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Desarrollo y caracterización de anticuerpos IgY contra péptidos sintéticos de las proteínas OMP25 y BP26 de *Brucella abortus*

Development and characterization of IgY antibodies against synthetic peptides from Brucella abortus *OMP25 and BP26 proteins.*

Claudia Moreno[1]*, Noraida Zerpa[1], Caridad Malavé[1], Henry Bermúdez[2], Jhonny Demey[1], Rodolfo Fernandez-Gomez[1] & Olaf Ilzins[1]

SUMMARY

Brucellosis is a zoonose produced by bacterial species from the *Brucella* genus. Its isolation and identification in food using classical microbiological techniques is not practical due to its slow growth rate. Therefore, it is necessary to establish fast and specific methods for the detection of the bacteria in food. The goal of this work was the production and characterization of monospecific polyclonal antibodies in chicken (IgY) against synthetic peptides from *Brucella abortus* OMP25 and BP26 proteins, suitable for an antigen-capture assay. Conformational as well as antigenic predictions were performed using the ANTHEPROT package. Chemical synthesis was carried out by the multiple manual synthesis using the t-boc strategy. The peptides were used as antigens for the preparation of polyclonal antibodies in chicken. Experimental animals produced specific antibodies against the OMP25 and BP26 peptides constructs determined by ELISA and MABA assays showing correspondence between the predictive study and the immunogenicity obtained in chicken. The IgY proved to be able to recognize *B. abortus* by MABA assays. The binding activity and specificity of antibodies was determined by Western blot with cell extract from *B. abortus*. In this study, we demonstrated that OMP25 and BP26 peptides constructs are good candidates for production of specific IgY antipeptide antibodies capable of recognizing proteins from sonicated *B. abortus* strain S19, indicating the potential usefulness of the IgY antibody for development of immunoassays for detection of *Brucella abortus*.

Key words: *Brucella* sp., Immunoglobulin Y, Synthetic peptides, Immunodiagnostic.

RESUMEN

La brucelosis es una zoonosis producida por especies del género Brucella*. El aislamiento e identificación de la bacteria en alimentos usando las técnicas clásicas de microbiología no es práctico debido a su lenta tasa de crecimiento. Por lo tanto, es necesario establecer métodos rápidos para la detección de la bacteria en alimentos. En el presente trabajo se desarrollaron y caracterizaron anticuerpos policlonales monoespecíficos en gallinas (IgY) contra péptidos sintéticos de las proteínas OMP25 y BP26 de* Brucella abortus*, que puedan ser utilizados en un ensayo de captura. Para ello, se realizaron estudios conformacionales y de predicción de epítopes en la selección de los péptidos, los cuales se utilizaron como antígenos para la producción de las IgY. Los animales desarrollaron anticuerpos específicos contra los péptidos, mostrando correspondencia entre los estudios predictivos y la inmunogenicidad obtenida. Las IgY reconocieron a* B. abortus *en un ensayo de MABA y la actividad de unión y especificidad fue determinada por western blot con extracto celular de* B. abortus*. En este estudio, demostramos que los péptidos de las proteínas OMP25 y BP26 de* B. abortus *son buenos candidatos para la producción de anticuerpos IgY especificos capaces de reconocer proteínas de extracto de* B. abortus *cepa S19, indicando el potencial uso de anticuerpos IgY para el desarrollo de inmunoensayos para la detección de* Brucella abortus.

Palabras clave: Brucella *sp., inmunoglobulina Y, Péptidos sintéticos, Inmunodiagnóstico.*

INTRODUCTION

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella*, which has injurious effects in humans and several animal species with serious social and economical impacts. It is considered to be a Food Borne Illness (FBI), caused mainly by *B. melitensis* and *B. abortus* and *B. suis*, and it is transmissible to humans through consumption of contaminated food as raw milk and

[1] Fundación Instituto de Estudios Avanzados (IDEA), Carretera Nacional Hoyo de la Puerta, Valle de Sartenejas, Baruta, Venezuela.
[2] Instituto de Medicina Tropical. Universidad Central de Venezuela, Caracas, Venezuela.

*Autor de correspondencia: morenoclaud@gmail.com

cheese from bovines, caprines and ovines, infected or carrying a subclinical disease (Arimi *et al.*, 2005; Lang *et al.*, 1995; Leclerc *et al.*, 2002). Brucellosis affects also individuals such as veterinarians, farmers, and laboratory personnel who have close contact with secretion from infected animals and by inhalation in contaminated environments with *Brucella*. Therefore, it is considered one of the main zoonoses in the world (Kuplulu & Sarimehmetoglu, 2004; Wareth *et al.*, 2014).

