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## Cellular and Humoral Immune Responses and Antigen Recognition in Sprague-Dawley Rats Experimentally Infected with *Brucella abortus* Biotype 1

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**Abstract:** The study was undertaken to investigate the cellular and humoral immune responses as well as antigen recognition in the acute and sub-acute stages of *Brucella abortus* biotype 1 infection in Sprague-Dawley (SD) rats. The SD rats were infected intraperitoneally with  $1 \times 10^{10}$  colony forming unit (cfu) of *B. abortus* biotype 1 Korean bovine isolate. The cellular and humoral immune responses were measured at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection against Crude Brucella Protein (CBP) by Lymphocyte Proliferation Assay (LPA) and Indirect Enzyme-linked Immunosorbent Assay (IELISA). The experimentally infected rats developed specific lymphoproliferative and humoral immune response within 1 week post infection. A significant increase in the proliferative response to CBP was recorded on day 28 post infection. *Brucella abortus* specific IgG responses were initiated in SD rats at 3 days after infection. The highest IgG antibody titers were recorded at 35 days after infection and then the titer gradually decreased until the end of the experiment. Recognition of immunodominant antigens in CBP of *B. abortus* was performed by Western Blot (WB) assay using infected rat sera collected at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection. Western blot assay of the sera using CBP antigens revealed a wide array of protein bands between molecular weight of 19 and 125 kDa. Proteins of 125, 105, 82, 66, 54, 46, 32, 24, 22, 21 and 19 kDa were frequently recognized by the sera of infected rats during the experiment. The 82, 46, 32, 24, 22, 21 and 19 kDa proteins were intensely recognized during the course of infection. These antigens should be considered useful for the diagnostic of *B. abortus* infection.

**Key words:** *Brucella abortus*, immune response, antigen recognition, crude brucella protein, sprague-dawley rats

### INTRODUCTION

*Brucella* are Gram-negative, facultative intracellular bacteria which cause disease in humans, domesticated animals and wild mammals (Nicoletti, 1980; Young, 1983). Undulant fever, chills, sweating, anorexia, fatigue, weight loss, depression, arthralgia and myalgia are common clinical manifestations in human brucellosis patients. It causes abortion, infertility, stillbirth and retention of placenta in animals leading to huge economic losses (Radostits *et al.*, 2007). Humans are generally infected through direct contact with infected animals or by the consumption of the contaminated food especially unpasteurized milk and milk products (Nicoletti, 1992; Pappas *et al.*, 2006). Brucellosis mainly spread from one animal to another through contaminated materials during abortion, using infected bull or semen during natural breeding or artificial insemination (Lim *et al.*, 2005).

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Brucellosis remains endemic in many developing countries where it undermines animal health and productivity (Trujillo *et al.*, 1994). At present, brucellosis is an emerging public and animal health problem in many countries as well as Korea despite animal brucellosis control program. The other most likely source of introduction of brucellosis in domesticated animals is from free ranging wildlife (Davis and Elzer, 2002). Rats are known to harbor *Brucella* in many parts of the world (Oliakova and Antoniuk, 1989) and found to be infected with *B. abortus* on farm where cattle are infected (Moore and Schnurrenberger, 1981). The control of brucellosis in animals as well as humans could not be achieved without eradicating the disease in primary reservoir hosts as well as free ranging wildlife (Davis and Elzer, 2002; Godfroid, 2002).

There are three clinical stages of brucellosis in humans according to the duration of the disease: acute, sub-acute and chronic. Acute brucellosis persists less than 8 weeks, duration of sub-acute stage is from 8 to 52 weeks and chronic brucellosis is more than 52 weeks (Gotuzzo and Cellillo, 1998). An acute phase of brucellosis can progress either to recovery or to a chronic form. The diagnosis of brucellosis in domesticated animals is based on the detection of antibodies against O polysaccharide of smooth *Brucella* sp., in the sera by the routine serological tests. Antibodies against the O polysaccharide of smooth *Brucella* are known to react with the closely related bacteria which hamper accurate diagnosis of *Brucella* (Kittelberger *et al.*, 1998).

