Polymerase chain reaction assay for the diagnosis of experimentally infected pregnant Sprague-Dawley rats with *Brucella abortus* biotype 1

M. S. Rahman

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

ABSTRACT: In order to diagnose the experimentally infected pregnant Sprague-Dawley (SD) rats with *Brucella abortus* biotype 1 using polymerase chain reaction (PCR) assay, the SD rats were injected subcutaneously at the dose of 1.0×10^9 colony forming units (cfu) at different stages of gestation period. The maximum rectal temperature was recorded as 38°C in the infected group within 3 days, whereas in the control group the temperature remained normal (36°C). There were no stillbirths, abortions or premature birth and relapsing fever in the infected SD rats. The pathological findings of infected SD rats were splenomegaly, metritis, swelling of lymph nodes, placentitis associated with lymphocytic and macrophage infiltration. Four hundred ninety-eight base pair DNA was detected in infected tissues through AMOS (*Brucella abortus, Brucella melitensis, Brucella ovis, Brucella suis*) PCR assay. The AMOS PCR assay was shown to be a valuable tool for diagnosis of infected pregnant Sprague-Dawley rats with *B. abortus* biotype 1.

Keywords: *Brucella abortus* biotype 1; Sprague-Dawley rats; polymerase chain reaction; South Korea

Brucellae are gram-negative bacteria that are pathogenic to a variety of livestock animals and humans. Many species of wild animals including rats are susceptible to brucellosis and may serve as natural reservoirs of brucellosis for domestic animals and human beings (Moore and Schnurrenberger, 1981; Young, 1995). The potential role of wild rodents as *Brucella* reservoirs was also reported by many authors (Nielsen and Duncan, 1990).

In the present study, the polymerase chain reaction (PCR) assay was used for the diagnosis of experimentally infected pregnant Sprague-Dawley (SD) rats with *B. abortus* biotype 1.

MATERIAL AND METHODS

Culture of *B. abortus* biotype 1. *B. abortus* biotype 1 isolated from the bovine supra mammary lymph node in South Korea was used in this study. For experimental infection, the strain was cultured in *Brucella* broth (Difco Co., USA) for 48 hours at 37°C with 5% CO_2. The bacteria were washed with saline 3 times and suspended in physiological saline before use.

Experimental rats and inoculation. Healthy 6–10 months old SD rats ($n = 48$) weighing 200–250 grams with no history of exposure to *Brucella* species were used in this experiment. Before starting the experiment, the primiparous and multiparous female rats were kept with male rats for mating (two females with one male) and to see the vaginal plug (on day 1 after gestation, the vaginal plug was observed). Rats were classified into infected group ($n = 30$) and control group ($n = 18$). The infected group was equally divided into 3 subgroups representing early gestation (7 days) period, mid gestation (14 days) period and late gestation (18 days) period consisting of

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5 primiparous and 5 multiparous rats in each group, respectively. The control group was also equally divided into 3 subgroups according to period of early gestation, mid gestation and late gestation but consisting of 3 primiparous and 3 multiparous rats in each group, respectively. 500 microlitres containing 1.0 × 10^9 colony forming unit (cfu) suspension of *B. abortus* biotype 1 in physiological saline solution were injected subcutaneously to each of 30 rats. Eighteen rats were given only 500 microlitres of physiological saline each, housed separately and not exposed to *B. abortus* biotype 1 organisms (control). The rats were maintained under hygienic conditions and provided with commercial feed and water *ad libitum*.

Clinical examination. All of the rats were examined carefully to record the clinical signs and rectal temperatures every day until the completion of the experiment. The reproductive status of infected rats was compared with those of control rats.

Gross and histopathological examination. The spleen, uterus, lymph nodes and placentas from infected (n = 30) and control (n = 18) SD rats were examined grossly and histopathologically within 12 hours after parturition. The cervical and mesenteric lymph nodes, spleen, uterus, and placenta from rats were fixed in 10% neutral-buffered formalin for at least 24 hours. Tissues were dehydrated in graded alcohols, cleared with xylene, and infiltrated and embedded in paraffin. Tissues embedded in paraffin were cut at 4 to 6 micrometer and mounted on glass slides. Sections were stained with haematoxylin and eosin (H & E) and examined for histopathological changes under light microscope (Meador et al., 1988).