The global burden of human Brucellosis disease varied significantly within regions and within countries (Dean *et al.,* 2012). *Brucella abortus* is the most important biovar in Venezuela (Vargas, 2003). Official data from Venezuelan Ministry of Health provided an incidence rate of 0.11 cases per 100,000 persons in 2012 (Boletin Epidemiologico No. 52, 2012).

For diagnosis purposes of human and animal brucellosis, serological assays and bacterial isolation are routinely carried out. Although serological diagnosis is rapid and simple, these assays use *Brucella* lipopolysaccharide (LPS) as an antigen to detect antibodies, but sometimes false positive reactions occur because its cross-reactivity with other Gram-negative bacteria like *Yersinia enterocolitica* O:9 (Munoz *et al.,* 2005). Nowadays, most research on brucellosis have been focused on the detection of non LPS antigens, some external-membrane proteins have been pointed out as possible immunogens and protective antigens, among these, the Outer Membrane Proteins (OMPs) (Corbel *et al.*, 1984; Padilla *et al.*, 2003; Al Dahouk *et al.*, 2006). There are seven homologous proteins in the *Brucella* genus, of which OMP25 and OMP31 have been the most widely studied (Cloeckaert *et al.*, 2002). Using monoclonal antibodies and techniques such as ELISA, flow cytometry and electron microscopy, it has been determined that these proteins are exposed on the surface of the cell wall, which favors its use as antigens (Bowden *et al.*, 1995; Cloeckaert *et al.*, 1990; Cloeckaert *et al.*, 1991). However, on the *Brucella* smooth strains; these antigens are masked by the presence of the O-chain. It has been suggested that the gene that codifies OMP25 is highly conserved in the different species, varieties, and strains of the genus (Cloeckaert *et al.*, 1995), while OMP31 is absent in *B. abortus* (Vizcaíno *et al.*, 1997; Vizcaíno *et al.*, 2004). On the other hand, BP26 located in the periplasm of *Brucella* is another protein that has been identified as an important diagnostic antigen in brucellosis. BP26 is highly conserved among *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae* and *B. melitensis*, and was sensitive and specific for diagnosis of *Brucella* infection in animals by enzyme immunoassays (EIAs) (Qiu *et al.*, 2012).

Common laboratory procedures of food Microbiological analysis are conventional culture, immunological testing (ELISA test, Lateral Flow Technology, Immuno-magnetic separation, Biochips) and molecular methods (PCR) (Fung, 2002; Mukherjee *et al.*, 2015). However, there is no standardized method for *Brucella* detection in foods of animal origin (French Agency for Food, Environmental and Occupational Health & Safety, 2014). Even though, the bacteria isolation is routinely carried out it requires three weeks of incubation before considering a sample as negative due to the slow growth of *Brucella* in food. Also, it represents a danger to laboratory personnel and requires Class-III containment facilities (Coelho & Garcia, 2015). Therefore, it is necessary to establish fast and specific methods for the detection of the bacteria in food.

The use of chicken antibodies (IgY) has increased significantly in recent years, mainly because producing antibodies in eggs is both cost effective and minimizes animal welfare concerns (Alvarez, *et al.*, 2013; Schade *et al.*, 2005). In the same way, the employ of synthetic peptide as immunogen to generate specific antibodies for a variety of purposes has increased markedly in recent years. The goal of this work was the production and characterization of monospecific polyclonal antibodies in chicken (IgY) against synthetic peptides from *Brucella abortus* OMP25 and BP26 proteins, suitable for a sensitive and rapid immunological testing without bacteria isolation.

MATERIALS AND METHODS

Peptide design and synthesis

The OMP25 and BP26 proteins from *B. abortus* were analyzed using the ANHEPROT 6.0 software (Deléage *et al.*, 2001) and by Hoops & Woods (1981). One peptide was synthesized from the whole sequences of the OMP25 protein (GenBank Accession AAS84593.1) and another

from BP26 protein (GenBank Accesion Q44642.1). These peptides were synthesized by the multiple manual synthesis described by Merryfield (1963) and modified by Hougten *et al.* (1986), using the t-boc strategy (Laboratorio de Síntesis de péptidos, Instituto de Medicina Tropical (IMT), Universidad Central de Venezuela). Cys-Gly and Gly-Cys at the amino- and carboxy-terminal ends was added to allow peptide co-polymerization.

