Journal of Animal and Veterinary Advances 11 (10): 1564-1569, 2012
ISSN: 1680-5593

Kinetics and Cross-Reactivity of the Antibody in Sheep Inoculated with Virulent and Avirulent Brucella

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Abstract: Brucellosis is a zoonosis which is caused by *Brucella* species and pruduces severe economic losses and a public health problem. At present, the diagonsis of Brucella infection mainly depends on serological tests to detect antibodies in sera and the animal brucellosis is prevented via vaccine. In this study, the kinetics and cross-reactivity of antibodies in sera were evaluated in Small Tail Han sheep (*Ovis arie*) infected with a virulent field strain of *Brucella melitensis* (BmF) and ones inoculated with a vaccine strain S2 of *B. suis* under laboratory conditions. Serum samples were collected at 0, 3, 7, 14, 21, 30, 40, 44, 50, 60 and 75 days post-challenge (dpc) and were analyzed by Rose Bengal Plate Agglutination Test (RBPT) and indirect Enzyme-Linked Immunosorbent Assay (iELISA). Sera samples of BmF-challenged and S2-challenged sheep groups at 40, 44, 50, 60 and 75 dpc were tested positive to Brucella by the RBPT, nevertheless the earliest positive reaction results were observed in sera at 21 dpc by iELISA. The virulent field strain BmF initiated a higher level of antibody titer than vaccine strain S2 without statistic significant difference ($p>0.05$). The cross-reactivities with the virulent and the vaccine stains were confirmed in serum antibodies between the BmF-challenged group and the S2-challenged group. The results indicated that the serodiagnosis is hard to distinguish the brucella-infected sheep from the vaccine-inoculated sheep. Diagnosis methods of identifying between the healthy and the infected animals need to further be studied in future.

Key words: Small Tail Han sheep, *Brucella melitensis*, *Brucella suis*, kinetics of antibody, cross-reactivity, iELISA, RBPT

INTRODUCTION

Brucella which is the intracellular pathogen, consists of nine recognized *Brucella* sp. on the basis of host specificity and microbiological characteristics such as *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. microti*, *B. ceti* and *B. pinnipedialis* (Osterman and Moriyon, 2006; Huber *et al*., 2009) and causes the zoonotic brucellosis in humans and animals (Vemulapalli *et al*., 2004). Infection with Brucella usually leads to undulant fever, general malaise and reproductive failure with infertility and abortions in mammal species including humans. Brucellosis is the worldwide common bacterial zoonosis (Manthur and Amarnath, 2008) and pruduces severe economic losses and a public health problem. Humans usually are infected by brucella via directly contacting the infected animal secretions or consuming the contaminated animal products. Eradication of brucellosis in animal population has shown to be an effective strategy to prevent human brucellosis (Vemulapalli *et al*., 2004).

In areas where animal brucellosis is prevalent, vaccine inoculation is suitable for controlling the infection in animals and reducing the contamination of humans. Live attenuated derivatives of *Brucella* sp. have been successfully employed (Schurig *et al*., 2002) for instance that *B. suis* strain S2, *B. abortus* strain RB51 and 19 and *B. melitensis* strain Rev1 vaccines are available for

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domestic animals. Brucella vaccine strain S2 was isolated from swine fetus. The S2 vaccine was developed in China and introduced into the other countries, e.g., Spain, Turkey, Libya, Britain, France, Germany and Zambia (Deqiu *et al*., 2002). Now it is one of recommended vaccines to prevent animal brucellosis in China. Serological techniques are the mainstay of brucellosis diagnosis and widely applied testing programs (McGiven *et al*., 2003; Mohamed, 2007; Hawari, 2012). Traditional and well-documented serodiagnoses include the Rose Bengal Plate Agglutination Test (RBPT), Serum Agglutination Test (SAT), Complement Fixation Test (CFT) and so on (Junaidu *et al*., 2006; Ghazy *et al*., 2007).

However, serological diagnosis was somewhat confounded by the vaccination of brucellasis. In spite of the failures reported on differentiating antibodies induced by Brucella vaccine attenuated strains for animals from ones induced by pathogenic strains (Nielsen, 2002; Garin-Bastuji *et al*., 2006) evidences of antibody cross-reactivities between the infected sheep and the vaccinated sheep are insufficient. In this study, serological techniques were used to analyze the kinetics and cross-reactivities of antibodies from sheep inoculated with virulent or avirulent Brucella for confirming that the serodiagnoses had no effect on differentiation of ones infected or vaccinated and the time-courses of antibody responses to virulent field strain of *Brucella melitensis* (BmF) or avirulent *B. suis* S2 strain (S2) in Small Tail Han sheep had been characterized by iELISA for the first time.