Bacteriological examination. Liver and spleen specimens for bacteriological examination were stored not longer than for 48 hours at 4°C prior to culturing. For bacterial culture *Brucella*-selective medium was used (Rahman, 2003). The tissue was streaked onto the agar and the plates were incubated for 5 days at 37°C with 5% CO_2. *B. abortus* biotype 1 was identified on the basis of colony morphology and growth characteristics (Alton et al., 1988) and later confirmed by AMOS (*Brucella abortus, B.melitensis, B. ovis, B. suis*) PCR assay (Bricker and Halling, 1995).

Extraction of genomic DNA. Tissue extracts of spleen and liver were made separately through 2 microlitres of DNase, RNase free distilled water (Life Technology, UK) using a Masticator (IUR Instrument, Spain) and centrifuged for pellets. Genomic DNA from the pellets was extracted using InstaGene matrix (Bio-Rad Co., USA). Briefly, 1.5 microlitre of extract was centrifuged at 13 000 rpm for 3 minutes in Eppendorf tube. After the removal of supernatant 200 microlitres of InstaGene matrix were added to the pellet and after vortexing it was incubated at 56°C for 20 minutes. The supernatant was vortexed again at a high speed for 10 seconds and on a heat block at 100°C for 8 minutes. The sample was finally spun at 13 000 rpm for 3 minutes and 50 microlitres of the resulting supernatant were stored at –20°C until used.

AMOS PCR assay. Two microlitres of genomic DNA suspension and 2 microlitres of each primer were added to AccuPower^{TM} PCR Premix (Bioneer Co., Korea) containing 1 IU of thermostable DNA polymerase, 250 μM of each dNTP, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer, loading dye and distilled water for the total volume of 20 microlitres. The reaction mixture was dissolved by vortexing and centrifuged briefly. The sequence of oligonucleotide primers for AMOS PCR is shown in Table 1. Amplification reaction was performed in PTC-100TM Programmable Thermal Controller (MJ Research Inc., USA) under conditions described by Bricker and Halling (1995). Briefly, the samples were cycled (1.15 min at 95°C, 2.0 min at 55.5°C, 2.0 min at 72°C) 35 times and after the last cycle, the reaction mixtures were incubated for additional 5 min at 72°C before they were stored at 4°C. The products (5 microlitres from each reaction mixture) were analysed by electrophoresis through a 1.5% agarose gel (Sigma Co., USA) for 1 hour at 100 volts (V) with 0.5×TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8). Gels were stained with ethidium bromide (Sigma Co., USA), and bands

Table 1. Sequence of oligonucleotide primers for AMOS PCR assay of *B. abortus* biotype 1 infected Sprague-Dawley rats

Primer	Sequence
B. abortus specific primer	5'-GACGAACGGAATTTTTCCAATCCC-3'
IS*711* specific primer	5'-TGCCGATCACTTAAGGGCCTTCAT-3'

were detected with a UV transilluminator, and photographed with a Polaroid camera equipped with Polaroid 667 Film-Pack (Polaroid Ltd., UK).

RESULTS

Clinical findings. All of the SD rats inoculated with *B. abortus* biotype 1 developed lethargic, anorectic and febrile conditions, but control rats remained normal. The highest rectal temperature of the infected group reached 38°C within 3 days, whereas in the control group 36°C remained and there was no presence of relapsing fever. In addition, there were no differences between the rectal temperatures of primiparous and multiparous groups, and between different gestation stages of inoculation.

Reproductive findings. There were no stillbirths, abortions or premature birth either in the infected group or in the control group. All of the rats in infected and control groups delivered normally and did not show any abnormality of the foetuses.

Pathological findings. The pathological findings of all of the SD rats of infected group included splenomegaly, metritis, swelling of lymph nodes and placentitis, whereas there was no remarkable sign in the control group. The grossly cut surface of enlarged spleen of infected SD rat had diffuse, random, slightly elevated foci 1–2 mm in diameter. Lymph nodes from all SD rats infected with *B. abortus* biotype l were markedly enlarged.

AMOS PCR assay. Only one band of 498 base pair DNA was detected in PCR products of spleen and liver of infected SD rats as shown in Figure 1. There was no DNA of *B. abortus* biotype 1 from PCR products of control SD rats.