Immunization of chickens

Four Isabrown-laying chickens (twenty three weeks old, 2kg body mass) with an average laying of 6-7 eggs per week were immunized intramuscularly in the breast region at multiple sites with 125 µg from the peptides emulsified with saline and complete Freund's adjuvant (CFA) (1:1 v/v). Half of that amount of peptide was dissolved in incomplete Freund's adjuvant (IFA) and used in three boosts (Ten days apart). The eggs were collected every day for three days before the immunization and until week 12 after it; they were individually identified, and stored at 4ºC. Animals were bred and maintained at the animal facility from Instituto de Estudios Avanzados (IDEA) and cared for in accordance with the information contained in the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC) and the Code of Ethics for Life (Ministry of Popular Power for Science, Technology and Innovation, 2011).

Isolation and purification of IgY

Isolation and purification of IgY from the yolk of preimmune and hyper-immunized eggs was done according to the modified Polson's method (1985). Briefly, 3.5% (w/v) polyethileneglycol (PEG 6000 Scharlau Chemie SA; European Union) was added to yolk diluted in 3 volumes (1 volume = 1 weight of yolk) of PBS (10mM pH 7.4) under stirring. The supernatant containing IgY was collected by centrifugation at 3000g for 20 min at 4ºC, filtered through sterile gauze, followed by the addition of 20% chloroform, and centrifugation at 2000g for 15 min. Finally, an IgY precipitation step with 8.5% PEG using the same conditions was performed. Purified IgY pellets were resuspended in PBS and stored at -20ºC. The purity of these preparations was evaluated by 10% SDS-PAGE (Laemmli, 1970).

Cell extracts of Brucella abortus strain S19.

Brucella abortus strain S19 treated according to the protocol of Alton *et al.* (1975) was provided by INIA (National Institute of Agricultural Research, Venezuela). Cell extract of *B. abortus* strain S19 were obtained by sonication for 15 minutes (15). A dilution of 1/100 of a cocktail of protease inhibitors was added, to prevent the degradation of proteins (Calbiochem Protease Inhibitor Cocktail Set III). Subsequently BCA assay for quantification of proteins and an SDS-PAGE electrophoresis was performed.

ELISA measurements

The level of antibodies to peptide constructs were measured by enzyme linked immunosorbent assay (ELISA) according to Voller *et al.* (1974). Briefly, microtiter plates (Greiner Microlon 2HB) were coated with 5 µg/mL of peptides in PBS (10mM pH 7.4). Plates were incubated overnight at 4ºC and then, blocked with skimmed milk 5% w/v in PBS pH 7.4-Tween 20 at 0.05% v/v (PBST) for 2 h. Purified IgY (10 µg/mL) were added in duplicates and incubated at 37ºC for 90 minutes. Plates were washed three times with PBST between each incubation step. Detection was developed with HRP-conjugated rabbit anti-chicken IgY (α-IgY-HRP THERMO; Illinois, USA) diluted 1:20000 in PBST followed by 0.01% H_2O_2 with o-phenylenediamine (Pierce Biotechnology, Rockford, IL) in phosphate-citrate buffer (0.1M pH 5.0). Absorbance was read at 440 nm on a Synergy™ HT reader (Bio-Tek; Vermont, USA).
Multiple Antigen Blot Assay (MABA)

To evaluate the specificity of the anti-peptides antibodies and to verify their capacity to recognize the bacterium, a blot assay MABA (Multiple Antigen Blot Assay) (Noya & Alarcon de Noya, 1998) was performed. Peptides as well as the cell extracts of *Brucella abortus* strain S19 were immobilized onto a nitrocellulose membrane, using an acrylic template (Minibloter® 28 S-L Immunetics Inc., Cambridge, MA). The concentration peptide used was 5 µg/mL on PBS (10 mM pH 7.4), and the cell extracts of *Brucella abortus* strain S19 was 10 and 20 µg/mL on PBS (10 mM pH 7.4). After blocking with skimmed milk 5% w/v, 2 mm strips were cut perpendicular to the chamber's channels. Each strip was incubated with IgY antibodies, diluted 1:100 in blocking solution

for 90 minutes at room temperature. Detection was developed with HRP-conjugated rabbit anti-chicken IgY (α-IgY-HRP THERMO, Illinois, USA) diluted 1:20,000 in PBST, was added and developed by a chemiluminescent substrate (Luminol® Amershan, Life Sciences, UK). Positive reactions to the different antigens were recorded as small black squares in a film (ECL Western Blotting Analysis System, Amersham, UK).

