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Transplacentally Transmitted Congenital Brucellosis due to *Brucella abortus* Biotype 1 in Sprague-Dawley Rats

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Abstract. In the investigation on the transplacentally transmitted congenital brucellosis due to *Brucella abortus* biotype 1 in Sprague- Dawley rats, neither any stillbirth, abortion or premature birth nor any abnormality of fetus was observed in the infected group or in the control group. *B. abortus* biotype 1 was isolated from the fetus of infected rats only. Only one band of 498 base pair DNA was obtained in polymerase chain reaction products from DNA of the fetuses of infected SD rats.

Keywords: *Brucella abortus* biotype 1, transplacental transmission, congenital brucellosis

Introduction

Brucellosis is one of the world's major zoonoses, alongside bovine tuberculosis and rabies (Boschiroli *et al*., 2001). Major reservoirs of diseases include goats and sheep (*Brucella melitensis*), swine (*Brucella swis*), cattle (*Brucella abortus*) and dog (*Brucella canis*). *Brucella abortus* has been isolated from various tissues of rats in various parts of the world and were associated with proximity to *Brucella* infected-cattle (Moore and Schnurrenberger, 1981). Transmission to humans occurs through direct contact with infected animals or consumption of infected animal products. Human to human transmission is rare and has been described only after blood transfusion (Wood, 1955) and bone marrow transplantation (Ertem *et al*., 2000; Naparstek *et al*., 1983) and possibly during sexual intercourse (Ruben *et al*., 1991). Furthermore, some evidences are available that brucellosis may be transmitted to the neonatal infant through breast milk (Palanduz *et al*., 2000). In cattle, brucellosis acquired through suckling infected dam has been suggested as one of the possible modes of transmission (Cheville *et al*., 1995). In this study, the transplacentally transmitted congenital brucellosis due to *Brucella abortus* biotype 1 in Sprague-Dawley (SD) rats has been reported through bacteriological examination and polymerase chain reaction (PCR).

Materials and Methods

Culture of *B. abortus* biotype 1 isolated from bovine supra mammary lymph node in South Korea (Rahman and Baek, 2007) was used in this study for the experimental infection. *B. abortus* biotype 1 was grown in Brucella broth (Difco, USA) for 48 h at 37 °C with 5% CO_2. The bacteria were washed with saline thrice and suspended in physiological saline before use.

Experimental rats and inoculation protocol. Healthy (disease free) 6 to 10 month old pregnant female SD rats (n=25) weighing 200 to 250 gwith no history of exposure to *Brucella* species were used in this experiment. Before starting the experiment, the 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=15) and control group (n=10). A 500 µl containing 1.0 x 10^9colony forming units suspension of *B. abortus* biotype 1 in physiological saline solution was injected subcutaneously at shoulder region to each of the 15 rats of infected group at the 7th day of gestation. Ten rats injected with only 500 µl of physiological saline served as control. These were following the same procedure, housed separately and not exposed to *B. abortus* biotype 1 organisms. The rats were maintained under hygienic conditions and were provided with commercial feed and water *ad libitum*.

Clinical examination and collection of fetuses. All of the rats were examined to record the clinical signs and rectal temperature every day until completion of the experiment. All of the rats were sacrificed after 20 days of gestation (i.e. the day before parturition). The fetuses were collected directly from uterus without any contamination and were examined.

Bacteriological examination of fetuses. Fetuses for bacteriological examination were stored not longer than 48 h at 4 °C prior to culturing. For bacterial culture Brucella-selec-

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tive medium was used (Rahman, 2003). The fetal 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.*, 1975) and later confirmed by PCR as per method of Bricker and Halling (1995).

Extraction of genomic DNA. The extracts of whole fetuses were made separately through 2 µl of DNase, RNase free distilled water (Life Technology, UK) using a Masticator (IUR Instruments, Spain). 1.5 µl of the extract was centrifuged at 13000 rpm for 3 min in eppendorf tube, the supernatant was removed and the genomic DNA was prepared from the pellets using Insta Gene matrix (Bio-Rad, USA). Briefly, 200 µl of InstaGene matrix were added to the pellet and after vortexing it was incubated at 56 °C for 20 min. The supernatant was vortexed again at 13000 rpm for 10 seconds and on a heat block at 100 °C for 8 min. The sample was finally spun at 13000 rpm for 3 min and 50 µl of the resulting supernatant (DNA) was stored at -20 °C until use.

Polymerase chain reaction. Two microlitres of genomic DNA suspension and 2 µl of each primer (Table 1) were added to AccuPowerTM PCR Premix (Bioneer, 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. 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 and 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 µl from each reaction mixture) were analysed by electrophoresis through a 1.5% agarose gel (Sigma, USA) for 1 h at 100 volts (V) with 0.5 x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8). Gels were stained with ethidium bromide (Sigma, USA), and bands were observed with a UV transilluminator, and photographed,with a Polaroid camera equipped with, Polaroid 667 Film-Pack (Polaroid Ltd., UK).

Table 1. Sequence of primers for polymerase chain reaction of *B. abortus* biotype 1 in infected and control Sprague-Dawley rats

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

Results and Discussion

All of the SD rats inoculated with *B. abortus* biotype 1developed 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 36 °C in the control group (Fig. 1) and without relapsing fever symptoms.