Antibody responses directed against proteins of *Brucella* is known to specific for the genus *Brucella* (Diaz and Moriyon, 1989). Test detecting antibodies to the *Brucella* proteins are considered specific (Cherwonogrodzky *et al.*, 1990). *Brucella* proteins elicit cell-and antibody-mediated immune responses which have been investigated in animals and humans for diagnostic purpose. However, there is no study concerning diagnosis of brucellosis in free ranging wildlife such as rats using proteins of *B. abortus*. The aim of this study was to investigate the cellular and humoral immune responses against Crude *Brucella* Protein (CBP), during the acute and sub-acute stage of brucellosis in Spragne-Dawley (SD) rats and to investigate the specific antigen recognitions.

## MATERIALS AND METHODS

### Rats

Adult SD rats (n = 44) of 8 weeks old, were purchased from a commercial laboratory animal company (Koatech, Korea). The rats were housed in cages and provided with food and water *ad libitum*. The experimental protocol was approved by the local ethic committee of the Chonbuk National University, Korea. The animals were culture as well as seronegative for *Brucella* infection, prior to experimental infection, as ascertained by routine bacteriological examination of blood samples on brucella agar media (BBL, Becton, Dickinson and Company, Sparks, MD, USA) incubated at 37°C for 7 days under 5% CO<sub>2</sub> and screening of sera by the Rose Bengal plate agglutination test (RBPT).

### Bacterial Strain

*Brucella abortus* biotype 1 Korean cattle isolate was used for the experimental infection. *Brucella abortus* biotype 1 lyophilized stock culture was obtained from the laboratory repository. Stock culture was revived on brucella agar media (BBL, Becton, Dickinson and Company, Sparks, MD, USA) by incubating at 37°C for 7 days under 5% CO<sub>2</sub>. The grown bacteria were harvested in normal saline.

### Experimental Inoculation

Forty rats were injected intraperitoneally with 0.1 mL sterile pyrogen free solution containing  $1 \times 10^{10}$  cfu mL<sup>-1</sup> of *B. abortus* biotype 1. Four uninfected control rats were injected intraperitoneally with 0.1 mL sterile pyrogen free solution.

### **Clinical Examinations**

Rectal temperature, food and water intake as well as other abnormal clinical signs of all infected and control rats were recorded daily for 2 weeks.

### **Preparation of Crude *Brucella* Protein Antigen**

The CBP was prepared from *B. abortus* biotype 1 according to the previously described methods (Onate *et al.*, 2000) with modifications. Briefly, *B. abortus* biotype 1 was inoculated onto brucella agar and incubated at 37°C for 7 days under 5% CO<sub>2</sub>. The culture was harvested in sterile Phosphate Buffered Saline (PBS) after 7 days of incubation. Bacteria were washed three times with the sterile PBS at 8000 rpm for 10 min at 4°C. Finally, it was resuspended in 50 mL of sterile PBS and inactivated by heating at 60°C for 1 h. The inactivated culture were sonicated at melting ice temperature, applying 6 cycles at 100 W, each cycle was of 1 min duration. The sonicated cell lysate was centrifuged at 1200 g for 20 min at 4°C. The supernatant was collected in 2 mL aliquots and stored at -20°C until tested. The protein concentration of the antigen was measured by using Bradford kit (Bio-Rad, Hercules, USA).

### **Lymphocyte Proliferation Assay**

At 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection, four rats from each group were euthanized and their spleens were removed under aseptic condition. Single-cell suspensions were prepared from these spleens according to a standard procedure (Onate *et al.*, 2003). Briefly, single cell suspensions were prepared from rat spleens by dispersion of the tissue through a sterilized stainless steel. The spleen was meshed with Roswell Park Memorial Institute Medium (RPMI). The meshed spleen was centrifuged at 2000 rpm for 3 min. The supernatant was discarded. Lysis of the red blood cells in the sedimented spleenocytes was performed by Tris-buffered ammonium chloride (pH 7.2). After lysis of red blood cells the spleenocytes were centrifuged at 2000 rpm for 3 min and supernatant was discarded. Finally, 10 mL of complete RPMI-1640 media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU mL<sup>-1</sup> of penicillin, 100 µg mL<sup>-1</sup> of streptomycin and 2 µg mL<sup>-1</sup> of fungizone were added in the spleenocytes suspension. The concentration of the spleenocytes was adjusted in RPMI media at a concentration of 2×10<sup>5</sup> viable cells mL<sup>-1</sup>.