SDS-PAGE and Western Blot Assay

The cell extracts of *B. abortus* strain S19 at a final concentration of 11.6 μg/μL was used as source of native proteins and separated by SDS–PAGE under reducing and dissociating conditions in 12% mini-gels (Precision Plus Protein Standards, BIO-RAD) in parallel with molecular weight markers (Precision Plus Protein Standards, BIO-RAD). Gels were stained with Coomasie or electrophoretically transferred to nitrocellulose membranes for recognition of proteins by Western blot according to Towbin's method (1979). After blotting, membrane was blocked overnight at 4ºC with blocking solution (5% w/v nonfat milk in PBST) and cut into strips that were then incubated for 90 minutes at room temperature with the IgY antibodies diluted (1/100) in blocking solution. After three washes, the strips were incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-chicken IgY (α-IgY-HRP THERMO, Illinois, USA) diluted 1:20,000 in PBST. Finally, immunoreactivity was identified by chemiluminescence (ECL Western Blotting Analysis System, Amersham, UK).

Statistical Analysis

Statistical analysis was performed using the InfoStat professional version (Di Rienzo *et al.*, 2013). In order to quantify the relationships between kinetic production antibody against peptides from OMP25 and BP26 proteins in the time, a linear mixed models (LMMs) were fixed. Mean squared error (MSE), confidence interval and parameters functions was estimate on the parametric bootstrap methods for bias correction in linear mixed model (n=250) (Kubokawa & Nagashima, 2012). Values of $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Bioinformatics analysis and peptides selection

OMP25 and BP26 proteins were analyzed using Antheprot software and were selected those peptides with the high score of peptide antigenicity (Parker & Hodges, 1986), hydrophilicity (Hoop & Woods, 1981) and accessibility to solvent. One region comprised between amino acids 159-174 of the OMP25 protein was selected and synthesized. In this region the polar amino acids represent the 63%, which correlates with the presence of antigenic determinants, especially when arginine, lisine and glutamine are present (Novotny *et al.*, 1987). The analysis was performed in the same way for protein BP26, identifying as possible antigenic determinants a region in the 174-190 position of the amino acidic sequence with 37% of polar amino acids and one constructs of this region was synthesized (Table I). The selected peptide sequences showed a high epitope prediction score according to bioinformatics analysis tools, and could induce a strong and specific immune response in chicken models.

Immunogenicity of OMP25 and BP26 peptides from Brucella abortus *proteins*

In order to determine the immunogenicity of the peptides derived from OMP25 and BP26 proteins of *Brucella abortus*, polyclonal antibodies obtained from chickens immunized with the synthetic peptides were tested by ELISA and MABA assays. Experimental animals produced specific antibodies against the OMP25 and BP26 peptides constructs. Antibodies responses were evaluated weekly in order to determine the kinetic of antibody production by ELISA.

Fig. 1 shows the adjusted models for the effect on kinetic of chicken IgY anti-peptide production for the two proteins. The linear mixed models (LMMs)

Table I. Peptides of the proteins OMP25 and BP26 used for the immunization of the chickens.

Proteins	Peptides	peptide sequence	aminoacid	Immunized Chickens	Reference
OMP25	IMT-1182	CGSLRIGGEESSKSKTQTGC	159-174	10B-10C	(Vizcaíno *et al.*, 2004)
BP26	IMT-1185	CGSAVITRGKRAVANAIACGC	174-190	11A-11B	(Rossetti *et al.*, 1996)