There were no stillbirths, abortions or premature birth either in the infected group or in the control group (Table 2) and no abnormality of the fetuses was noticed in any of the fetuses. *B. abortus* biotype 1 was isolated from fetuses of infected rats only while the organism was not isolated from fetuses of the control SD rats.

The results of PCR are shown in Fig. 2. Only one band (498 base pairs) DNA was observed in PCR products from DNA of all fetuses of the infected SD rats. However, the similar band (498 base pairs) DNA was not observed in the fetuses of control SD rats.

The diagnosis of brucellosis is confirmed by isolation and identification of *Brucella* by pure culture technique. The diagnosis of brucellosis based exclusively on *Brucella* isolation however, presents several drawbacks. The slow growth of *Brucella* may delay diagnosis for more than 7 days (Yagupsky, 1999; Ariza, 1996; Rodriguez Torres and Fermoso,

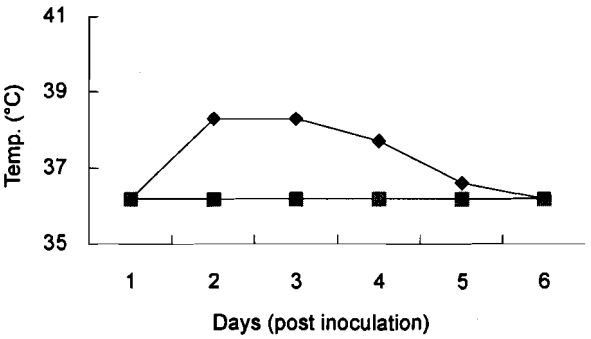

Fig. 1. Change of rectal temperature in *B. abortus* biotype 1 infected (-◆-) and control (-■-) Sprague-Dawley rats.

Table 2. Reproductive findings of *B. abortus* biotype 1 in infected and control Sprague-Dawley rats

Groups	No. of rats	No. of fetuses (Mean ± SD)	Still birth	Abortion
Infected	15	11.31±1.15	None	None
Control	10	11.32± 1.16	None	None

SD = standard deviation

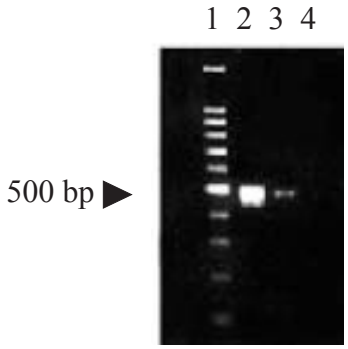

Fig. 2. Amplification of DNA in polymerase chain reaction. 1, 100 base pair DNA marker; 2, DNA extracted from standard bacterial colony; 3, DNA extracted from fetus of infected SD rat; 4, DNA extracted from fetus of non-infected SD rat.

1987). Also, the sensitivity of the method is often low, ranging from 50 to 90% depending on disease stage, *Brucella* species, growth medium, concentration of bacteria and culture technique employed (Yagupsky, 1999; Gotuzzo *et al*., 1986).

PCR assay has been a useful approach for the diagnosis in various diseases (Adone *et al*., 2001; Leal-Klevezas *et al*., 2000; Guarino *et al*., 2000). It is both quick and inexpensive tool for detection of the organism. Additionally, for PCR assay specimens in which the pathogenic organisms have been rendered biologically safe can be used and it can identify *B. abortus*, *B. suis*, *B. melitensis* and *B. ovis.* However, in the present study PCR assay on has helped identification of the *B. abortus* biotype 1 in infected fetuses based on the presence of repetitive genetic element IS*711*. This was supplemented by conventional bacteriological method. The PCR assay described in the present study offers several advantages over the bacteriological method to identify *B. abortus* biotype 1. A major advantage is the short time (one day) required for the assay while the bacteriological methods require several days. Moreover, no live *Brucella* organisms are required for this assay. This is significant because *B. abortus* is a human pathogen. The PCR assay remains unaffected even by contamination by other microbes that might be present in the fetuses (non issue). After collection of the sample, the bacteria can be killed/inactivated and sent for identification.

The course of pregnancy in affected woman and the mode of transmission to the fetus are not well documented. The question whether infection with *Brucella* sp. in women during pregnancy leads to abortion still remains controversial (Mohamed *et al*., 1985; Sarram *et al*., 1974) but abortion is a common sequel of *Brucella* infection in cows, swine and many other animals (OIE, 2000). Nevertheless, Bosseray (1980) challenged the pregnant mice on days 3, 7, 11, and 15 of pregnancy with *B. abortus* strain 544 using several routes and observed neither abortions nor foetal deaths. There is also evidence of using *Brucella* to induce abortion in mice (Tachibana *et al.*, 2008). In our study no stillbirth, abortion or premature birth were observed in the infected SD rats. With regard to mode of transmission during pregnancy in humans, it seems that in most of cases *Brucella* sp. cross the placenta and infect the fetus (Carbajo-Ferreira *et al*., 1995). The transmission of *B. abortus* from infected dam to the offspring has been well documented in cattle while the calves remain seronegative for months or even years (Grillo *et al*., 1997). In this study, the fetuses were collected on the day before parturition, therefore, the infection of fetuses through infected feed or infected placenta or vaginal discharge can be excluded and the transplacentally transmitted *Brucella* to the fetuses, seems to be the only route/mode of acquiring of the infection.

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