The lymphocyte proliferation response was determined by the colorimetric Thiazolyl Blue Tetrazolium Bromide (MTT) assay according to the method described by Mosmann (1983), with modifications. Briefly, 100 µL of splenic cell suspension was placed in triplicate (2×10<sup>5</sup> cells well<sup>-1</sup>) in 96-well tissue culture plates with 50 µL of medium alone or medium containing CBP (2 µg well<sup>-1</sup>) and Concanavalin A (ConA) 0.5 µg well<sup>-1</sup>. The cultures were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 48 h, after which supernatant was removed by pipette. Then 50 µL of the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; Sigma] was added to each well. The cells were further incubated at 37°C for 4 h and then 100 µL of acid isopropanol (0.04 N HCl) was added to each well. The absorbance was measured at the wavelength of 570 nm with a microplate Enzyme-Linked Immunosorbant Assay (ELISA) reader (Tecan, Austria). Cellular proliferation was expressed as mean Stimulation Index (SI), calculated by dividing the mean Optical Density (OD) of the stimulated cultures by the mean OD of unstimulated control cultures.

### **Rose Bengal Plate Test**

Sera collected from SD rats at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection were tested by RBPT using a commercial whole cell antigen of *B. abortus* 1119-3 (Deasung Microbiological Labs, Korea) according to the previously described method (Alton *et al.*, 1988).

### **Standard Tube Agglutination Test**

Two-fold serial dilutions of sera collected at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection were tested by Tube Agglutination Test (TAT) using the whole cell antigen of *B. abortus* 1119-3 (National Veterinary Research and Quarantine Service, Anyang, Korea) according to the method of Alton *et al.* (1988). Reciprocal of the highest dilution of serum that showed clearing of the suspension and formation of a distinct agglutination mat at the bottom of the tube was expressed as the agglutination titer.

### **Enzyme-Linked Immunosorbent Assay**

The presence of serum total immunoglobulin G (IgG) was determined by an indirect ELISA. The CBP was diluted to  $10 \mu\text{g mL}^{-1}$  in 0.05 mM sodium bicarbonate buffer (pH 9.6) and used to coat the wells ( $100 \mu\text{L well}^{-1}$ ) of a flat-bottomed 96-well microtitre plate (Nunc, Deumark). Affinity purified Rat IgG (Bethyl laboratories, Inc., USA) were used to coat the 96-well plate starting from 500 to  $7.8 \text{ ng well}^{-1}$  for generation of standard curve. After overnight incubation at  $4^\circ\text{C}$ , plates were washed three times with wash solution (PBST: PBS pH 7.4) with 0.05% (V/V) Tween 20 and blocked with 1% Bovine Serum Albumin (BSA) (Sigma Aldrich Inc., St. Louis, MO, USA) in PBS for 30 min at  $37^\circ\text{C}$ . After three washes with PBST,  $100 \mu\text{L}$  of control and test sera samples, diluted 1:500 in sample diluent (50 mM tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) were added to each well in duplicate. The plates were sealed and incubated at  $37^\circ\text{C}$  for 1 h. After five washing cycles with PBST, each well was incubated with  $100 \mu\text{L}$  of 1: 100000 dilution of goat anti-rat IgG antibodies conjugated to horseradish peroxidase (Bethyl laboratories Inc., USA) diluted in conjugate diluent (50 mM tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0). After 1 h incubation at  $37^\circ\text{C}$ , plates were washed 5 times as described above and the color reaction was developed by adding  $200 \mu\text{L well}^{-1}$  of a solution containing  $1.0 \text{ mg mL}^{-1}$  of O-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis, USA) in 0.05 M citrate buffer (pH 4.0) with 0.04%  $\text{H}_2\text{O}_2$ . After 30 min of incubation at room temperature, the enzyme reaction was stopped by addition of  $50 \mu\text{L}$  of 3 M sulfuric acid/well and the absorbance of the developed color was measured at 492 nm, using an automatic ELISA plate reader (Tecan, Austria). The standard curve describing the relation between the concentration of standards and their absorbance value was generated and the concentration of antibody for each sample was expressed as  $\text{ng mL}^{-1}$ .

