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Rose Bengal and Mercaptoethanol Tests for the Diagnosis of *Brucella abortus* Biotype-1 Infection in Sprague-Dawley Rats

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Abstract. Rose Bengal test (RBT) and mercaptoethanol test (MET) were conducted for the diagnosis of *Brucella abortus* biotype-1 infection. For this purpose, female Sprague-Dawley (S-D) rats were subcutaneously infected at the dose rate of 1.0×10^9 colony forming unit suspension of *B. abortus* in physiological saline. The S-D rats were monitored at regular intervals using serological and bacteriological methods. The reciprocal antibody titer after the first week was 1: 800 with RBT, whereas it was 1: 400 with MET. The reciprocal antibody titer of 1: 25 through RBT and 1:100 through MET, respectively, were noted in infected rats of 24 weeks post-infection. Bacteremia was detected untill 24 weeks post-infection. RBT, using *B. abortus* strain S1119-3 whole cell antigen, appears to be a potential candidate, as a useful diagnostic method, for brucellosis detection in rats.

Keywords: brucellosis, *Brucella abortus* biotype-1, Sprague-Dawley rats, Rose Bengal test, mercaptoethanol test

Introduction

Brucellosis is an economically important disease of domestic and pet animals. Methods for its diagnosis have been extensively investigated (Baek *et al.*, 2003; Kakoma *et al.*, 2003; Plommet and Fensterbank, 1984). Although wild and laboratory animals are crucial in the economy of developing countries, studies on the prevalence of brucellosis in these animals have received comparatively little attention. With respect to serological diagnosis, studies have been carried out on conventional tests using whole cell antigens and complement fixation test (CFT), as well as agar immunodiffusion tests using uncharacterized trichloroacetic acid or sonic extracts (Diaz-Aparicio *et al.*, 1994; Waghela *et al.*, 1980; Falade, 1978; Bell *et al.*, 1976). *Brucella abortus* biotype-1 has been isolated from cattle from different provinces of South Korea and studied extensively (Rahman, 2003; Baek *et al.*, 2001; Park *et al.*, 1998; Chung *et al.*, 1988). Purpose of the present study was to investigate the Rose Bengal test (RBT) and mercaptoethanol test (MET) for the diagnosis of *B. abortus* biotype-1 infection in Sprague-Dawley (S-D) rats using whole cell antigen of *B. abortus* strain 1119-3 (S1119-3). Such studies, using rat sera from brucellosis infected, or non-infected rats, have not been performed preveously. The present study, therefore, is an attempt to establish the actual diagnostic value of these tests, not reported so far, for practical applications.

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Materials and Methods

Culture of *Brucella abortus*. *Brucella abortus* biotype-1, isolated in South Korea, was used in this study for experimentally induced brucellosis infection in rats. For the preparation of antigens for RBT and MET, S1119-3 strain of *B. abortus* was used. *B. abortus* biotype-1 was cultured in *Brucella* broth (Difco Co., USA) for 48 h at 37 °C with 5% CO_2. The master inoculum of S1119-3 was grown on *Brucella* agar (Difco Co., USA) for 72 h at 37 °C, followed by growth in *Brucella* broth in a rotary shaking incubator at 180 rpm at 37 °C. After 30 h incubation, the cell culture was inactivated by heating at 95 °C for 1 h, washed with physiological saline 3 times, and kept suspended in the physiological saline until use.

Experimental design. Healthy and disease-free, 6 to 10 month old female S-D rats (n = 45), weighing 200 - 250 g with no history of previous exposure to *Brucella* species were used in the present investigations. Rats were classified into brucellosis infected (n = 27) and control groups (n = 18). The infected group was equally divided into nine sub-groups, each comprising three animals, for 0, 1, 2, 4, 8, 12, 16, 20 and 24 weeks of post-infection studies. Similar sub-groupings were followed for the control group, however, each sub-group consisted of 2 rats. The rats were maintained under hygienic conditions and were fed on commercial feed and water *ad libitum*. 500 µl suspension, containing 1.0×10^9 colony forming unit (cfu) of *B. abortus* biotype-1, suspended in physiological saline solution, was injected subcutaneously to each of the 27 rats. Eighteen rats were sub-cutaneously

injected with only 500 µl each of the physiological saline, to serve as the control. All rats were carefully examined daily for one week to record the appearance of clinical symptoms, while rectal temperatures after infection were recorded daily untill the completion of the experiment.

Bacteriological culture of blood and collection of serum. One ml blood was collected from the heart of each rat with heparin (100 IU), after every 7-day post-infection period, under anaesthesia (ketamin, 25 mg, intra-muscular). The collected blood was cultured in glass tubes containing 3 ml of tryptose soy broth (Difco Co., USA) containg 5% bovine serum, with 5% CO_2 at 37 °C for 3 days. The contents of this tube were inoculated again in petri plates, containing tryptose soy agar (Difco Co., USA) with 5% bovine serum and 5% CO_2 at 37 °C for 3 days for determining *B. abortus* cfu. Another 1 ml blood sample was also collected, following the same procedure but without heparin, and the blood serum was separated, frozen and stored at -20 °C until later use.

