Immunoblotting analysis of Immunoglobulin G antibody response against cytosoluble antigens of *Brucella melitensis* strain Rev 1 in naturally infected sheep*

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SUMMARY

In this study, Immunoglobulin G (IgG) antibodies against Brucella melitensis cytosoluble antigens (CSA) in sera of Brucella infected sheep were examined by immunoblotting. CSA were extracted from Brucella melitensis strain Rev 1 by heat treatment of Brucella suspension in 1 mol/l NaCl containing 0,1 mol/l Na-Citrate (salt extractable CSA) and by ethanol precipitation after autoclaving of Brucella suspension in 0,15 mol/l NaCl (ethanol precipitable CSA) respectively. The analysis of the both extracts in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resulted mainly in similar protein bands at molecular masses (MM) of 62-66 kDa, 42 kDa, 33 kDa , 27-29 kDa, 20.5-22 kDa and the protein bands below 18.7 kDa. In addition, a distinct protein band in salt extractable CSA was observed at MM of 50 kDa . In immunoblot analysis using a pool of sera from different Brucella infected sheep, IgG antibodies reacted mainly with CSA between 33 kDa and 50 kDa MM of both exracts. In addition, antibody response of infected sheep against 23 to 25 kDa, 52 to 60 kDa and 62 to 66 kDa ethanol precipitable CSA was also detected. Antibody reactivity against CSA of the both extracts below 20 kDa wasn't observed.

Key words: *Brucella melitensis* Rev 1, Immunoblotting, Sheep

ÖZET

Brucella ile enfekte koyunlarda *Brucella melitensis* Rev 1 sitosoluble antijenlerine karşı oluşan immunoglobulin G antikorlarının immunoblot yöntemi ile analizi

Bu çalışmada Brucella ile enfekte koyunların kan serumlarında, *Brucella melitensis* sitosoluble antijenlerine karşı oluşan immunoglobulin (IgG) antikorlar immunoblot yöntemi ile incelendi. Sitosoluble antijenler, *Brucella melitensis* Rev 1 suşundan 0.1 mol/l sodium sitrat'lı 1 mol/l NaCl çözeltisi içinde hazırlanan Brucella süspansiyonunun ısı ile muamelesi (tuzla ekstrakte edilebilen sitosoluble antijenler) ve 0.15 mol/l NaCl içinde hazırlanan Brucella süspansiyonunun otoklavda muamele edilmesinden sonra etanol'le çöktürme (etanol ile çöktürülebilen sitosoluble antijenler) işlemleri ile ayrı ayrı ekstrakte edildi. Her iki ekstrakt sodyum dodesil-sülfat poliakrilamid jel elektroforezde analize edildiğinde, 62-66 kDa, 42 kDa, 33 kDa, 27-29 kDa ve 20.5-22 kDa ile 18,7 kDa'dan daha küçük moleküler ağırlığa sahip benzer protein bandları içerdiği gözlendi. Ayrıca tuzla ekstrakte edilebilen 50 kD moleküler ağırlıkta farklı bir protein bandı tespit edildi. İmmunoblot analizde, Brucella ile enfekte koyunların kan serumlarının karıştırılması ile elde edilen serumdaki IgG antikorların her iki ekstraktdaki 33-50 kDa arasında moleküler ağırlığa sahip sitosoluble antijenlerle reaksiyon verdiği gözlendi. Ayrıca, antikorlar, etanol ile çöktürülebilen, 23-25 kDa, 52-60 kDa, 62-66 kDa moleküler ağırlıktaki sitosoluble anijenlerle reaksiyon verdi. Her iki ekstraktda 20 kDa moleküler ağırlığın altındaki sitosoluble antijenlere karşı antikorlarla reaksiyon görülmedi.

Anahtar kelimeler: *Brucella melitensis* Rev 1,İmmunoblot, Koyun

INTRODUCTION

Bacteria belonging to Brucella genus are Gram-negative and facultative intracelluler pathogens causing serious diseases both in human and animals. *Brucella melitensis* is a member of the genus of *Brucella* which commonly causes abortion in sheep and goats (1, 2).

Since in most cases of Br. melitensis infection of sheep no clinical sign except for abortion is seen, the

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infection could be mainly diagnosed by serological tests (3). It is generally accepted that the Br. melitensis infection can not be eradicated in many countries by the detecting of sero-positive sheep and slaughtering alone and that the vaccination programme should be done to reduce the spread of the disease. *Br. melitensis* Rev 1 is the best vaccine available for such a purpose (3). The antibody response to this strain, mainly to its Lipopolysaccharide (LPS) component, is detectable for a long time and the serological reactions following vaccination with strain Rev 1 interfere with diagnosis of brucellosis (3, 4).

