucella* strains. These strains represent all 6 *Brucella* species and all their biovars. According to published data, OMP19 is an immunoreactive outer membrane lipoprotein.^{28–33} The appearance of OMP19 has been proven to be necessary for the acceptance of a defensive reaction by the vaccine strain *B. abortus* S19, since the abrogation of its gene in this strain leads to the destruction of its protective ability in heifers,³⁴ showing that OMP19 should be a key component of a subunit vaccine against brucellosis. Furthermore, we have previously reported that recombinant OMP19, when injected with the mucosal adjuvant cholera poison, is a protective mucosal antigen that confers protection against an oral challenge with virulent *Brucella*.³⁰ For this reason, in this research, we expressed OMP19 for the creation of a polyclonal antibody to use it in an immunofluorescence assay (IFA).

The study was endorsed by Animal Experimentation Ethics Committee of Kerman University of Medical Sciences, Iran.

Material and methods

Cloning and expression

The OMP19 gene was synthesized based on the existing gene sequence of *B. abortus* (accession No. U35742) and cloned into the pET-28a expression vector by Genray Biotechnology Company (Shanghai, China). The pET-28a vector allowed the expression of the cloned gene as a fusion protein with a 6-histidine residue at the amino terminus. The recombinant plasmid was transformed into an *E. coli* strain BL21 (DE3) competent cell by the heat shock method (42°C for 90 s), according to standard protocols.³⁵ The transformant bacterium was chosen from Luria Bertani (LB) agar medium containing 30 µg/mL of kanamycin. The transformant was grown in 10 mL LB containing 50 mg/mL kanamycin at 37°C, shaken at 200 rpm overnight. Then, 1 mL of the medium was supplemented to 100 mL of fresh LB. The transformant was grown at 37°C until the optical density (OD) at 600 nm achieved 0.6, and after being prompted by including Isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and incubation at 25°C overnight with shaking. The transformant was amassed by centrifugation at 5000 × g for 10 min, then the cell was resuspended in the (5-pellet volume) lysis buffer pH 8.0 (300 mM NaCl, 10 mM Imidazole, 50 mM NaH₂PO₄) and incubated at 4°C for 30 min. The lysate was also sonicated (10 s pulse on followed by 10 s pulse off, total time: 4 min). The resulting lysate was centrifuged at 5000 × g for 30 min at 4°C. The supernatant including the recombinant protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 5% stacking and 15% resolving gels, dot blot and western blot analyses. The recombinant OMP19 was purified using metal chelate affinity chromatography by Ni-NTA column (Qiagen, Hilden, Germany).

Immunization

A New Zealand white female rabbit was immunized intramuscularly with the purified recombinant OMP19 protein (0.5 mg/mL) mixed with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, USA) (1:1) administered into the back legs of the rabbit, followed by 2 injections emulsified with Freund's incomplete adjuvant and without adjuvant, respectively, at 2-week intervals.

Antiserum collection

The rabbit was bled 4 times (before each injection and 2 weeks after the 3rd injection). Blood was obtained from the marginal vein of the ear and kept at 4°C overnight. Serum was isolated by centrifugation at 3000 × g for 10 min and put away at –20°C until utilized.

To evaluate the serum titer, 2 enzyme-linked immunosorbent assay (ELISA) tests were carried out. In the

1st ELISA test, the reaction of serum during immunization against the purified recombinant protein was tested. In the 2nd ELISA test, final serum was diluted from 1/100 until 1/100000 to determine the serum titer.

Enzyme-linked immunosorbent assay

Sera were assessed by ELISA for antibody reactivity vs the recombinant OMP19 as follows: 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark) were covered with the recombinant OMP19 (100 µL per well) in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, USA) at a density of 25 µg/mL and incubated overnight at 4°C. The wells were washed 3 times with PBS and blocked with 200 µL of blocking buffer PBS containing 3% bovine serum albumin (BSA) (Sigma-Aldrich) for 2 h at 37°C. After 3 washes, the diluted sera were added and the plates were incubated for 1 h at 37°C. The plates were then washed 3 times with 0.1% (v/v) Tween-20 in PBS (PBST). Antibodies attaching to OMP19 were visualized using 100 µL polyclonal goat peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Inc., Hercules, USA) diluted 15,000-fold in blocking buffer. After 1-h incubation at 37°C, the plates were washed with PBST and developed with 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (1 mL TMB, 9 mL of citrate-phosphate buffer, pH 5, and 3 µL of 30% H₂O₂). The reaction was stopped after 15 min by the addition of 50 µL 1.0 M H₂SO₄ to each well and read at 450 nm by the ELISA plate reader.