MATERIALS AND METHODS

Animals: A total of 6, 10 months old male Small Tail Han sheep (*Ovis arie*) from brucellosis-free and S2-unvaccinated flock of Sangang farm (Jilin province, P.R. China) were randomly selected from for experimental inoculation. The flock of origin was also free from the most relevant sheep infectious diseases (paratuberculosis, chlamydiosis, mycoplasmosis, scrapie and salmonellosis). All of the selected sheep were verified by regular clinical examinations and laboratory testing and every 3 sheep were divided into one group in total two groups. Two groups were separately kept in two isolated pens with a natural daylight cycle and normal feed. Clinical examination was carried out every day. All sheep were killed at 75 days post-challenge (dpc) by exsanguination via the carotid artery and visual inspection of pathology was performed during necropsy.

Bacteria: The lyophilized avirulent *B. suis* S2 strain was obtained from commercial available Brucellosis Vaccine, Live (Strain S2) (Harbin Pharmaceutical Group Co., Ltd. Haerbin, China). The virulent *B. melitensis* Field strain (BmF) was isolated and identified from the blood of sheep infected *B. melitensis* by Institut of Zoonosis (Jilin University, Changchun, China). The freeze-dried S2 strain was rehydrated and then, S2 and glycerol-preserved BmF were separately cultured on Trypticase Soy Agar (TSA) plates containing 5% sterile bovine serum at 37°C in 10% CO_2 for 48 h. Single colony were randomly selected to be inoculated in Trypticase Soy Broth (TSB) for 72 h at 37°C. For challenge, BmF and S2 were respectively harvested by centrifugation of 3000 g for 10 min at 4°C and suspended in sterile saline solution (0.85% NaCl) at a concentration of 2.2×10^{10} cfu mL^{-1} to form bacterial suspension for challenge as shown by viable cell counts, made before challenge.

Bacterial challenge and serum sample collection: Three sheep were randomly selected and challenged by the virulent *B. melitensis* field strain (BmF) with a total dose of 2.2×10^{9} cfu in 100 μL bacterial suspension (50 μL administered conjunctivally and 50 μL intrapreputially) per sheep as a BmF-challenged group. Similarly, other three sheep were inoculated with a equal quantity of the avirulent *B. suis* vaccine strain S2 and the same administration as a S2-challenged group. Serum samples before inoculation served as the negative and the others were collected at 3, 7, 14, 21, 30, 40, 44, 60 and 75 days post-challenge (dpc) for serological studies. All serum samples were stored at -80°C until used.

Rose bengal plate agglutination test: All serum samples were analyzed by Rose Bengal Plate Agglutination Test (RBPT) which was performed with RBPT antigen (Harbin Pharmaceutical Group Co., Ltd. Haerbin, China) according to the manufacturers' instructions. In short, equal volumes (30 μL of each) of colored antigens and test sera were mixed on a clean glass slide with a sterilized toothpick. The slide was observed for the formation of clumps during 5 min. The formation of clear clumps was considered a positive test while the absence of clear clumps was considered as a negative reaction.

Indirect enzyme-linked immunosorbent: A whole-cell sonicate was prepared from heat-killed BmF or S2. Cells were sonicated at 30 Hz for 15 min with a Sonifier JY92-2D (Ningbo Scientz Biotechnology Co., Ltd. Ningbo, China.) (Funk *et al*., 2005). The suspension was centrifuged at 12,000 g at 4°C for 15 min and the supernatants were then freeze-dried, adjusted to a concentration of 0.1 μg mL^{-1} BmF or S2 sonicate resuspended in 0.05 M Na_2CO_3 for coating in ELISA procedure. For kinetics analysis of antibodies, ELISA plates coated with BmF sonicate were

used for detecting BmF-stimulated antibodies and with S2 sonicate for S2-stimulated antibodyies. However, for antibody cross-reactivity analysis, ELISA plates coated with BmF sonicate was used for detecting antibodies stimulated by S2 and with S2 sonicate for antibodies stimulated by BmF. Indirect Enzyme-Linked Immunosorbent (iELISA) was performed according to the previously described procedure (Jacques *et al*., 1998). In short, ELISA plates (Jet Bio-Filtration Products, Co., Ltd. Guangzhou, China) were coated with BmF or S2 sonicate (100 μL well^{-1}) for one night at 4°C. The wells were washed three times with washing buffer (0.1 M phosphate-buffered saline plus 0.05% Tween 80) for 5 min each time and then filled with blocking buffer (0.1 M phosphate-buffered saline plus 10% (w/v) skim milk powder) and incubated for 1 h at 37°C.