Rose Bengal plate test (RBPT) and complement fixation (CF) tests using whole smooth cells of Brucella as antigen are the main serological tests for the diagnosis of Brucella infection in sheep. Enzyme immunoassay (EIA) using unpurified Br. melitensis smooth LPS (5) and salt exractable proteins of Brucella abortus (6) as antigen has also been employed for the diagnosis of ovine and bovine brucellosis. But, the distinction of the infected sheep from vaccinated ones is difficult by these tests in vaccinated sheep population. Other serological tests such as radial immundiffusion test (RID) and EIA using purified O-polysaccharide chain from Br. abortus have been used for differentiating of cattle infected with Br. abortus from cattle vaccinated with Br. abortus strain S19 (7, 8). However these tests do not completely differentiate sheep infected with *Br. melitensis* from sheep subcutaneously vaccinated with Br. melitensis Rev 1 (5).

The antibody response in brucellosis is not only directed to the LPS component of the *Brucella* cell but also to the proteins and other macromoleculer components (9). Recent studies using immunoblot have focused on the identification of immunogenic *Brucella* outer membrane proteins (OMP) and cytosoluble proteins (CSP) in human (10), cattle (11), and sheep (12, 13, 14, 15). It has been suggested that identification of certain OMP and CSP could be useful to develop serological tests for differentiating of Br. melitensis infection from vaccination.

The prupose of the present study was to examine IgG antibody response of Brucella infected sheep to the cytosoluble antigens of *Br. melitensis* strain Rev 1 after SDS-PAGE by immunoblotting. Two different extracts of *Br. melitensis* were used to determine the possible differences between sero-reactivity of the CSA.

MATERIAL AND METHODS

Preparation of cytosoluble antigens (CSA):

Brucella melitensis strain Rev 1 was obtained from Veterinary Research Enstitute Pendik / İstanbul / Türkiye and cultivated in Triptic Soy Agar for 72 h at 37°C. Bacteria were harvested with 0.15 mol/l sodium chlorid containing 0.5 % Phenol and washed with the same buffer. After autoclaving of the Brucella suspension in 0.15 mol/l sodium chlorid, the supernatant was precipitated with 3 vols of cold ethyl alcohol. The resulting precipitate was dissolved in distilled water and used as ethanol precipitable CSA (5, 7). To prepare of salt extractable CSA, Brucella supension in 1 mol/l NaCl containing 0.1 mol/l sodium citrate was heated at 60°C for 8 h, cooled to 5°C and centrifuged. The supernatant was used as salt extractable CSA (16). Protein contents of both extracts was measured as described previously (17).

SDS-PAGE and Immunoblotting of cytosoluble antigens

SDS-PAGE

SDS-PAGE was performed as described previously (18), on a 1.5 mm thick slab gel (12x16) containing 15 % acrylamide and 2,7 bisacrylamide with a 4% acrylamide stacking gel. Tank and sample buffers were prepared as recomended (Hoeffer Scientific Instruments, San francisco, CA, USA). Antigen samples containing 20 µg of protein were boiled under reducing conditions in the sample buffer for 3 min and cooled at room temperature. Samples were applied to a vertical slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) and electrophoresed constantly at 30 mA . Protein bands were visualized by staining with 0.025 % Coomassie Brilliant blue R-250 in 40 % methanol and 7 % acetic acid. Moleculer masses of the proteins were determined by comparing their electrophoretic mobility with standard molecular weight marker (Sigma) containing a mixture of proteins between 14.2 kDa - 66 kDa (19) .

Immunoblotting

For immunoblotting analysis, electrophoretic transfer of CSA to Nitrocellulose membranes (BA-85, Schleicher and Schull, Dassel, Germany) was carried out on a blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) in blotting buffer at 20 V and 0.3 A for 12 h. Blotting Buffer is

