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Original Article

Immunogens of *Brucella Abortus* S19 Identified By Two-Dimensional Gel Electrophoresis and Immunoblotting

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Abstract

Background: Lipopolysaccharides (LPSs) and several antigenic proteins of Brucella have been considered for preparation of diagnostic reagents and subunit vaccines. The objective of this study was to identify and compare immunogens of B. abortus S19 which induce humoral immune responses in human, goat and rabbit.

Material and Methods: The bacterial whole cell extract was prepared in extraction buffer and resolved using two-dimensional gel electrophoresis (2-DE). The resolved antigens were reacted against human, goat and rabbit sera using western blotting.

Results: At least 19, 14 and 16 immunogenic proteins were recognized in western blotting with human, goat and rabbit sera, respectively. The most abundant proteins of the bacterium with immunogenic properties in goat and rabbit but not in human, were a group of 5-6 proteins with molecular masses of 32-34 KDa and isoelectric point (pI) ranging from 4.5 to 5.7. In contrast, a group of 5 proteins with molecular weight of 45 KDa and pI in the range of 4.5 to 5.4 as well as several low molecular weight proteins were immunogenic in human. Furthermore several proteins of Brucella had similar reactions against all sera.

Conclusion: These results showed that some of the antigenic proteins of Brucella could be candidates for more accurate diagnosis of Brucellosis in humans and domestic animals.
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Keywords ● Brucella abortus ● Two dimensional gel electrophoresis ● Immunoblot ● Immunogens

Introduction

Brucellosis is one of the most important zoonotic diseases caused by the genus of Brucella (B).[1] B. abortus and B. melitensis are responsible for most cases of Brucellosis in` human and cattle in many developing countries including Iran.[2,3] Brucellae are facultative intracellular bacteria lacking spores, flagella, pilli, capsule or specified exotoxines.[3,4] LPSs, outer membrane proteins and several ribosomal and/or cytosolic proteins have so far been considered as the main immunogens for development of subunit vaccines and diagnostic reagents.[5-11] LPSs are the most abundant glycolipids of outer membrane of gram negative bacteria which act as potent

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inducers of humoral immune responses. The current serologic tests for recognition of Brucella infections mostly measure anti-LPS antibodies.[12,13] These antibodies usually cross-react with some other gram-negative bacteria such as Yersinia enterocolitica and E coli,[14,15] and remain in high levels after treatment in many patients.[16] In addition, they can not distinguish between some naturally infected and vaccinated animals.[17,18] Because of these limitations, a great deal of investigations has been focused on the immunogenic proteins to improve specificity of diagnosis,[10,19,20] and vaccination.[5-8,21,22]

In this research, proteins of B. abortus S19, an attenuated standard strain that is antigenically similar to virulent strains, were resolved using 2-DE. Using immunoblotting, the reactivity of sera from naturally infected humans and goats and immunized rabbits were then analyzed against the resolved proteins. The main objective of this study was to compare immunogenic proteins which induce humoral response in the foregoing species and if possible to find protein candidates to be used for diagnosis of Brucellosis in quantitative tests such as enzyme linked immunosorbent assay (ELISA).

Materials and Methods

Bacterial preparation

B. abortus S19 was obtained from Pasteur Institute, Tehran, Iran and cultured on Brucella agar (Becton Dickinson). After 48 hrs of cultivation, the bacterial suspension was collected by centrifugation (5000xg), washed with phosphate buffered saline (PBS), resuspended in 20 mM tris-HCl (pH 7.2) and killed with cold acetone.

Serum samples

The human serum sample comprised pooled serum samples of 14 patients with clinical diagnosis of brucellosis, a symptomatic treatment for less than two months and titers of at least 1/320 and 1/160 for Wright tube test and 2-mercaptoethanol (2-ME) test respectively.

The goat serum sample comprised pooled sera from 34 goats with brucellosis with corresponding titers of at least 1/320 and 1/160 for Wright tube test and 2-ME tests and supplied by Veterinary Research Center, Kermanshah, Iran. Negative control serum was a pool of at least 10 normal serum samples from humans and goats with Wright tube test titer of less than 1/20.