### **Western Blot Analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a mini-gel system (Bio-Rad, USA) under reducing conditions as described by Laemmli (1970). Briefly, the antigen was solubilized in sample buffer and subjected to SDS-PAGE using 12% polyacrylamide gels and analysed with Coomassie blue (2.5% brilliant blue in 50% methanol, 10% acetic acid) staining. For Western blotting, electrophoresed antigen was transferred to nitrocellulose membranes (0.45 nm pore size, Bio-Rad, USA) at 100 V for 1 h under conditions essentially similar to that described by Towbin *et al.* (1979). Unbound sites on the membrane were blocked with PBS containing 0.2% Tween 20 at  $4^\circ\text{C}$  overnight. The blocking solution was poured off and the membrane was washed with PBST (0.1% Tween 20 in PBS) three times. The membrane was then reacted with sample serum diluted in PBS containing 0.05% Tween 20 for 1 h at rt. After three times rinse with PBS containing 0.1% Tween 20 for 30 min, phosphatase-labeled affinity purified antibody rat IgG (KPL, Europe) diluted in PBS at 1:1000 were reacted for 1 h at room temperature. After 3 times rinse as described above, the membrane were immersed in BCIP/NBT substrate (KPL, Europe) and the reaction was developed in darkness at rt for 10-15 min or until desired color is achieved. A last washing step was performed once with distilled water. The membrane was allowed to dry in the air. The thoroughly dried membrane was scanned for taking the image.

### Statistical Analysis

The data were analyzed for statistical significance using Student's two-tailed t-test. The p-value of <0.05 was considered to be significant.

## RESULTS AND DISCUSSION

### Clinical Observations

All of the rats inoculated with *B. abortus* biotype 1, developed lethargic, anorectic and febrile conditions within 24 h. The uninfected control rats did not manifest any of the abnormal clinical signs. The highest rectal temperature was  $38.30 \pm 0.152^\circ\text{C}$  in the rats of inoculated group and  $36.5 \pm 0.05^\circ\text{C}$  in the uninfected control group.

### Cellular Immune Response

Sequential monitoring of lymphocytes responses to CBP revealed a progressive increase of the SI in the infected rats. The antigen specific cellular response increased from 7 days after infection with a SI of  $3.25 \pm 0.25$ . This was followed by a persistent rise in the lymphocyte response of infected rats, with a peak SI of  $6.5 \pm 0.28$  at 28 days after infection, which were significantly different from lymphocyte responses recorded in the control group. The proliferative response showed a decrease on 60 and 90 days after infection followed by a second one on 120 days after infection ( $p < 0.05$ ). The ConA mitogen as positive control was able to induce T-cell proliferation in all cases (data not shown). The results of cellular immune responses are presented in Fig. 1.

### RBPT Screening

Sera collected from rats at 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection were tested positive for *B. abortus* by the RBPT. On the other hand, RBPT was negative for sera collected from rats at 0 and 3 days after infection.

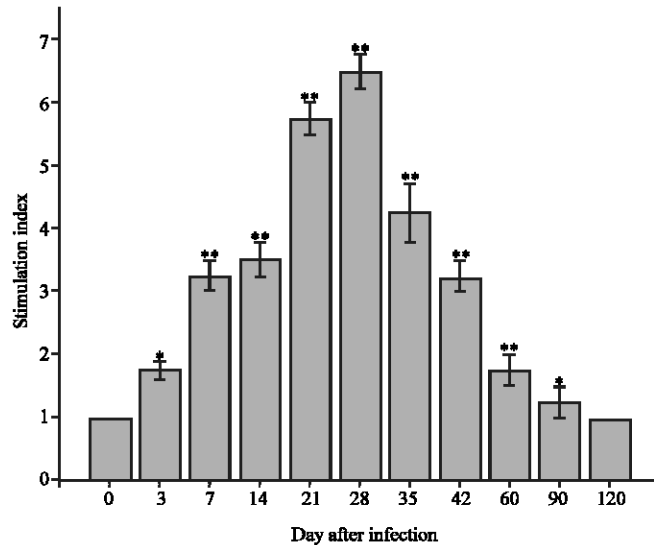


Fig. 1: Lymphocyte proliferation assay in *B. abortus* biotype 1 infected SD rats at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection. Results are expressed as the Mean±SD. Statistically significant difference of stimulation index between uninfected control and infected rats at different time points of infection are indicated by asterisks (\* $p < 0.05$  and \*\* $p < 0.001$ )

### TAT Antibody Titers

The data on TAT antibody responses of infected rats showed a remarkable increase in antibody titers after experimental infection. The TAT antibody titers at 7 days after infection was  $125 \pm 25$ , but exhibited a five-fold increase to  $625 \pm 50$  on 35 days after infection. The antibody titers began declining until the end of the experiment. No antibody responses were noted in the control group. The mean reciprocal serum antibody titers measured by TAT are presented in Fig. 2.