Rose Bengal test (RBT). The preparation of diagnostic antigen for RBT was done in accordance with the procedure of Baek *et al.* (2002). Briefly, inactivated *B. abortus* S1119-3 whole cells were washed with 0.5% phenol saline (0.85%) and suspended in 0.5% phenol saline (0.85%) at the rate of 1 g in 22.5 ml. This suspension (35 ml) was added to 1 ml of 1% Rose Bengal (Sigma, USA) and stirred for 2 h at room temperature. The stained cells were uniformly resuspended at the rate of 1 g of cells to 7 ml of diluent (21.1 g NaOH dissolved in 353 ml phenol saline, followed by 95 ml of lactic acid, and adjusted to 1,056 ml with phenol saline). After filtration through cotton wool, it was adjusted to a packed cell volume of 8%. 30 µl of serum was mixed with equal volume of antigen on a white enamel plate, circled approx 2 cm in dia with manicure. The mixture was shaken gently for 4 min at room temperature, and then observed for any sign of agglutination, as the positive sign of detection.

Mercaptoethanol test (MET). The MET was performed as described by Rahman (2003). Briefly, fresh 0.1 M mercaptoethanol solution made in normal saline (NaCl, 8.5 g; 7.14 ml, 2-mercaptoethanol; distilled water to 1 liter) and stored at 4 °C. Test sera (ml), 0.08, 0.04, 0.02, 0.01, 0.005, 0.0025, and 0.00125 were separately transferred to 1 ml of 0.1 M mercaptoethanol in saline and 1 ml of concentrated tube agglutination test antigen (diluted 1: 50 in normal saline solution) in test tubes. The tubes were then shaken and incubated at 37 °C for 48 h. A positive reaction was indicated when the serum-antigen mixture was clear and gentle shaking did not disrupt the flocculi. A negative reaction was indicated when the serum-antigen mixture was not clear and gentle shaking revealed no flocculi.

Results and Discussion

Clinical findings. All rats infected with *Brucella abortus* biotype-1 developed lethargic, anorectic and febrile conditions. The highest rectal temperature of rats in the infected group was 38 °C for 3 days, whereas temperature remained 36 °C in rats in the control group. The temperature before infection was also 36 °C. No other adverse reactions or clinical symptons were observed after infection.

Bacteriological findings. *Brucella abortus* biotype-1 colonies were observed to develop on the tryptose soy agar plates cultured from blood samples of all infected rats during the entire 24 weeks of post-infection investigation period.

Rose Bengal test. The reciprocal antibody titers of RBT in the sera of infected S-D rats, using S1119-3 whole cell antigen, have been presented in Table 1. The average reciprocal antibody titer was 1: 800 during 1st and 2nd week of post-infection observations, which increased to 1:1600 after the 4th week of post-infection. The reciprocal antibody titers gradually decreased to 1: 25 after the 24th week of post-infection. Reciprocal antibody titers were not detected before the infection of *B. abortus* biotype-1 and in the control rats. The pattern of reaction observed in RBT is shown in Fig. 1.

Mercaptoethanol test. The reciprocal antibody titers of MET in sera of S-D rats infected by S1119-3, against whole cell antigen, have been presented in Table 1. The average recipro-

Fig. 1. Rose Bengal test as observed in the sera of Sprague-Dawley rats, infected by *Brucella abortus* biotype-1 strain S1119-3, whole cell antigen; N = serum before inoculation; P = serum of the 4th week post-infection; arrow = indicating the agglutinated particles.

Table 1. Results of Rose Bengal test (RBT) and mercaptoethanol test (MET) for the diagnosis of brucellosis in Sprague-Dawley rats infected with *Brucella abortus* biotype-1 strains S1119-3 whole cell antigen

Method of diagnosis	Reciprocal antibody titers post-infection (weeks)							
	1	2	4	8	12	16	20	24
RBT	1:800	1:800	1:1600	1:200	1:200	1:50	1:50	1:25
MET	1:400	1:400	1:1600	1:800	1:200	1:200	1:100	1:100

cal antibody titer was 1: 400 during 1st and 2nd week of post-infection observations, which increased to 1:1600 after the 4th week of post-infection. The titers gradually decreased to 1:100 after the 24th week post-infection. Antibody titers were not detected before the infection of *B. abortus* biotype-1 and in the control rats. The pattern of reaction observed in MET is shown in Fig. 2.