IMT: Instituto de Medicina Tropical

adjusted shows that exists significant differences in the kinetic antibody production between proteins, times and its interaction ($P \leq 0.05$). The experimental animals produced specific antibodies against both peptides. In the case of the OMP25 protein the antibody response is higher with intercept at the origin. The lowest antibody response was found for the BP26 protein and an intercept significantly different from 0. The maximum of kinetic response was found at 5 and 7 weeks to proteins BP26 and OMP25, respectively. In both proteins, three groups of activity were detected: group 1 were present in the time frame 0 to 4 weeks (maximum slope), group 2 in the times from 5 to 7 weeks (maximum response) and group 3 in the time 8 to 12 weeks with decrease response. According to the reported by Schade *et al.* (2005), the most frequently observed antibodies titer kinetics in chicken after the first immunization show a transient increase in IgY in the first phase, and the second phase (booster immunization) is characterized by an initial increase in the antibodies titer within approximately 10 days, followed by a plateau for a further 10 days and a decline thereafter, as in our case. These results indicate a correspondence between the predictive study and the immunogenicity obtained in chickens as shown by the antibody response. However, we obtained lower response in the chicken immunized with IMT-1185 peptide (BP26). It is very important to underline that the immunogenic capacity of a peptide not only depends on intrinsic chemical properties of the peptide but also on many complex interactions with various elements of the host immune system, such as the host immunoglobulin repertoire, self-tolerance, and various cellular and regulatory mechanisms definable only in the context of the host (Van Regenmortel, 2001). On the other hand, since the chickens were not raised in Specific Pathogen Free (SPF) conditions, the nonspecific recognition of the pre-immune IgYs (intercept different from 0) is probably the consequence of epitopes shared with other Gram-negative bacteria from previous exposures.

Similar results were obtained with MABA (Fig. 2); the post-immune IgYs recognized specifically the peptides with which the chickens were immunized, without any cross-reactivity with the other peptide. The anti-peptide IgYs were able to recognize the bacterium *Brucella abortus* sonicated with a more intense signal than the pre-inmune (at 20 µg/mL) demonstrating that the peptides were able to mimic the antigenic determinants of the OMP25 and BP26 selected in the in silico analysis. It should be highlighted that the bacterial suspension was sonicated to expose the proteic core of OMP25 and BP26 which is masked by the O-chain in *B. abortus*. It would be advisable to select the specific IgYs by means of an immunoaffinity column to reduce unspecific signals and noise (Tini *et al.*, 2002) and to evaluate his specificity with other bacteria.

Fig. 1. Adjusted models for the production kinetic of IgY anti-peptides antibodies from OMP25 and BP26 proteins.

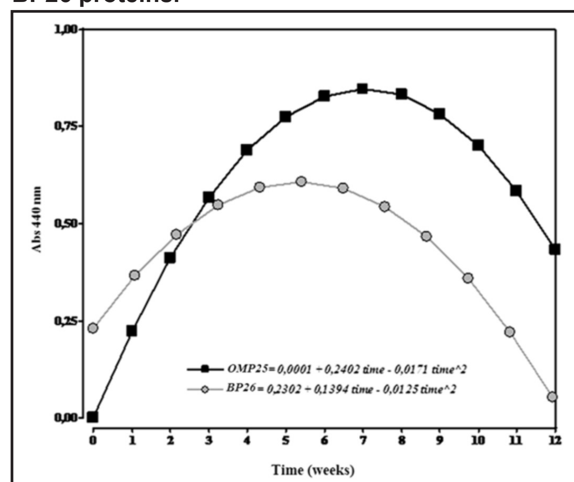
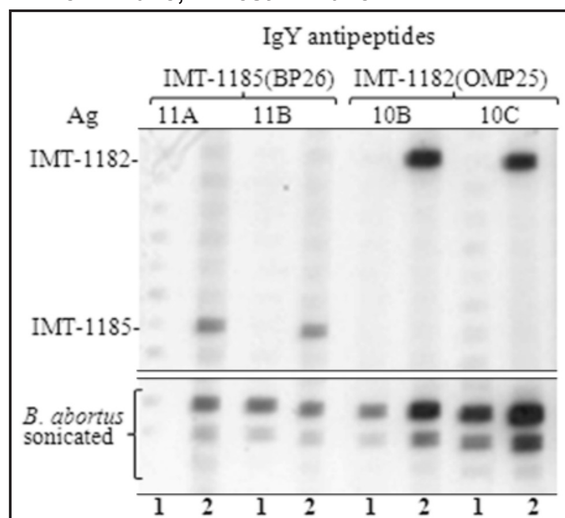


Fig. 2. Immunogenicity and cross reactivity of synthetic peptides constructs IMT-1182 and IMT-1185 from OMP25 and BP26 respectively. Anti-BP26 (chickens: 11A and 11B) and anti-OMP25 (chickens: 10B and 10C) IgY evaluated by MABA. 1: Pre-immune; 2: Post-immune.