### ELISA Antibody Titers

In the infected rats, *B. abortus* specific IgG antibodies response started at 3 days after infection ( $150.9 \pm 14.67 \text{ ng mL}^{-1}$ ). The IgG response rose significantly from 7 days after infection ( $509.26 \pm 26.07 \text{ ng mL}^{-1}$ ). The highest serum IgG titer recorded at 35 days after infection ( $1213.27 \pm 9.06 \text{ ng mL}^{-1}$ ) then the antibody titers decreased gradually until the end of the experiment. The results of IgG responses in the sera of infected rats are presented in Fig. 3.

### Western Blot Analysis

The transfer of *B. abortus* antigens separated by electrophoresis onto a nitrocellulose gel revealed a large array of proteins with apparent molecular weight between 19 and 125 kDa. Pre-infection sera reacted weakly with the 24, 22 and 21 kDa proteins. Three days after infection 105, 54, 32 and 19 kDa proteins were recognized by the sera. At 7 days after infection, sera of the infected rats recognized 82, 32 and 19 kDa proteins. At 14 days after infection bands of 125, 46, 32 and 19 kDa protein were observed in the sera. Sera of 21 days after infection reacted with 82, 46, 32 and 19 kDa proteins. Protein bands of the 82, 46, 32 and 19 kDa reacted with sera collected after 28 days after infection. Sera collected at 35 days after infection reacted with 125, 105, 82, 46 and 32 kDa proteins. Five

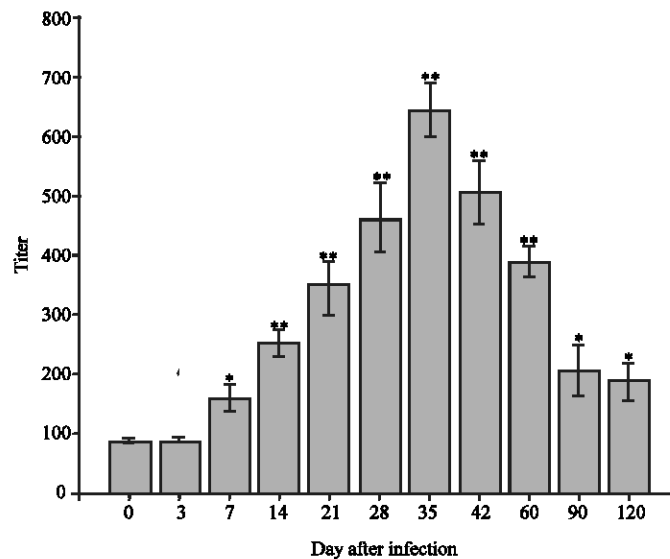


Fig. 2: Serum antibody titers in *B. abortus* biotype 1 infected rats measured by TAT at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection. Results are expressed as the Mean±SD. Statistically significant difference of antibody titers between uninfected control and infected rats at different time points of infection are indicated by asterisks (\* $p < 0.05$  and \*\* $p < 0.001$ )

