Bangl. J. Vet. Med. (2004). 2 (1) : 63–67

PLATE AND TUBE AGGLUTINATION TESTS FOR DIAGNOSIS OF *BRUCELLA ABORTUS* BIOTYPE 1 INFECTION IN SPRAGUE- DAWLEY RATS

M. S. Rahman[1]

College of Veterinary Medicine, Chonbuk National University, Jeonju 561 756, South Korea

ABSTRACT

The plate and tube agglutination tests were evaluated for the diagnosis of experimentally induced *Brucella abortus* biotype 1 infection in 45 female, 6 to 10 months old Sprague- Dawley (SD) rats during the period from 2001 to 2002. These 45 rats were divided into two groups A and B, of which group A consisting of 27 rats used for experimental infection, whereas 18 rats of group B served as uninfected control. Each rat of group A was injected subcutaneously @ 1.0×10^9 colony forming units (CFU) in 500 µl of bovine pathogenic strain of *B. abortus* biotype 1 suspension in physiological saline. The SD rats were monitored at regular intervals by serological and bacteriological methods. The reciprocal antibody titer was 1:400 through tube agglutination test (TAT) whereas it was 1:800 through plate agglutination (PAT) at first week of post-infection. There was no reciprocal antibody titer in sera of 24 weeks of post-infection both through PAT and TAT despite the presence of bacteremia and these tests were evaluated for the first time using sera from rat with brucellosis. PAT using *B. abortus* strain 1119-3 (S1119-3) whole cell antigen was a potential candidate as an improved diagnostic method for field diagnosis of brucellosis in wild animals.

Key words : *B. abortus* biotype 1, plate and tube agglutination tests, Sprague-Dawley rats

INTRODUCTION

Brucellosis is one of the major zoonoses in South Korea and has been recognized as a cause of reproductive failure in dairy cattle, thereby causing significant economic losses through calf loss and in costs for regulatory and eradication programs. Because of the economic importance of brucellosis in domestic animals, diagnosis and prophylaxis of brucellosis in domestic animals have been widely investigated (Alton *et al.*, 1988; Nicoletti, 1990). Although wild animals are crucial in the economy of developing countries, the brucellosis of wild animals have received comparatively little attention. With respect to serological diagnosis there have been studies on conventional tests using whole cell antigens and complement fixation test and also on agar immunodiffusion tests using uncharacterized trichloroacetic acid or sonic extracts (Bell *et al.*, 1976; Falade, 1978; Waghela *et al.*, 1980; Diaz-Aparicio *et al.*, 1994). However, those studies have not been performed using rat sera from infected or free of brucellosis, and therefore, the actual value of those tests are unknown. *B. abortus* biotype 1 has been isolated from cattle in different provinces of South Korea following extensive studies (Chung *et al.*, 1988; Park *et al.*, 1998; Baek *et al.*, 2001; Rahman, 2003). The purpose of the present study was to investigate the plate (PAT) and tube (TAT) agglutination tests for diagnosis of *B. abortus* biotype 1 infection in Sprague- Dawley (SD) rats using whole cell antigen of *B. abortus* strain 1119-3 (S1119-3).

MATERIALS AND METHODS

Culture of *B. abortus*

B. abortus biotype 1 isolated in South Korea was used in this study for infection. For the preparation of antigens for PAT, TAT, S1119-3 was used. *B. abortus* biotype 1 was cultured in *Brucella* broth (Difco Co., USA) for 48 hours at 37°C with 5% CO_2. The master seed of S1119-3 was grown on *Brucella* agar (Difco Co., USA) for 72 hours at 37°C. Then growth was performed in *Brucella* broth (Difco Co., USA) in shaking incubator at 37°C with 180 rpm. After 30 hours culture, the organisms were heat inactivated at 95°C for 1 hour. The bacteria were washed with saline for 3 times and suspended in physiological saline before use.