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 programmes. The diagnosis of brucellosis is confirmed by the isolation of *Brucella* by bacteriological culture or by the detection of an immune response to its antigens by serological tests (Orduna *et al.*, 2000; Ewalt, 1989). 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 (Yagupsky, 1999; Ariza, 1996; Rodriguez-Torres and Fermoso, 1987). Further problems in the diagnosis may appear on account of the often low sensitivity, which may range between 50 to 90% depending on the disease stage, the *Brucella* species involved in the infection, culture medium used, the quantity of bacteria isolated, and the culture technique employed (Yagupsky,1999; Gotuzzo *et al.*, 1986).

The RBT is used as a standard screening test for serological diagnosis of *Brucella* infection, which is more sensitive than the complement fixation test when testing culture-positive animals (Blasco *et al.*,1994). In some countries, the *Brucella* positive serum samples are referred for further testing, using MET as the confirmatory test (Samartino *et al.*, 1999).

The MET depends on the ability of 2-mercaptoethanol to split the disulfide bonds in proteins. In the absence of urea, the chemical selectively inactivates IgM, leaving the IgG intact. MET originally developed for use in cattle, is seldom used for the testing of brucellosis in cattle now (George, 1994). It has been widely used as the preferred method for the testing of dogs for *B. canis* infection and for diagnosis of brucellosis in humans, although its reliability for the diagnosis of brucellosis in human patients is now regarded as questionable (George, 1994).

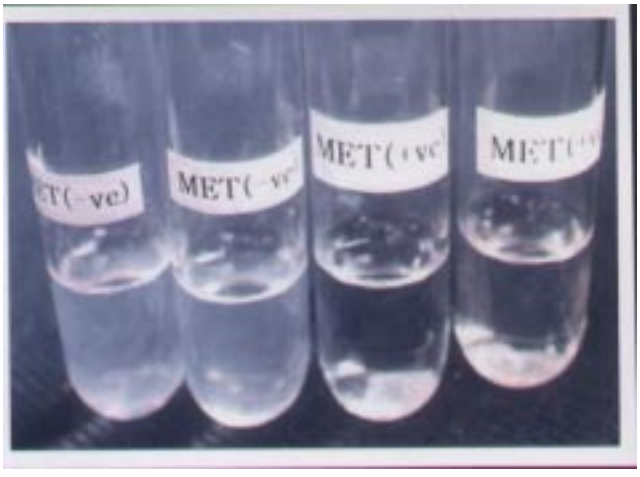

Fig. 2. Mercaptoethanol test as observed in the sera of Sprague-Dawley rats infected by *Brucella abortus* biotype-1 strain S1119-3, against whole cell antigen; MET (-ve) = negative reaction for brucellosis; MET (+ve) = positive reaction for brucellosis.

Brucella species are facultative intracellular pathogens, which survive within a variety of cells including macrophages. The virulence of these species and the establishment of long-time infections by *Bruclla* species are thought to be essentially due to their ability to avoid the killing mechanisms within macrophages (Sangari and Aguero, 1996; Baldwin and Winter, 1994). These macrophages, in the peritoneum of *Brucella*-infected rats, produce nitric oxide, which can contribute to the persistance of the infection for a long time (Urrutia *et al.*, 2000). The involvement of nitric oxide in the antibrucella activities of macrophages has also been suggested by pharmacological investigations (Jiang *et al.*, 1993). In the present study, bacteremia was noted throughout the 24-week post-infection period of studies. This phenomenon may be due to the relatively high resistance to macrophage killing of *B. abortus* biotype-1 in the infected S-D rats. Similar phenomenon was also observed in mice infected with *B. abortus* (Cheers and Pagram, 1979).

Bacteremia due to brucellosis may persist for varying periods of time depending on the host and the *Brucella* species. For example, in the goats infected with *B. melitensis*, bacteremia

was detectable for more than 300 days; in cattle infected with *B. abortus*, the onset of bacteremia may last for 5 months or more; in swine infected with *B. suis*, bacteremia may persist for more than 3 years while bacteremia with *B. canis* has been shown to be dose dependent and may remain detectable for 1,120 days (George, 1994). In the present study, *B. abortus* biotype-1, when inoculated to the S-D rats subcutaneously at the dose rate of 1.0×10^9 cfu, bacteremia was detected for the 24-week period of post-infection study.

The present study, furthermore, has evaluated for the first time RBT and MET using S1119-3 whole cell antigen using brucellosis infected sera in rats. The study has revealed, that after the first-week of post-infection, the reciprocal antibody titer was 1: 800 with RBT, whereas it was 1: 400 with MET. The reciprocal antibody titers were 1: 25 with RBT and 1:100 with MET, respectively, in infected rats after 24-week of post-infection studies. It may also be noted that MET required 48 h incubation at 37 ^{o}C to complete the reaction and procedure of skilled dilution of the antigen. Mercaptoethanol, furthermore, is toxic to laboratory personnel, RBT on the other hand required only 4 min with no incubation, no dilution of antigen and is also not toxic to laboratory personnel. Therefore, RBT using *B. abortus* biotype-1 strain S1119-3 whole cell antigen appears to be a potential candidate as the preferred diagnostic method of brucellosis detection in rats.

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