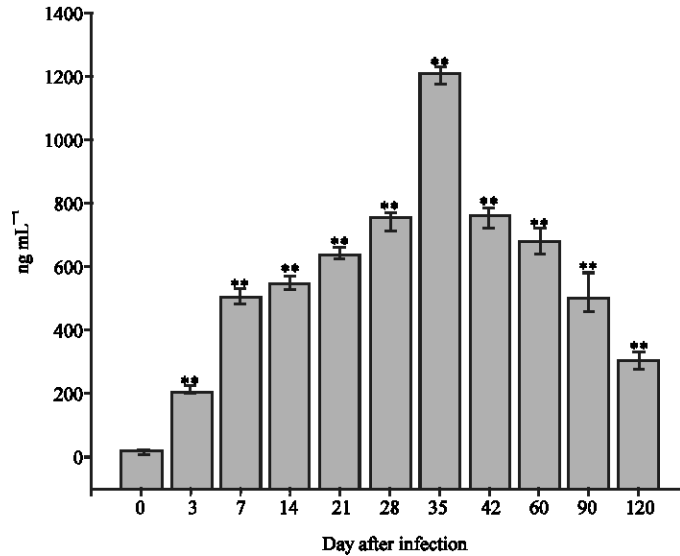


Fig. 3: Serum IgG antibody titers in the *B. abortus* biotype 1 infected rats measured by the indirect ELISA at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection. Results are expressed as the Mean±SD. Statistically significant difference of antibody titers between uninfected control and infected rats at different time points of infection are indicated by asterisks (\* $p < 0.05$  and \*\* $p < 0.001$ )

protein bands at the molecular weight of 125, 105, 82, 46 and 32 kDa reacted with sera collected at 42 days after infection. Sera at 60 days after infection reacted with 82 and 19 kDa proteins. Protein bands around 82, 66, 46 and 19 kDa molecular weight were observed in sera collected at 90 days after infection. Sera collected at 120 days after infection reacted with 46, 32 and 19 kDa proteins. The result of WB assay is shown in Fig. 4.

A wide range of wildlife is known to be the reservoir of *B. abortus*. Domesticated livestock animals might get infected with *B. abortus* by contact with the infected wildlife. Control of brucellosis in wildlife is necessary for eradication of bovine brucellosis. The cellular and humoral immunity in *Brucella* infection has always been a matter of interest for the researchers. The current understanding of immune response and antigen recognition against *B. abortus* has been arisen from the studies either in mouse or cattle. However, very little systemic information seems to be available on the measurement of immune response and antigen recognition in free ranging wildlife. We measured the cellular and humoral immune responses as well as antigen recognition induced by a virulent *B. abortus* biotype 1 in SD rats.

In the present study, experimentally infected rats mounted a lymphoproliferative and humoral response within one week after infection. We noticed lymphocyte stimulation response before the development of antibodies. Kaneene *et al.* (1979) using an extract of autoclaved *B. abortus* cells as antigen found that experimentally infected cattle developed cellular responses by 1 to 2 weeks post exposure and several days earlier than seroagglutinins could be detected. A study in a naturally infected herd confirmed that lymphocyte proliferative response preceded development of antibodies (Kaneene *et al.*, 1978).

This study demonstrates that intraperitoneal infection of rat with *B. abortus* biotype 1 generates a strong specific humoral as well as T-cell responses. The induction of T-cell immune responses following *B. abortus* infection was evaluated by measuring T-cell-proliferative responses after *in vitro*



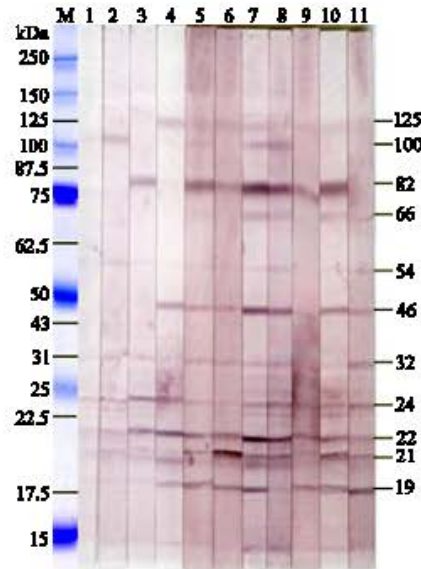


Fig. 4: Western blot analysis of the rats sera using IgG.M. protein marker (kDa), lanes 1-11, corresponding to the sera collected at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection with *B. abortus* biotype 1, respectively

stimulation of splenic cells with CBP. Data of the lymphocyte proliferation assay suggests an initial high T-cell-proliferative response followed by a gradual decreased up to the end of the study. Onate *et al.* (2000) used CBP for measuring cellular immune response in mice and observed a higher lymphoproliferative response following single injection of CBP in the footpads of mice. Cabrera *et al.* (2009) measured lymphocyte proliferation response in *B. abortus* strain RB51 vaccinated mice by *in vitro* stimulation with CBP.