Experimental design

Healthy disease free 6 to 10 months old female SD rats (n = 45) weighing 200 to 250 grams with no history of exposure to *Brucella* species were used in this experiment. Rats which were classified into infected group (n = 27) and control group (n = 18), antibody by PAT, TAT were negative for brucellosis (Alton *et al.*, 1975). The infected group was equally divided into 9 subgroups for 0, 1, 2, 4, 8, 12, 16, 20 and 24 weeks post-infection consisting of 3 in each subgroup. Similar procedure was followed in control group but each subgroup consisting of 2 rats (Table 1). The rats were maintained under hygienic conditions and provided with commercial feed and water *ad libitum*.

[1]Present address : Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University Mymensingh-2202, Bangladesh.

Copyright © 2004 Bangladesh Society for Veterinary Medicine All rights reserved 1729-7893/0025/04

A 500 μl containing 1.0×10^9 colony forming unit (CFU) suspension of *B. abortus* biotype 1 in physiological saline solution was injected subcutaneously to each of 27 rats of infected group. Eighteen rats of control group were injected subcutaneously only with 500 μl of physiological saline each, housed separately and not exposed to *B. abortus* biotype 1 organism. All of the rats were examined carefully daily for 1 week to record the clinical signs, rectal temperatures after infection.

Table 1. Sprague-Dawley rats used in plate and tube agglutination test for diagnosis of *B. abortus* biotype 1 infection

Groups	Number of rats at weeks post-inoculation								
	0	1	2	4	8	12	16	20	24
Infected	3	3	3	3	3	3	3	3	3
Control	2	2	2	2	2	2	2	2	2

Bacteriological culture of blood and collection of serum

One ml of blood was collected from the heart of each rat with heparin (100 IU / ml) at every 7 days post-infection after anaesthesia with ketamine hydrochloride (25 mg IM / rat) and was cultured at 37°C with 5% CO_2 for 3 days in glass tube containing 3 ml of tryptose soy broth (Difco Co., USA) with 5% bovine serum. From this tube, cultured content was cultured again at 37°C with 5% CO_2 for 3 days on tryptose soy agar (Difco Co., USA) plate with 5% bovine serum to see bacterial colony (Alton *et al.*, 1988). Another 1 ml of blood was collected following the same procedure without heparin and serum was separated, frozen and stored at -20°C until used.

Tube agglutination test

The preparation of diagnostic antigen and procedure were conducted as described by Hur *et al.* (2001). The prepared antigen was standardized according to procedure of OIE, 2000. Briefly, inactivated S1119-3 whole cells were washed with 0.5% phenol saline (0.85%) and suspended in 0.5% phenol saline (0.85%) containing preservative, at the concentration of 4.5% (v / v). This concentrated antigen was diluted in phenol saline for use at 1:100 dilution. Thereafter, quantities of 0.08, 0.04, 0.02, 0.01, 0.005, 0.00125 ml of serum samples were placed in different tubes and mixed with 2 ml of diluted antigen. The results were read after incubation at 37°C for 48 hours. A positive reaction was one in which the serum-antigen mixture was clear and gentle shaking did not disrupt the flocculi. A negative reaction was one in which the serum-antigen mixture was not clear and gentle shaking revealed no flocculi.

Plate agglutination test

The preparation of diagnostic antigen and procedure were conducted according to the procedures of Ryu *et al.* (1997). The prepared antigen was standardized according to procedure of OIE (2000). Briefly, inactivated S1119-3 whole cells were washed with 0.5% phenol saline (0.85%) and suspended at 11.0% instead of 4.5% of TAT. Crystal violet brilliant green staining solution was prepared by dissolving 2.0 gram brilliant green and 1.0 gram crystal violet in 300 ml of distilled water. Then 6 ml of this staining solution was added into 1,000 ml of cell suspension. 0.03 ml of antigen solution was added to 0.08, 0.04, 0.02, 0.01, 0.005, 0.00125 ml of each sample serum on a glass plate and then incubated for 8 min at room temperature. The plate was hand rotated three times, at 4 and 8 min after mixing and just before reading. Any sign of agglutination was considered positive (Alton *et al.*, 1988).

RESULTS AND DISCUSSION

Clinical signs

All of the rats infected with *B. abortus* biotype 1 developed lethargic, anorectic and febrile conditions. The highest rectal temperature of infected group was 38°C until 3 days, whereas in control group the temperature remained 36°C. There were no other adverse reactions or clinical signs after infection.