Cultural findings. *B. abortus* biotype 1 was isolated only from the culture of spleen of infected rats. No colony of *B. abortus* biotype 1 was observed in the culture of liver. Neither was there any *B. abortus* biotype 1 in the cultures of liver and spleen of control SD rats.

Histopathological findings. The endometrium of the uterus of rats infected with *B. abortus* biotype 1 was characterized by moderate, diffuse, but multifocally prominent accumulation of lymphocytes and macrophages in the superficial lamina. Neutrophils were also observed in some areas. In the spleen, there was diffuse congestion of the red pulp and diffuse infiltration of macrophages with increased giant cell numbers and there was a prominent germinal

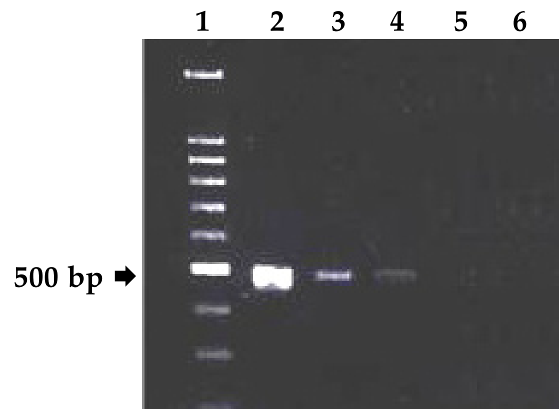

Figure 1. AMOS PCR assay in tissue extracts of *B. abortus* biotype 1 in infected and control Sprague-Dawley rats; 1 = 100 base pair DNA marker; 2 = DNA extracted from standard bacterial colony; 3 = DNA extracted from spleen of infected rat; 4, DNA extracted from liver of infected rat; 5 = DNA extracted from spleen of non-infected rat; 6 = DNA extracted from liver of non-infected rat

centre. In the placenta, there were areas of necrosis in the periplacentomal chorionic epithelium and adjacent interstitium accompanied by infiltration of underlying connective tissue with moderate numbers of macrophages. Severely affected placentas had vasculitis characterized by intimal and medial infiltrates of neutrophils and macrophages, intimal oedema, and fibrin deposition. In lymph nodes, there were infiltrations of macrophages and giant cells. There were vacuolated and engorged macrophages scattered in necrotic debris.

DISCUSSION

Brucellosis is an important zoonosis (Matyas and Fujikura, 1984; WHO, 1986). It is a systemic infection in which the bacteria initially localize in the regional lymph node, then disseminate haematogenously to the organ of the reticuloendothelial system to multiply within phagocytic cells (Memish et al., 2000). The release of bacterial endotoxin from phagocytic cells produces the constitutional symptoms and signs of disease.

Brucellosis has been an emerging disease since the discovery of *B. melitensis* as the cause of Malta Fever by Bruce in 1887 and the isolation of *B. abortus* from aborted cattle by Bang in 1897 (Nielsen and Duncan, 1990) and it is an important disease with a predilection for placenta and foetal membrane. The most common clinical features of brucellosis are placentitis and abortion (Silva et al., 2000). The

genus *Brucella* has six recognized species on the basis of host specificity. Among all six species of *Brucella*, the greatest economic impact results from bovine brucellosis caused by *B. abortus* (Bricker and Halling, 1994) and *B. abortus* biotype 1 was isolated from cattle in different provinces of South Korea following extensive studies (Chung et al., 1988; Rahman, 2003).

Guinea pigs and mice have been the principal animals used in laboratory experiments for brucellosis. All brucellae, with exceptions such as *B. ovis*, were pathogenic to guinea pigs (Nielsen and Duncan, 1990). The findings from this study confirmed the generalized infection of SD rats by *B. abortus* biotype 1 with the uterus, placenta, spleen and lymph node being the main target organs. The pathological findings as well as histopathological lesions in the SD rats were similar to those that develop in naturally infected cows (Jubb et al., 1985). The cellular nature of the present study was consistent with bovine brucellosis; inflammatory cells were primarily macrophages, lymphocytes, and lesser number of neutrophils. *B. abortus* biotype 1 organism was detected in tissue extracts of liver and spleen of *B. abortus* biotype 1 infected SD rats and there were pathological lesions in internal organs. The prominent histopathological changes seemed to induce macrophages in spleen as reported by other authors (Riglar and Cheers, 1980; Lauderdale et al., 1990) and the increased number of macrophages in spleen contributed to splenomegaly as reported by Palmer et al. (1996).