After washing three times again, 100 μL of serum samples diluted (from the beginning of 1:800) in dilution buffer (0.1 M phosphate-buffered saline) were added into each well. Following incubation for 1 h at 37°C, the wells were again washed three times and then, filled with 100 μL of a 1:5000 dilution of horseradish peroxidase-conjugated rabbit polyclonal anti-sheep IgG (H+L) (Wuhan Boster Bio-engineering, Co., Ltd. Wuhan, China) and incubated for 1 h at 37°C. The plates were then washed with washing buffer four times and filled with 100 μL of the substrate solution (0.04% (w/v) diamino-benzene crystals, 0.045% H_2O_2, citric acid 20 mM and citrate 30 mM) each well. After incubating the plates for 10 min at 37°C, the reaction was stopped by adding 50 μL of 11.2% (w/v) H_2SO_4 per well and the Optical Density (OD) at 490 nm was immediately recorded using Multi-function plat reader (Biotek instruments, Highland Park, USA). All samples were test in triplicate and were considered as positive with average absorbance values showing >2.1 times above those of the negative controls. The maximal diluted time in the positive samples was recorded as titer.

Statistical analysis: To evaluate the significance of the differences in serological response between BmF-challenged and S2-vaccinated sheep, Student's t-test was used to compare the result obtained by iELISA. Values were considered significantly different at $p<0.05$. Geometric Mean Titers (GMT) were calculated using SPSS 13.0.

RESULTS AND DISCUSSION

During the trial in BmF-challenged group. The abnormal states were not clearly observed however, necropsy at 75 dpc showed clear-cut differences between the BmF-challenged group and the S2-challenged group. Splenomegaly, hemorrhagic necrosis of deep inguinal lymph nodes and increased synovial fluid in tarsal joint (unilateral or bilateral) were found in 100% sheep (3/3) infected with virulent field strain BmF. Contrastively, in the S2-inoculated sheep, a few of the lymph nodes appeared slightly enlarged but that was highly variable and did not appear to correlate with inoculation and other tissues and apparatuses did not show macroscopic pathological changes.

Kinetics of antibodies against Brucella: All of the serum samples were tested positive from the beginning of 40 dps by RBPT in the BmF-challenged or the S2-innoculated groups and 21 dps by iELISA with GMT value 8063 in the BmF-challenged group or with GMT value 2016 in the S2-innoculated group (Fig. 1). RBPT is characteristics of simplicity and affordability that make it close to the ideal test for brucellosis in small and understaffed hospitals and laboratories (Diaz *et al*., 2011).

Although, brucellosis vaccine to human has been applied, it is rarely administrated to human, especially in China. Hence, RBPT is very useful for diagnosis of human brucellosis. However, in animal brucelloses, it did not differentiate infection of the virulent field Brucella strain from that of the vaccine strain because bacteria-killed vaccines induce unacceptable levels of antibodies interfering with serodiagnostic tests (Schurig *et al*., 2002) and RBPT was not more sensitive than ELISA. As observed in this study, serum samples could not be detected positive before 40 dps by RBPT.

As shown in Fig. 1, positive serum reactions to Brucella were found at 3 weeks after inoculation in groups challenged with the virulent field strain BmF of *B. melitensis* or the vaccine strain S2 of *B. suis* and antibody titers of two groups increased in the similar pattern without significant difference ($p>0.05$). The BmF-challenged sheep produced the anti-Brucella antibodies reaching at the highest level at 44 dpc and then the titers of antibodies against Brucella began to decline rapidly.

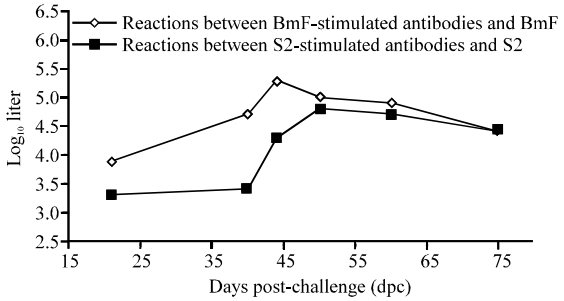

Fig. 1: Kinetics of antibodies in serum samples under laboratory conditions

While the level of anti-Brucella antibodies in the group inoculated with S2 maintained increasing until 50 dpc. Geometric mean values of antibody titers were between 8063 (21 dpc) and 204800 (44 dpc) in the BmF-challenged group and between 2016 (21 dpc) and 64508 (50 dpc) in the S2-vaccinated group. The GMT values was 25600 at 75 dpc in both groups. According to the variation of curves, the time course of antibody titers could be divided into 3 phases including a primitive term with slow growth of initial antibody titers, a medium term with rapid growth and rapid decrease of antibody titers and a terminal term with slow decrease of antibody titers. In BmF-challenged group, the primitive term lasted from 21-40 dpc, the medium term from 40-50 dpc and the terminal term from 50-75 dpc. By contrast, S2-inoculated sheep produced lower antibody titres in sera than BmF-challenged sheep with the primitive term from 21-40 dpc, the medium term from 40-60 dpc and the terminal term from 60-75 dpc. Antibody titers in serum samples are shown in Fig. 1.