Immunofluorescence test

The polyclonal antibody was purified using the Proteus Protein Mini Purification Spin Column Pack (Bio-Rad Laboratories, Inc., Hercules, USA). The antibody was conjugated to fluorescein isothiocyanate (FITC) using standard protocols. Briefly, 40 µL of antibody (100 µg/mL) was mixed with 80 µL of borate buffer (0.1 M). Then, 4 µL of FITC (1 mg/mL) was added to 120 µL of the mixture and incubated at 37°C for 30 min. The conjugated antibody was stored at 4°C until use.

The detection of *B. abortus* by IFA: The amount of 500 µL of culture medium, which included *B. abortus*, *E. coli*, *Salmonella*, and *Klebsiella* (negative controls), was centrifuged at 4000 × g for 10 min. The resulting pellets were washed 3 times with PBS and suspended in 200 µL of PBS. Then, 20 µL of the conjugated antibody was added and the suspensions were incubated at room temperature overnight with shaking. Subsequently, the suspensions were centrifuged, washed 3 times with PBS, and then 10 µL of the samples were examined under a fluorescence microscope.

In this study, 29 bacterial isolates including 19 *Brucella* isolates, confirmed with specific primers in the polymerase chain reaction (PCR), and 10 non-*Brucella* isolates without a specific amplicon in the PCR, were used to determine the sensitivity and specificity of the above-mentioned immunofluorescence (IF) test, using the anti-OMP19 antibody.

The results of variables, including truly positive (TP), truly negative (TN), false positive (FP), and false negative (FN) ones, were analyzed using the following formulas:

$$\text{Sensitivity} = [\text{TP}/(\text{TP} + \text{FN})] \times 100$$

$$\text{Specificity} = [\text{TN}/(\text{TN} + \text{FP})] \times 100$$

Results

Expression and purification

The induced transformant was examined using SDS-PAGE and immunoblotting. The SDS-PAGE examination of extracted total proteins indicated that OMP19 was expressed successfully with an estimated molecular weight of 19 kDa (Fig. 1, 2). The protein was transferred to the nitrocellulose membrane and the specificity of the expressed 6×His-tagged recombinant OMP19 protein was determined using a commercial anti-His-tag antibody (Sigma-Aldrich, St. Louis, USA) by an immunoblot assay (Fig. 3, 4). The recombinant protein was purified with an affinity chromatography and subsequently used for the immunization of the rabbit.

Fig. 1. SDS-PAGE analysis of total cell lysate of *E. coli* producing the OMP19 protein

OMP19 – the recombinant outer membrane protein 19 kDa; lane 1 – OMP19; lane M – protein prestained ladder; the arrowhead shows the location of the *Brucella* recombinant protein.

Fig. 2. SDS-PAGE analysis of the purified recombinant *Brucella* OMP19 protein

OMP19 – the recombinant outer membrane protein 19 kDa; lane M – protein prestained ladder.

Fig. 3. Dot blot analysis of the expressed His-tagged OMP19 protein, using a commercial anti-His-tag peroxidase-conjugated antibody

OMP19 – the recombinant outer membrane protein 19 kDa; lane 1 – OMP19; lane 2 – negative control.

Fig. 4. Western blot analysis of the expressed His-tagged OMP19 protein, using a commercial anti-His-tag peroxidase-conjugated antibody

OMP19 – the recombinant outer membrane protein 19 kDa; lane 1 – OMP19; lane M – protein prestained ladder.

Production of a polyclonal antibody

The immunization of a New Zealand white female rabbit led to the production of a specific polyclonal antibody against the OMP19 protein. As Fig. 5 shows, during the immunization procedure the titer of the antibody against this protein rose gradually. Figure 6 indicates that a high affinity antibody with a strong reaction was achieved after the last immunization.