The rabbit serum sample was prepared from two Newzeland rabbits, immunized by four subcutaneous and intramuscular injections, in a period of two months, of killed and sonicated B. abortus S19 with a titer of at least 1/320 for 2-ME test.

Sample preparation for electrophoresis

All materials used in sample preparation and electrophoresis, except some such as lysozyme or phenyl methyl sulfonyl fluoride (PMSF), were obtained from Amersham Pharmacia Biotech. Complete solubilization of the bacterial suspension was performed by lysozyme, urea and 3-[(cholamidopropyl)-dimethyl-amminio]-1-propane sulphonate (CHAPS). PMSF (1mM), EDTA (1mM) and lysozyme (1mg/100mg of bacterial dry weight) were added to 0.25gr of washed bacterial pellet resuspended in 1 ml of Tris-HCl buffer. The sample was then incubated overnight at 37°C with gentle shaking. This preparation was then supplemented with 9 M urea, 4% (w/v) CHAPS, 0.8% (v/v) carrier ampholytes (ampholine,4-6 and pharmalyte,5-8) in equal volumes) and 65 mM DTT. The mixture was kept for 2 h at room temperature followed by centrifugation at 50000 g for 60 min. The supernatant was divided in small volumes and stored at -20°C until used.

Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out in rehydrated gels according to the method of Westermeire,[23] with some modifications. Polyacrylamide gels (4%T) with thickness of 0.5 mm were prepared over gel bond sheets. The prepared gels were washed extensively in deionized water and dried overnight at room temperature. They were then rehydrated overnight in 20mM Tris-HCl (pH 7.2) and containing 8 M urea, 2% (w/v) CHAPS, 6% (v/v) carrier ampholytes (ampholine 4-6 and pharamalyte 5-8 in equal volumes), 18mM DTT, 7.5% (v/v) ethylene glycol and sufficient amount of the sample.

IEF was performed at 16°C using Multiphore II system at several stages including; 60min at 0.0-500 volts for prefocusing, 20min at 0.0-2500 and 6 hrs at 2500 volts for separation and 15 min at 3000 volts for sharpening. After IEF, the gels were cut into strips (width of 5-6 mm) and equilibrated for two intervals of 15min in 50mM Tris-HCl (pH 8.8) containing 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and 0.01% (w/v) bromophenol blue. The strips were applied on the top of 12% vertical gels prepared for sodium dodecyle sulfate polyacrylamide gel elctrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli,[24] at a constant voltage.

Immunoblotting

The resolved proteins were transferred to nitrocellulose membranes by tank eletroblotting,[25] in constant current (1 h at 100 mA and 2 h at 300 mA). The membranes were blocked with 0.5% tween 20 in PBS for 15 min and

washed 3 times with 0.05% tween 20 in PBS (PBS-T) for 15 min.

The membranes were incubated for 1.5 hr in the human, goat or rabbit sera with dilution of 1/50, 1/50 or 1/200 respectively, followed with the secondary antibodies (anti-human, anti-goat or anti-rabbit IgG conjugated with peroxidase, Sigma) for 1.5 h with dilutions of 1/1,000,1/1,000 and 1/3000 respectively. Before and after addition of the secondary antibodies, the membranes washed 5 times, each time for 5 min, in PBS-T. To visualize antigenic components, the membranes were incubated in substrate solution (diaminobenzeden, Dako) and H_2O_2 for 10-15 min.

Gel staining

IEF gels were stained by colloidal solution of Commassi brilliant blue G-250 after fixation in trichloroacetic acid for 2 hrs.[26] SDS-PAGE and 2-DE gels were stained by the sensitive silver method introduced by Hochstrasser and co-workers.[27]

Results

The extraction method used in this study completely solubilized the bacterial proteins. This method benefited from the effect of lysozyme, urea, CHAPS and DTT. The molecular masses of bacterial proteins extracted by this method varied mostly between 10 to 100 KDa of which 32-34 were more intensively stained (Fig 1). The extracted proteins were predominantly acidic with isoelectric points (PIs) ranging from 4.5 to 6. Some protein groups of B. abortus resolved by 2-DE included several proteins with similar molecular masses but substantial isoelectric point (pI) differences (Fig 2A). In this connection, the most abundant proteins were a group of 5-6 proteins with masses between 32 and 34 KDa and pIs in the range of 4.5 to 5.6.