Characterization of IgY Antibodies

Purity of IgY anti-OMP25 and anti-BP26 peptides

As seen in Fig. 3, the collected IgYs (lanes 2-3) show two predominant protein bands at approximately 70 and 25 kDa just as the IgY standard (lane 1), which match the expected heavy and light chains of these antibodies (Narat, 2003). However, some other contaminating proteins were also observed which could correspond to vitellogenins cleavage products, apolipovitellin II (35 kDa) identified by Klimentzou *et al.* (2006) and phosvitin (40 kDa) (Mann and Mamm 2008). In fact, the presence of these impurities did not affect the effectiveness of the IgYs (Alvarez *et al.*, 2012).

Immunoreactivity of IgY anti-peptide antibodies evaluated by Western blot

To determine whether IgY polyclonal antibodies were able to recognize molecules from cell extract of *B. abortus* strain S19, Western blot assays were carried out using anti-OMP25 (IMT-1182) and anti-BP26 (IMT-1185) IgY antibodies (Fig. 4). The anti-OMP25 IgY antibody recognized bands of apparent molecular masses of 12, 26-36, 32-49 and 76-119 kDa. However, these did not correspond to the molecular weight OMP25 protein (25-27 kDa). The sodium dodecyl sulfate insoluble (SDS-I) fraction of *B. abortus* is strongly associated with peptidoglycan (PG) (Sowa *et al.*, 1991). Probably the different molecular mass observed in SDS-PAGE would to correspond to PG fragments of different sizes in association with the OMPs. In addition, was suggested by Dubray & Charriaut (1983) that the 25-27 kDa OMP could possibly be a glycoprotein containing 1,2-diol groups. Thus Cloeckaert *et al.* (1992) suggests that, the multiple band patterns observed for the 25 kDa OMP could also be due to variation in the size of the oligosaccharide portion.

On the other hand, the anti-BP26 (IMT-1185) IgY antibody recognized specifically a band of apparent molecular masses of ~ 46 kDa. But, these not correspond to the molecular weight BP26 protein. This protein was recently crystallized by Kim *et al.* (2013) and a new sequence was reported (GenBank Accesion 4HVZ_D). The crystal structure of BP26 reveals a structure with 16 BP26 molecules that form a channel-like assembly, which eight form a ring-shaped structure containing a hole in the center and the others molecules interacts each other to form the channel having a large internal cavity (Kim *et al.*, 2013). It is very important to underline, that in our study the bacteria were sonicated without membrane protein extraction and this process might not be sufficient to completely the separation of the protein. Therefore, is possibly that due to the structural complexity of the BP26 protein, fragments of the complex with a different molecular weight are obtained.

In summary, we demonstrated that IMT-1182 from OMP25 and IMT-1185 from BP26 are good candidates for production of specific antipeptide antibodies capable of recognizing proteins from sonicated *B. abortus* strain S19, indicating the potential usefulness of the IgY antibody for development of antigen-capture assay. Furthermore, the use of synthetic peptides as immunogen to produce IgY anti-*Brucella* emphasizes its importance in this work, since it avoids the risk involved in the handling of a bacteria of high virulence and infectivity. The peptide is easy, cheaper, and safe to prepare as compared to purified and recombinant protein in the production of antibodies. It is conceivable that the test can be optimized by using more than one peptide which will often improve the sensitivity and reliability of a test. This technology could be extended for the detection of other pathogens responsible for FBIs (Food Borne Illness).

Fig. 3. Analysis of IgY antibodies purified from egg yolks by SDS-PAGE. Lane 1: Molecular weight marker. Lane 2: commercial standard IgY. Lane 3: IgY anti-BP26 peptide, Lane 4: IgY anti-OMP25 peptide. Arrow indicated the heavy (68 kDa) and light (25 kDa) chains of IgY antibody.