Bacteriological findings

Colony of *B. abortus* biotype 1 was observed on the tryptose soy agar plate cultured from blood of all infected rats until 24 weeks post-infection.

Tube agglutination test

The reciprocal antibody titers of TAT in sera of infected SD rats using S1119-3 whole cell antigen have been presented in Table 2. The average reciprocal antibody titer was 1:400 at 1st and 2nd weeks of post-infection and it increased to 1:1600 at 4th week post-infection. The reciprocal antibody titers gradually decreased to 1:25 at 20th week post-infection, and there was no reciprocal antibody titer at 24th week post-infection (Table 2). Reciprocal antibody titers were not detected before infection of *B. abortus* biotype 1 and in control rats. The reaction (agglutination) pattern observed in TAT has presented in Fig. 1.

Table 2. Tube and plate agglutination tests of *B. abortus* biotype 1 infected SD rats using *B. abortus* strain 1119-3 whole cell antigen.

Tests	Reciprocal antibody titres at weeks post-infection								
	0	1	2	4	8	12	16	20	24
TAT	–	1:400	1:400	1:1600	1:200	1:200	1:50	1:25	–
PAT	–	1:800	1:800	1:1600	1:200	1:200	1:25	1:25	–

TAT = Tube agglutination test, PAT = Plate agglutination test, – indicates no agglutination.

Fig. 1. Tube agglutination test in sera of *B. abortus* biotype 1 infected Sprague-Dawley rats using *B. abortus* strain 1119-3 whole cell antigen; TAT (–ve), negative reaction for brucellosis, TAT (+ve), positive reaction for brucellosis.

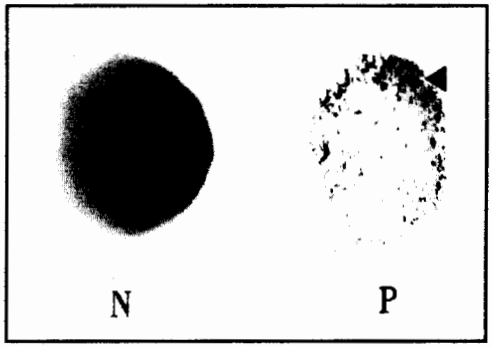

Fig. 2. Plate agglutination test in sera of *B. abortus* biotype 1 infected Sprague-Dawley rats using *B. abortus* strain 1119-3 whole cell antigen; N = Serum before inoculation, P = Serum of the 4th week of post-infection of *B. abortus* biotype 1, ▲Arrow indicates the agglutinated particles.

Plate agglutination test

The reciprocal antibody titers of PAT in sera of infected SD rats using S1119-3 whole cell antigen have been presented in Table 2. The average reciprocal antibody titer was 1:800 at 1st and 2nd weeks post-infection and it increased to 1:1600 at 4th week of post-infection. The titers gradually decreased to 1:25 at 20th week post-infection, and there was no reciprocal antibody titer at 24th week post-infection. Antibody titers were not detected before infection of *B. abortus* biotype 1 and in control rats. The reaction (agglutination) pattern observed in TAT presented in Fig. 2.

The diagnosis of brucellosis is confirmed by isolation of *Brucella* by bacteriological culture or by the detection of an immune response by serological test to its antigens (Ewalt, 1989; Orduna *et al.*, 2000). The diagnosis of brucellosis based exclusively on *Brucella* isolation presents several drawbacks. The slow growth of *Brucella* may delay diagnosis for more than 7 days (Rodriguez Torres and Fermoso, 1987; Ariza, 1996; Yagupsky, 1999). Also, the sensitivity is often low, ranging from 50 to 90% depending on disease stage, *Brucella* species, culture medium, quantity of bacteria

and culture technique employed (Gotuzzo *et al.*, 1986; Yagupsky, 1999). Thus, the serological tests play an important role in the diagnosis of brucellosis.