In immunoblotts of the bacterial proteins resolved by 2-DE, at least 19, 14 and 16 proteins were recognized in human, goat and rabbit sera, respectively (Fig 2B-D). Approximate molecular masses and pIs of the bacterial proteins are shown in Table 1. The immunoblots showed different immunoreactivity of the sera with different protein groups. In this regard, immunoblots of goat and rabbit but not that of human revealed a group of 5-6 proteins with masses between 32 and 34 KDa and pI from 4.5 to 5.6. In contrast, the immunoblotts of human and goat but not that of rabbit, showed a group of 5 proteins with mass of 45 KDa and pI from 4.5 to 5.4.

However, all immunoblotts displayed a large smear-like spot with molecular masses mainly in the range of 30 to 50 KDa and pI of 4 to 5.

Table 1: Immunogens of B. abortus S19 recognized by man, goat and rabbit serum.

Estimated		Reactive with scrum		
Mass (KDa)	**pI**	**man**	**goat**	**rabbit**
64-66	4.4-4.6	+	-	-
55-57	4.9	-	-	+
55-57	5.1	-	-	+
55-57	5.3	-	-	+
49-50	4.6-4.7	+	-	-
45	4.5	+	+	-
45	4.7	+	+	-
45	4.9	+	+	-
45	5.1	+	+	-
45	5.3	+	+	-
42-43	4.2	-	+	-
36-37	4.6-4.8	-	+	-
32-34	4.6	-	+	+
32-34	4.8	-	+	+
32-34	5.1	-	+	+
32-34	5.3	-	+	+
32-34	5.6	-	+	+
29-29	4.8	-	-	+
27	5.1	-	-	+
27	5.3	-	-	+
21	4.2-4.3	-	-	+
18-19	4.5-4.6	+	-	-
18-19	5.2	+	-	-
18-19	6.7	+	-	-
18-19	7.2	-	+	-
18-19	4.4	+	-	+
16	4.7	+	-	-
15-16	6.8	-	-	+
15-16	7.4	-	-	+
14-15	4.9	+	-	-
14-15	5.3	+	-	-
14	7.0	+	+	-
12-14	5.6	+	-	-
12-14	5.8	+	-	-
12-14	6.3	+	-	-
10-12	4.2-4.3	-	+	+

This pattern was stained more intensively against rabbit serum and reflected the electrophoretic mobility of B. abortus LPSs. The pattern of LPS was stained very weakly in the immunoblotts with negative control sera.

Discussion

Among the few studies using proteome or immunoproteome analysis to identify antigenic proteins in different species of Brucella,[3,28-32] the present study, to our knowledge, appears to be the first application of 2-DE to identify components of B. abortus that induce humoral immune response in human, goat and rabbit. Interestingly, the large portion of anti-Brucella antibodies in the sera of naturally infected human and goats and immunized rabbits reacted against Brucella LPSs. These reactions were seen as a large smear-like spot with a mass of more than 20 KDa in the central portion of immunoblots while pH was in the range of 4 to 5 (Fig 2B-D). In this connection, the electrophoretic pattern of LPS of S19 is more pronounced in the immunoblot with rabbit serum which is due

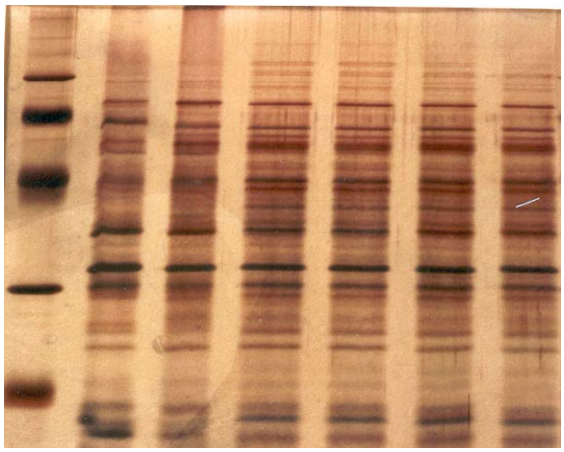

Figure 1: SDS-PAGE of the extracted proteins in 12% gel, stained by silver nitrate.