Although, sheep infected with *B. melitensis* show serum antibodies detected massively in 2 weeks (Fensterbank *et al*., 1982; Verger *et al*., 1995), the result of detecting a ntibody titers at 3 weeks post-challenge in this study was similar to that of sheep challenged with *B. melitensis* strain 53H38 (Duran-Ferrer *et al*., 2004). Researchers suspected that heterogeneity of animal species and virulence of *B. melitensis* contributed to the difference of opportunity for positive showed.

Along with Brucella infection, gene expression in host cells is modified and the immune responses are modulated which facilitates intracellular survival and the development of chronic infections (Adams, 2002; Maria-Pilar *et al*., 2005; Rajashekara *et al*., 2006; Galindo *et al*., 2009). Thus, essential disease-dependent host genes would be predicted for generating targeted genetic disease resistance or developing novel strategies to diagnose, treat and prevent brucellosis (Adams, 2002). Information about gene expression pattern has been obtained in mouse macrophages infected with *B. melitensis* (He *et al*., 2006). In this study, the time-courses of antibody response to *B. melitens* BMF strain and *B. suis* S2 strain in sheep were discussed and provided time reference for screening differentially expressed genes.

Antibody cross-reactivity: The cross-reactivities with the virulent *B. melitensis* stain BmF and the vaccine *B. suis* stain S2 were confirmed in serum antibodies between the BmF-challenged group and the S2-challenged group with similar patterns as shown in Fig. 2 and 3. In the S2-inoculated group, the antibodies against S2 showed the cross-reactivity with the virulent BmF and the titer with BmF (GMT value 258032) at 50 dpc was higher than one against S2 (GMT value 64508) but no significant difference ($p>0.05$) (Fig. 2). In the BmF-challenged group although, the titers of anti-sera with BmF were higher than that with S2 and the peak of titers with BmF (GMT value 204800, at 44 dpc) appeared more early than that with S2 (GMT value 64508 at 50 dpc), significant difference ($p>0.05$) was not found in titers of anti-sera with between BmF and S2 (Fig. 3).

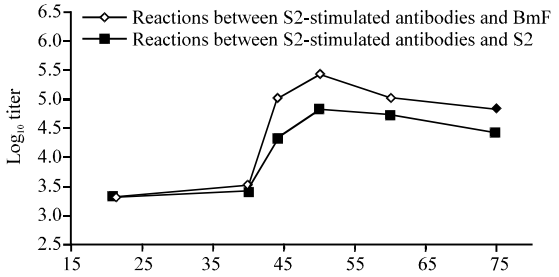

Fig. 2: S2-stimulated antibodies showed the cross-reactivity with BmF

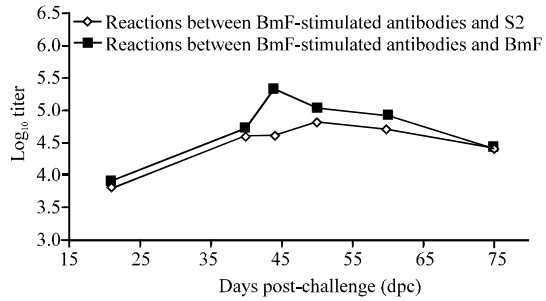

Fig. 3: BmF-stimulated antibodies showed the cross-reactivity with S2

In China, Brucella vaccines of strain S2 protect the animals from brucellosis at some extent (Deqiu *at al*., 2002). From the perspective of humoral immunity, it explains why S2 vaccines show the ability to prevent brucellosis that the anti-sera of animals vaccinated with S2 perform the cross-reactivity with field virulent Brucella strains. However, antibodies induced by vaccine S2 interfere with the serodiagnoses to brucellosis and lead to difficulties in differentiating healthy animals vaccinated from animals infected with brucellosis. Therefore, to study diagnoses techniques on differentiating infected animals from vaccinated ones is indispensable and important for preventing and controlling brucellosis.

CONCLUSION

In this study, the time-courses of antibody responses to virulent or avirulent Brucella in sheep had been

characterized and were regarded as indicators to different phase of humoral immune. In addition, the cross-reactivities of Brucella-induced antibodies with the virulent or the vaccine stains were confirmed between the BmF-challenged group and the S2-vaccinated group. The present study indicated that the serodiagnosis faced the difficulty in distinguishing the Brucella-infected sick sheep from the vaccine-inoculated healthy sheep. As a result, diagnosis methods of identifying between the healthy and the infected animals need to be further studied in future.

ACKNOWLEDGEMENTS

The project was supported by the National Natural Science Foundation of China (Grant No. 30901070). Feng Tang and Hong-Lin Ren made equal contributions. Shi-Ying Lu (lushiying1129@163.com) and Zeng-Shan Liu (zsliu1959@sohu.com), were corresponding authors, too.

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