Fig. 5. The production of the polyclonal antibody against the recombinant outer membrane protein 19 kDa (OMP19) of *Brucella* spp. during the immunization of a rabbit by the enzyme-linked immunosorbent assay (ELISA)

OD – optical density.

Fig. 6. Determination of the titer of the polyclonal antibody by the enzyme-linked immunosorbent assay (ELISA)

OD – optical density.

Recognition of *Brucella* with a fluorescence microscope

The anti-OMP19 polyclonal antibody conjugated to FITC was able to properly identify *B. abortus*, while the negative controls were not identified (Fig. 7). In evaluating sensitivity and specificity, 16 of 19 *Brucella* isolates and 5 of 10 non-*Brucella* isolates were IF-positive in the test. Therefore, the sensitivity and specificity of this test were 84.2% and 50%, respectively.

Fig. 7. Recognition of *Brucella* with a fluorescence microscope

A – *B. abortus*; B – negative controls.

Discussion

The diagnosis of brucellosis by serological examinations on the basis of the detection of antibodies against LPS of bacterial cell membranes causes false positive results in some cases because of cross-reactivity with other Gram-negative microorganisms. The OMPs of *Brucella* spp. have been widely considered as potential immunogenic and protective antigens.

In this research, 1 OMP of *Brucella*, a non-LPS group of immunogens, was used. The OMP19 was expressed as a fusion protein containing 6×His-tag. The recombinant OMP19 reacted positively with the antibody against His-tag in blotting assays. The immunization of a New Zealand white female rabbit by the recombinant OMP19 led to a high-titer polyclonal antibody, as showed in ELISA. The high-titer polyclonal antibody can be utilized for experimental biology, medicine, biomedical research, the development of a vaccine and improved diagnostic tests. Some other investigators have previously produced particular antibodies vs several OMPs of *Brucella*. For example, several mAbs to 7 *Brucella* outer membrane proteins were considered by Cloeckaert.¹⁴ These antibodies were achieved by vaccinating mice with sodium dodecyl sulfate-insoluble (SDS-I) portions, cell walls, or whole bacterial cells of *B. melitensis* or *B. abortus*. The OMPs detected by these antibodies were the proteins with a molecular weight of 25–27 kDa and 36–38 kDa (porin) (major proteins), and the proteins with a molecular weight of 10, 16.5, 19, 31–34, and 89 kDa (minor proteins).²⁶

The production of an mAb against OMPs with a molecular mass of 25–27 kDa (Ba-4, Ba-5), 36–38 kDa (Ba-6) of *B. abortus* S99 and rOMP31 of goat *Brucella* was performed by Farshad et al. and Zheng et al.^{36,37} It was proven by Ghasemi et al. that the immunized serum was achieved from a rabbit that was inoculated with attenuated *B. melitensis*. This serum contained antibodies against recombinant OMP31 (rOMP31).³⁸ The production of antibodies against OMPs of *B. ovis* in rams was performed by Riezu-Boj et al.³⁹ The production of immunoglobulins was investigated by Sung et al. in BALB/c immunized with Cu/Zn superoxide dismutase (SOD1) and OMP2b porin of *B. abortus*.⁴⁰ The production of an antibody against the minor 89-kDa OMP of *Brucella* in cattle was performed by Limet et al.⁴¹ Western immunoblot analysis using antisera raised against 3 strains of *B. abortus*, i.e., RB 51, S19 and a local field isolate of biotype 1 in buffaloes, indicated the production of antibodies against OMPs in a study by Munir et al.⁴² On the basis of previous studies, it can be stated that OMPs of *Brucella* are capable of inducing an immune response and antibody production. These antibodies can be utilized for the improvement of vaccine candidates and diagnostic tests of brucellosis.

The antibody produced against the recombinant OMP19 had a high titer. Therefore, it could be utilized for diagnostic tests of brucellosis and the improvement of vaccine candidates for humans and animals. Moreover, this antibody conjugated

to FITC was able to properly identify *B. abortus*. Therefore, it could be used for diagnostic tests of brucellosis in humans and animals. Although the sensitivity of antibodies was high, their specificity was moderate in an IF test. This could be due to some cross-reactions of the polyclonal antibody.

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