composed of 25 mM Tris base, 192 mM glycine, and 20% (v/v) of methanol (20). After electrophoretic transfer, nitrocellulose sheet was incubated in tris buffered saline (TBS; 50 mM Tris, 0.9% NaCl, pH 7.4) containing 2% skimmed milk powder for 2 h at room temperature (rt) to block the remaining protein reactive sites on nitrocellulose membrane. The blocking solution was removed, and nitrocellulose sheet was incubated for 2 h at rt with a pool sera from infected sheep diluted 1:100 in TBS containing 0.05 % Tween 20 (TBS-T) and then with horseradish peroxidase-conjugated anti ovine İmmunoglobulin G (IgG) from donkey (Sigma, immunochemicals) at a dilution of 1:500 in TBS for 2 h at rt. Between incubation periods, nitrocellulose sheet was washed five times for 5 min with TBS-T. After additional washing with TBS, nitrocellulose sheet was immersed in substrate solution. The substrate solution was prepared freshly by dissolving of three mg of 4-chloro-1-naphtol (Merck, chemicals) in 1 ml methanol and then by adding 5 ml TBS and 0.02 ml of %30 hydrogen peroxide to per ml of the solution. After colour development, the nitrocellulose sheet was washed with distilled water and dried.

Sheep serum used in immunoblotting was a pool of 10 sera from aborted sheep. In a previous work (21), we isolated *Br. melitensis* from five of these aborted sheep. Each serum gave positive reaction in RBPT and titers ranging from 1:40 to 1:160 in serum agglutination test (SAT).

RESULTS

SDS-PAGE of the extracts of Brucella melitensis Rev 1 is shown in Figure 1. Coomassie blue staining profiles of the both extracts yielded many protein bands between 13,7 kDa and 66 kDa. Major protein bands of both extracts were detected at molecular masses (MM) of 62 to 66 kDa, 42 kDa, 33 kDa , 27 to 29 kDa, 20.5 to 22 kDa and below 18.7 kDa. However, a distinct protein band at the MM of 50 kDa of salt extractable CSA was observed.

Immunoblot analysis of IgG antibody response of Brucella infected sheep to CSA of *Brucella melitensis* Rev 1 is shown in Figur 2. Antibodies developed in infected sheep sera recognized the antigens of both extracts at the MM between 33 kDa and 50 kDa where is stained intensely. Intensive sero-reactivity of ethanol precipitable CSA, but slightly salt exractable CSA, at the MM ranging from 52 to 60 kDa and 62 to 66 kDa were also detected. Reactivity of IgG antibodies with the ethanol precipitable CSA, but not with the salt extractable CSA, at the MM between 23 kDa - 25 kDa which lost in photographic reproduction, was observed. IgG antibodies did not react with antigens of both extracts at MM below 20 kDa.

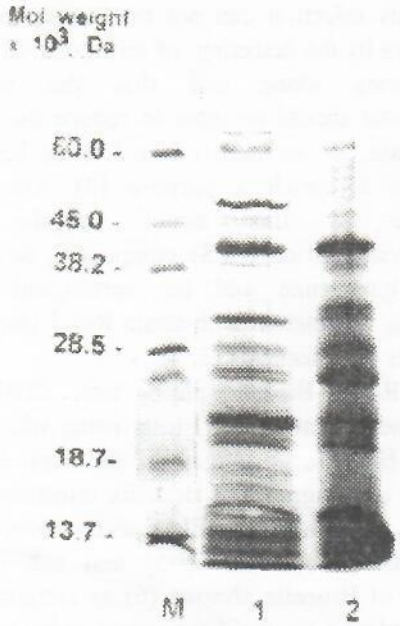

Figure 1: SDS-PAGE coomaassie blue staining of the salt extractable proteins (lane 1) and after autocklaving ethanol precipitable proteins (lane 2) of *Brucella melitensis* Rev 1. (M) indicates the molecular mass standard.

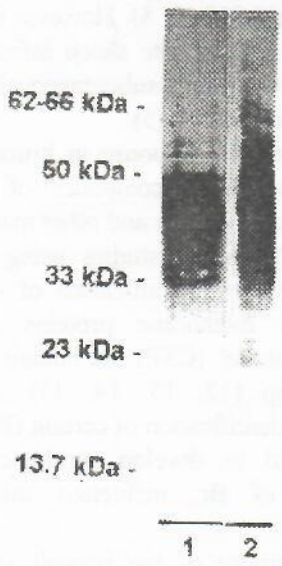

Figure 2: Immunoblotting analysis of pooled sera (n:10) from infected sheep to *Brucella melitensis* Rev 1 cytosoluble antigens. Lanes: (1) salt extractable antigens, (2) after autoclaving ethanol precipitable antigens

DISCUSSION

It has been generally accepted that host immune response was directed against surface or cell membrane antigens of pathogens (4, 9).