Among the serological tests, the TAT has become the standard method, is the test recommended for collection of quantitative information on immune responses, and is the most frequently used confirmatory serological test (Lucero and Bolpe, 1998). In many countries, the PAT, which may give false negative results, is the routine test and is sometimes the only used (Lucero and Bolpe, 1998). TAT was the first test used for the diagnosis of brucellosis in people and was soon adapted for use in animals (George, 1994).

Brucella species are facultative intracellular pathogens which survive within a variety of cells including macrophages, and the virulence of these species and the establishment of long time infections by them are thought to be essentially due to their ability to avoid the killing mechanisms within macrophages (Baldwin and Winter, 1994; Sangari and Aguero, 1996). These macrophages in the peritoneum of *Brucella* infected rats produce nitric oxide, which can contribute to persist the infection for a long time (Urrutia *et al.*, 2000). The involvement of nitric oxide in the anti-brucella activities of macrophages has also been suggested by pharmacological experiments (Jiang *et al.*, 1993).

Bacteremias due to brucellosis may persist for varying periods of time depending on the host and *Brucella* species. In goats infected with *B. melitensis,* bacteremia is detectable more than 300 days. In cattle infected with *B. abortus,* the onset of bacteremia may last 5 months or more. In swine infected with *B. suis,* bacteremia may persist for more than 3 years (Alton, 1990; George, 1994). Bacteremia with *B. canis* has been shown to be dose dependent and may detectable at 1,120 days (Carmichael, 1990; George, 1994). In the present study, *B. abortus* biotype 1 was inoculated into the SD rats subcutaneously at the dose rate of 1.0×10^9 CFU and bacteremia was detected until 24 weeks post-infection.

In the present work, PAT, TAT using S1119-3 whole cell antigen have been evaluated for the first time using brucellosis infected sera in rats. The reciprocal antibody titer was 1:400 through TAT whereas it was 1:800 through PAT at first week post-infection. There was no reciprocal antibody titer in sera of 24 weeks post-infection both through PAT and TAT but there were bacteremia until 24 weeks post-infection. The reciprocal antibody titer was 1:25 both by PAT and TAT on 20th week post-infection. Therefore, there was a close relationship between PAT and TAT. TAT requires 48 hours incubation and skilled dilution of the antigen to complete the reaction whereas PAT requires only 8 minutes with no requirements of incubation and dilution of antigen. Therefore, PAT using S1119-3 whole cell antigen can be recommended as a suitable test for field diagnosis of brucellosis in wild animals.

REFERENCES

1. Alton GG (1990). *Brucella melitensis.* In : *Animal Brucellosis,* Nielsen K and Duncan JR, CRC Press, Boca Raton, Florida, USA.
2. Alton GG, Jones LM, Angus RD and Verger JM (1988). Bacteriological method, serological method. In : *Techniques for the brucellosis laboratory,* Institut National de Recherche Agronomique, Paris, France.
3. Alton GG, Jones LM and Pietz DF (1975). Laboratory techniques in brucellosis. 2nd edn., World Health Organization, Geneva, Switzerland.
4. Ariza J (1996). Brucellosis. *Current Opinion and Infectious Diseases* 9 : 126-131.
5. Baek BK, Rahman MS, Matsuda K, Lee JY, Hur J, Lim CW and Kakoma I (2001). Infection of *Brucella abortus* biotype 1 to pregnant Sprague-Dawley rat. *Korean Journal of Veterinary Public Health* 25 : 301-308.
6. Baldwin CL and Winter AJ (1994). Macrophages and *Brucella. Immunological Series* 60 : 363-380.
7. Bell LM, Hayles LB and Chanda AB (1976). Evidence of reservoir hosts of *Brucella melitensis. Medical Journal of Zambia* 10 : 152-153.
8. Carmichael LE (1990). *Brucella canis.* In : *Animal brucellosis,* Nielsen K and Duncan JR, CRC Press, Boca Raton, Florida, USA.
9. Chung JS, Cho YJ and Park CK (1988). Isolation and biotyping of *Brucella abortus* from dairy cattle in Kyungbuk area, Korea. *Korean Journal of Veterinary Research* 28 : 339-343.
10. Diaz-Aparicio E, Marin C, Alonso-Urmeneta B, Aragon V, Perez-Ortiz S, Pardo M, Blasco JM, Diaz R and Moriyon I (1994). Evaluation of serological tests for diagnosis of *Brucella melitensis* infection of goats. *Journal of Clinical Microbiology* 32 : 1159-1165.
11. Ewalt DR (1989). Comparison of three culture techniques for the isolation of *Brucella abortus* from bovine supramammary lymph nodes. *Journal of Veterinary Diagnosis and Investigation* 1 : 27-230.
12. Falade S (1978). A comparison of three serological tests in the diagnosis of caprine brucellosis. *Research in Veterinary Science* 24 : 376-377.
13. George WB (1994). Handbook of Zoonoses, 2nd edn., Section A: Bacterial, Rickettsial, Chlamydial, and Mycotic. CRC Press, Inc., USA.
14. Gotuzzo E, Carrillo C, Guerra J and Liosa L (1986). An evaluation of diagnostic methods for brucellosis – the value of bone marrow culture. *Journal of Infectious Diseases* 153 : 122-125.