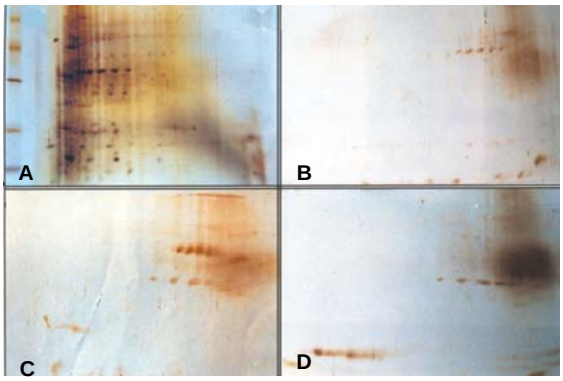

Figure 2: 2-DE map of B. abortus S19 proteins resolved by IEF in pH range of 4-8 and SDS- PAGE in 12% gel, stained by silver nitrate (A). Immunoblots of B. abortus proteins against human (B), goat (C) and rabbit (D) sera.

to the heterogeneity of O-polysaccharide chains length.

This heterogeneity has already been described.[16,33] LPSs of Brucella induce strong humoral immune responses in human and cattle. The anti-LPS antibodies have been used for the diagnosis of brucellosis in human and domestic animals, but this was not apparently an ideal candidate for diagnosis or vaccination.[16-18,33]

One of the most important differences in immunoreactivity of the sera against resolved proteins of Brucella was the presence of antibodies against a group of proteins with masses of 32-34 KDa and pI of 4.5-5.6 in goat and rabbit but not in human sera. (Fig 2B-D). This group represented most abundant proteins of S19 strain and comprised 5-6 protein spots. This group which seems to belong to group 3 of outer membrane proteins could be considered as a possible diagnostic antigen or subunit vaccine in domestic animals, a suggestion which needs to be confirmed.[34] An immunogenic group of 3 proteins with masses of 32-33 KDa and pI of 5.2, 5.6 and 6 was also reported in B. ovis.[28]

Furthermore, human and goat but not in rabbit sera, contained antibodies against a protein group with mass of 45 KDa and pI from 4.5 to 5.4 (Fig. 2B-D). This group which was composed of 5 proteins can be considered as important immunogens in human and goat that were naturally infected but not in rabbit immunized by the killed bacteria. The presence of antibodies against two proteins from B. ovis, with a mass of 45KDa and pIs of 5.1 and 5.4 had already been reported in sheep serum.[28]

Using the aforementioned sera, the immunoblots of B. abortus extract, showed several proteins with masses less than 20KDa (Table 1). Eleven proteins reacted against human sera, compared with 2 and 4 in the sera of goat or rabbit. A protein, immunogenic in animals, with mass of 18-20 KDa and pI 4.9 was isolated from B, melitensis.[35] A protein with mass of 18-20 KDa and pI 5.6 was isolated from B. melitensis and claimed to be a good marker for differentiation of active from non-active Brucellosis.[10] In addition, a protein with mass of 18-20 KDa and pI 8.6 were isolated from B. abortus that was able to protect mice against virulent B. abortus.[36] Rebecca and his colleagues reported a 14 KDa protein from B. abortus, which was able to induce cellular and humoral immune response in mice.[9] Our results, which were consistent with those of foregoing studies, showed that most of B. abortus proteins with masses less than 20 KDa were immunogenic and appeared to be ribosomal or cytosolic. The possible application of these proteins to diagnosis, development of vaccines or to distinguish between infected from vaccinated cattle needs further investigation.

Conclusion

Among the proteins of B.abortus S19 resolved by 2-DE, the presence of several protein groups with similar molecular mass and different pI, were of particular interest. Some of these protein groups reported herein deserve to be studied, in purified form, by appropriate quantitative methods before they are used as possible candidates for the diagnosis of Brucellosis in human and domestic animals.

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