Using ELISA, several studies have been conducted to analyze the antibody response against proteins of *Brucella* sp. (Letesson *et al.*, 1997). ELISA targeting selected cytoplasmic (Hemmen *et al.*, 1995), periplasmic (Rossetti *et al.*, 1996), or membrane (Cloeckeaert *et al.*, 1992; Zygmunt *et al.*, 1994) proteins has been described. In the present study, IgG antibodies mediated humoral immune response against *B. abortus* biotype 1 has been measured by an indirect ELISA using CBP during the course of infection. We detected IgG antibodies response in the sera of SD rats at 3 days after infection with *B. abortus* biotype 1. In cattle, Beh (1973) recorded IgG antibodies responses at 7 days after infection with *B. abortus*.

In acute brucellosis, serum IgG response initially becomes low but with the progress of the infection the IgG antibody titers increase. In this study, the serum IgG antibodies measured by ELISA showed maximal antibody titers at 35 days after infection. Then the antibody titers gradually decreased from 35 days of infection to the end of the experiment. Similar pattern of IgG antibody responses to *B. abortus* under experimental conditions have previously been documented in BALB/c mice (High *et al.*, 2007). On the contrary, in cattle, the IgG antibodies responses against *B. abortus* reached the peak value at 28 to 42 days after infection, after which they declined (Macmillan, 1990). The patterns of antibody responses recorded by the TAT in the sera of infected rat in our experiment were similar to that of ELISA.

The *Brucella* cell envelope is a three-layered structure in which an inner or cytoplasmic membrane, a periplasmic space and an outer membrane can be differentiated (Cloeckeaert *et al.*, 1990).

In this study, we disrupted the *B. abortus* by sonication and cell free crude extract was obtained by centrifugation. The crude extract contains outer membrane, periplasmic as well as cytoplasmic proteins. We performed WB analysis of sera collected at 0, 3, 7, 14, 21, 28, 35, 42, 60, 90 and 120 days after infection using CBP to identify the immunoreactive antigens. WB assay has been used to identify the immunoreactive proteins of *Brucella* sp., which could be used as diagnostic antigens for animal or human brucellosis (Letesson *et al.*, 1997). Among the immunodominant *Brucella* antigens identified by WB, some belong to the cell envelope and correspond to both major Outer Membrane Proteins (OMPs) (25 to 27 kDa and 36 to 38 kDa) and minor OMPs (10 kDa, 16.5 kDa, 19 kDa and 89 kDa) (Letesson *et al.*, 1997). In this study, IgG antibodies present in the sera were reacted with protein bands at a molecular weight ranging between 19 and 125 kDa in WB assay. Six protein bands of 82, 46, 24, 22, 21 and 19 kDa were intensively recognized by almost all sera of infected rats. Protein bands of 125, 105, 66, 54 and 32 kDa were weakly recognized by the sera of the infected rats. The pattern of recognition of immunodominant bands by the sera of infected rats was almost similar between acute and sub-acute infections.

In this study, a few bands were weakly recognized by pre-infection sera collected at 0 day after infection. Cross reaction and background binding were reported especially when soluble antigens were used (Dubey *et al.*, 1996; Harkins *et al.*, 1998; Nishikawa *et al.*, 2002; O'Handley *et al.*, 2002). In the present study, 105 kDa proteins were moderately recognized by sera collected at 3, 35 and 42 days after infection. Proteins band of 82 kDa was intensely recognized by sera collected at 7, 21, 28, 35, 42, 60 and 90 days after infection. The 46 kDa protein band was intensely recognized by the infected rat sera from 14 days after infection until the end of the experiment. Intense protein band of 19 kDa was identified by the sera collected at 21 days after infection and persisted up to 120 days after infection. Sera collected at 14 days after infection weakly recognized 125 kDa protein bands which were persisted until the end of the experiment. The protein band of 32 kDa was weakly recognized by sera collected at 7 days of infection and persisted until the end of the study.

Data of this study suggest that the anti-protein antibody responses were heterogeneous among infected animals and that only a combination of selected *Brucella* proteins could lead to a satisfactory diagnostic test (Limet *et al.*, 1993; Tabatabai and Heunager, 1994; Hemmen *et al.*, 1995). Based on frequency and intensity of recognition, the 82, 46, 32, 24, 22, 21 and 19 kDa proteins should be considered in rats as immuno-dominant *B. abortus* antigens which could be useful for the diagnosis of *B. abortus* infections.

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