15. Hur J, Lim CW and Baek BK (2001). Immune response of dogs immunized with inactivated *Brucella abortus* biotype 1. *Korean Journal of Veterinary Public Health* 25 : 89-95.
16. Jiang X, Leonard B, Benson R and Baldwin CL (1993). Macrophage control of *Brucella abortus* : role of reactive oxygen intermediates and nitric oxide. *Cell Immunology* 151 : 309-319.
17. Lucero NE and Bolpe JE (1998). Buffered plate antigen test as a screening test for diagnosis of human brucellosis. *Journal of Clinical Microbiology* 36 : 1425-1427.
18. Nicoletti P (1990). Vaccination. In : *Animal Brucellosis*, Nielsen K and Duncan JR, CRC Press, Boca Raton, Florida, USA.
19. OIE (2000). OIE Manual of Standards for Diagnostic Tests and Vaccines. 4th edn., 12 rue de Prony, 75017 Paris, France.
20. Orduna A, Almaraz A, Prado A, Gutierrez PM, Garcia-Pascual A, Duenas A, Cuervo M, Abad R, Hernandez B, Lorenzo B, Bratos MA and Torres AR (2000). Evaluation of an immunocapture-agglutination test (Brucellacapt) for serodiagnosis of human brucellosis. *Journal of Clinical Microbiology* 38 : 4000-4005.
21. Park NC, Kim SY, Cho KH, Do JC, Kim YH, Shin SH, Cho MH, Oh KH, Kim WH, Kim JH, Jyeong JS, Kim SW and Kim BH (1998). Studies on the brucellosis in Kyeongbuk area. *Korean Journal of Veterinary Service* 21 : 451-465.
22. Rahman MS (2003). Experimental infection and protective immunity of Sprague-Dawley rats with *Brucella abortus*. PhD thesis, College of Veterinary Medicine, Chonbuk National University, Jeonju, South Korea.
23. Rodriguez Torres A and Fermoso J (1987). Brucellosis. *Medicine* 76 : 3165-3177.
24. Ryu YS, Park CK and Chang CH (1997). *Diagnostic Manual for Animal Diseases*. 1st edn., Seoul, 1-Gong World, South Korea.
25. Sangari FJ and Aguero J (1996). Molecular basis of *Brucella* pathogenicity: an update. *Microbiologia* 12 : 207-218.
26. Urrutia LL, Alonso A, Nieto ML, Bayon Y, Orduna A and Crespo MS (2000). Lipopolysaccharides of *Brucella abortus* and *Brucella melitensis* induce nitric oxide synthesis in rat peritoneal macrophages. *Infection and Immunity* 68 : 1740-1745.
27. Waghela S, Wandera JG and Wagner GG (1980). Comparison of four serological tests in the diagnosis of caprine brucellosis. *Research in Veterinary Science* 28 : 168-171.
28. Yagupsky P (1999). Detection of brucellae in blood culture. *Journal of Clinical Microbiology* 37 : 3437-3442.