Thorpe et al. (1967) conducted experimental studies on the susceptibility of wild life and laboratory animals to experimental infection with *B. abortus*, *B. suis, B. melitensis,* and *B. neotomae*. Eight species of mice were generally found to be relatively susceptible to all strains of *Brucella,* while rats, lagomorphs and squirrels were more resistant. In a comparative study of susceptibility Taran and Rybasov (1971) inoculated guinea pigs, mice, rats and sheep with *Brucella*. Guinea pigs that were inoculated with 1.0×10^1 to 1.0×10^2 cells of *B. melitensis* and *B. suis* developed granulomatous lesions. In mice, it was necessary to administer 1.0×10^4 to 1.0×10^6 cells to produce lesions. Rats were infected with 1.0×10^9 cells, while sheep are resistant to the three strains used. In this study, SD rats were used with an inoculum of 1.0×10^9 cfu *B. abortus* biotype 1 for the infectivity in pregnant rats. The clinical findings agree with those obtained in similar trials in pregnant ewes (Ris, 1970) demonstrating that, despite of the induction of severe metritis, *B. abortus* biotype 1 has relatively no ability to induce abortion in infected SD rats.

Abortion is a common sequel of *Brucella* infection in cows, swine and many other animals (OIE, 2000). Nevertheless, Bosseray (1980) challenged pregnant mice on days 3, 7, 11, or 15 of pregnancy with *B. abortus* strain 544 using several routes and observed neither abortions nor foetal deaths. In the present study, the SD rats at different stages of pregnancy were injected experimentally with *B. abortus* biotype 1 isolated in South Korea, and there were no abortions or foetal deaths. Brucellosis did not affect pregnancy in mice although placental colonization occurred as early as 5 minutes post inoculation (Bosseray, 1983). In both cow and mice hosts *Brucella* colonization of the gravid reproductive tract can lead to severe placental damage, foetal infection and foetal death (Bosseray, 1982; Tobias et al., 1992). The pathogenesis of placental colonization found in mice is similar to that observed in rats (Bosseray, 1980). In this study, we observed necrosis in the periplacentomal chorionic epithelium of placenta with metritis in the rats infected on days 7, 14 and 18 of pregnancy but *B. abortus* biotype 1 did not affect the pregnancy of SD rats.

PCR assay was a useful approach to diagnosis in the various diseases (Ibrahim et al., 1992; Guarino et al., 2000; Leal-Klevezas et al., 2000; Adone et al., 2001). It is both quick and inexpensive tool for detection. Additionally, PCR assay can use specimens in which the pathogenic organisms have been rendered biologically safe. At least three PCR assays for *Brucella* species were described (Fekete et al., 1990a,b; Herman and Ridder, 1992). AMOS PCR assay can identify *B. abortus*, *B. suis*, *B. melitensis*, *B. ovis* but here the AMOS PCR assay identified the *B. abortus* biotype 1 in a tissue extract based on the observations that the repetitive genetic element 1S*711* (Halling et al., 1993).

In this study, the infection was diagnosed using AMOS PCR assay and additionally the conventional methods (clinico-pathological and cultural ones) were also applied. The AMOS PCR assay described in the present study has several advantages over the clinico-pathological as well as cultural method to identify *B. abortus* biotype 1 infection. A major advantage was the speed with which the assay can be performed, i.e. within a day. The clinico-pathological and cultural methods required at least several days. Another major advantage of AMOS PCR assay was that no live *Brucella* organisms were necessary

for this assay. This was significant because *B. abortus* was a human pathogen. This assay was unaffected by contamination by other microbes that might be present in the tissue sample used for isolation. After collection, the bacteria can be killed and sent for identification. AMOS PCR assay identified 498 base pair DNA in PCR products and showed to be a valuable tool for the diagnosis of infected pregnant Sprague-Dawley rats with *B. abortus* biotype 1.

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Corresponding Author

Md. Siddiqur Rahman, DVM, PhD, Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
Tel. +88 091 548 84, fax +88 091 520 60, e-mail: siddiquerahman@hotmail.com