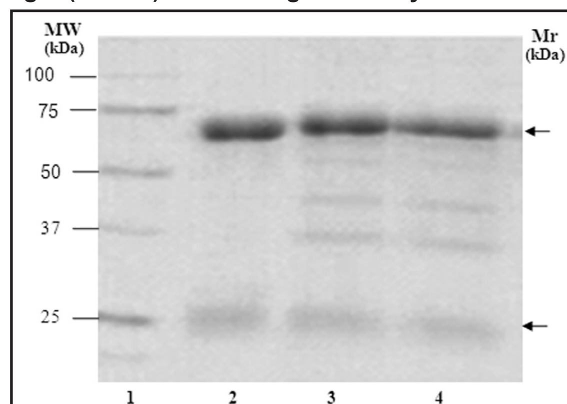
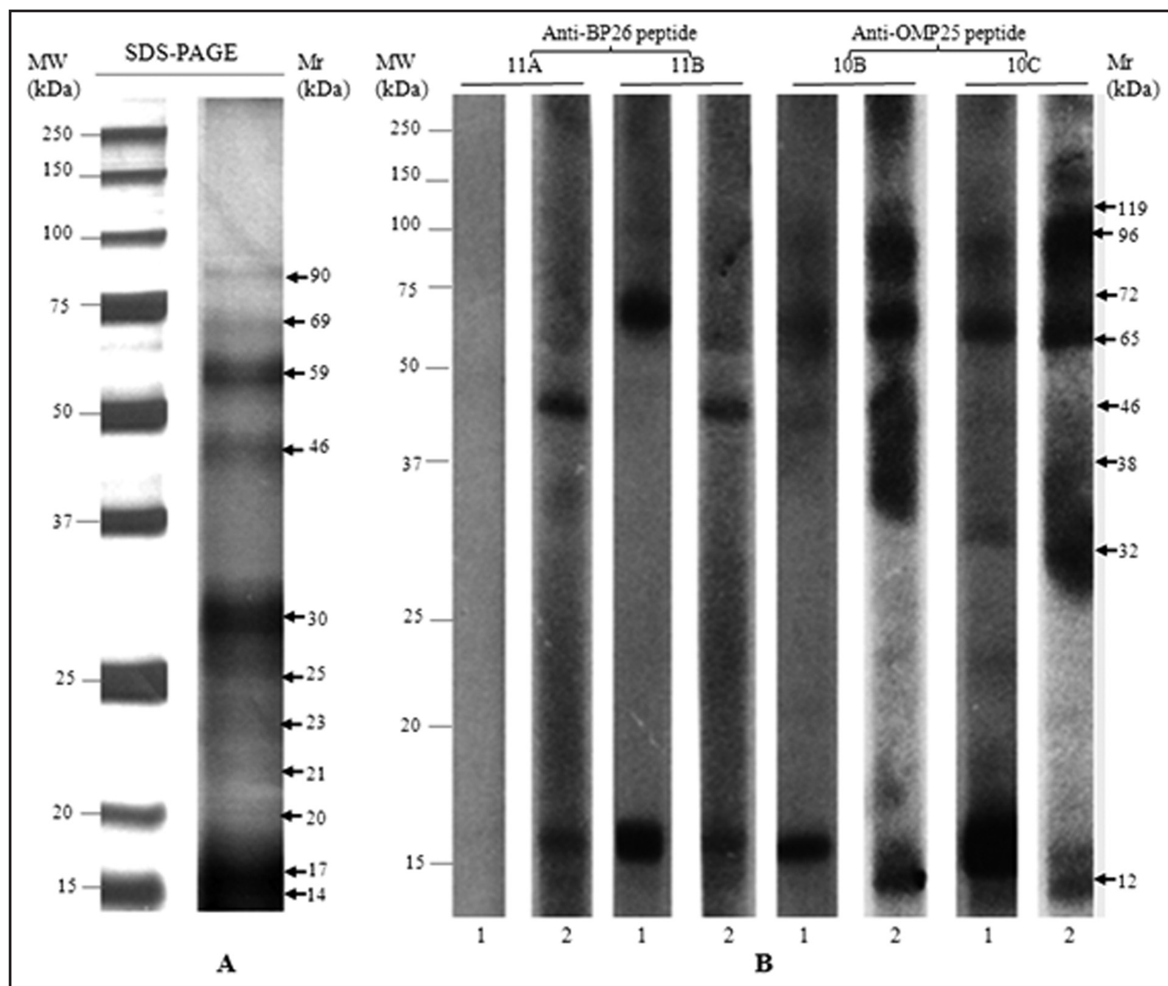


Fig. 4. Electrophoretic and Western blot profile of sonicated *B. abortus* strain S19. (A) Coomasie-stained gel (SDS-PAGE 12%). (B) Western blot analysis showing the reactivity of IgY anti-BP26 (chickens: 11A and 11B) and anti-OMP25 (chickens: 10B and 10C). Molecular weight (MW) markers are indicated on the left in kDa and the apparent molecular masses (Mr) are shown on the right. 1: Pre-immune; 2: Post-immune.



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