Recent studies using immunoblot have already shown the antibody response to cytosoluble antigens, mostly outer membrane proteins, of Br. melitensis (13, 15), *Br. ovis* (12, 14) and *Br. abortus* (11, 22, 23) in ovine and bovine brucellosis. Zygmunt et al. (15) investigated by immunoblot using cytosoluble protein (CSP) extract of *Br. melitensis* strain B115 that spesific antibodies in sera of naturally infected sheep was mainly directed against CSP at MM of 25 to 27 kDa, 31 to 34 kDa, 36 to 38 kDa, 55 to 62 kDa, 70 to 73 kDa and 89 to 94 kDa and CSP at MM lower than 20 kDa whereas antibodies in sera of sheep vaccinated with Br. melitensis Rev 1 reacted only with CSP at MM of 36 to 38 kDa, 60 kDa, 70 to 73 kDa and 89 kDa . In a similar study, Debbarh et al. (13) reported that spesific antibodies in sera of naturally infected sheep reacted with CSP at MM of 19, 23, 24, 28, 32, 38, 39, 50, 54, 68 and 80 kDa whereas spesific IgG antibodies in sera of sheep vaccinated with Br. melitensis Rev 1 were directed against CSP at MM of 39 kDa and 50 kDa. But, except 28 kDa, 39 kDa, 50 kDa and 54 kDa CSP, specific IgG antibodies in sera of infected sheep to 19 kDa, 23 kDa, 24 kDa, 25 to 27 kDa and 31 to 34 kDa CSP could not be detected in all sera of infected sheep. It has already been discussed if 28 kDa and 31 to 34 kDa CSP were identical or not.

In the present study, salt extractable CSA and ethanol precpitable CSA extracts from Br.melitensis strain Rev 1 were used and examined the IgG antibody response of infected sheep to CSA of these extracts. By immunoblotting using a pool of sera from Brucella infected sheep, IgG antibody reactivity in a broad stained smear beetween 33 kDa and 50 kDa CSA of both extracts were detected. CSA at these MM could be probably identical to the CSA of 31 to 34 kDa, 36 to 38 kDa and 39 to 50 kDa described previously (13, 15). We have also determined antibody response against ethanol precipitable CSA ranging from 52 to 60 kDa and 62 to 66 kDa and slight reactivity against the CSA of 23 to 25 kDa of the same extract. But antibody response to CSA at the MM of 19 and 27 kDa could not be detected. We could not rule out that reactivity of the IgG antibodies between 22 kDa and 25 kDa CSA observed in this study are identical to the CSP of 23, 24, 25 kDa mentioned above. Antibody response to salt extractable CSP at the MM of 52 to 66 kDa was not clear. Similar to our results, Belzer et al. (11) demonstrated by immunoblot using preparation of Br. abortus S 19 salt extractable CSP that IgG antibodies in sera from infected and from cattle vaccinated with Br. abortus S 19 bound to a common group of antigens ranging in MM of 31 to 45 kDa.

Previous study (15) has also shown by using a S-LPS spesific monoclonal antibodies that antibody response in sera of infected sheep between 38 kDa and 65 kDa corresponds to a reactivity against S-LPS like molecule but the adsorbtion of the sera with smooth Brucella cell did not alter the antibody response to the CSP at these range of MM. It is concluded that CSA stained at these MM represents a protein-LPS complex. Rev 1 used in this study is a smooth attenuated strain of Br. melitensis and may contain S-LPS in its outer membrane (3). It has also been reported that extracts prepared from Br. melitensis smooth strain by ethanol precipitation after autoclaving of the Brucella cells contains LPS and proteins (5). Using Coomassie blue staining after SDS-PAGE which detects only the proteins, we detected major proteins at MM of 33 kDa, 42 kDa, 50 kDa and 62 kDa. By immunoblotting using spesific antibody, both proteins and LPS are detectable. Therefore, immunostaining between 33 kDa and 66 kDa except the major proteins detected by coomassie blue staining in this study may be due to LPS component of the CSA preparations. Antibody response to CSP at MM of 27 - 29 kDa and 20,5 - 22 kDa and proteins below 20 kDa which were detected by coomassie blue stainning after SDS-PAGE could not be demonstrated by immunoblotting. This could be attributed that proteins at these MM may not be on cell surface or associated with outer membrane of Brucella.

The results presented in this study showed that IgG antibody reactivity in sera of naturally infected sheep could be mainly directed against CSA of Br. melitensis Rev 1 between 33 kDa and 50 kDa. In addition, depending on the preparation of antigen and use of different Brucella strains, spesific reactivity of IgG antibody with CSA at different moleculer masses will be detected, if the results of the other studies on this subject has been considered.

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