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Effects of *Brucella abortus* Biotype 1 Infection on the Reproductive Performance of Sprague-Dawley Rats

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Abstract: The aim of this study was to assess the impact of *Brucella abortus* biotype 1 infection on the reproductive performance using Sprague-Dawley (SD) rat model. Virgin female SD rats (n = 24) were infected intraperitoneally with 0.1 mL of saline containing 1×10^{11} Colony Forming Unit (CFU) of pathogenic *B. abortus* biotype 1 Korean bovine isolate. Control rats (n = 24) were inoculated with 0.1 mL of apyrogenic saline. Both inoculated and control rats were divided into six subgroups. Four rats in each subgroup were consistently bred at 3, 7, 14, 21, 28 and 60 days after infection. *B. abortus* infection induced 41.67% infertility in the infected rats. The mean number of offspring/litter was 8.71±2.01 for infected rats and 12.87±1.42 for control rats (p<0.001). The mean weight of the viable offspring was 6.10±0.36 g for infected rats and 7.15±0.40 g for control rats (p<0.001). The rate of stillbirth was 12.30% in the infected rats. *B. abortus* biotype 1 was isolated from the uteri of the infected rats. The data of this study indicate that *B. abortus* biotype 1 infections in SD rat model affect reproduction adversely by causing infertility, stillbirth and loss of number and weight of offspring.

Key words: *Brucella abortus*, infertility, stillbirth, abortion, endometritis, Th1 immune response

INTRODUCTION

Brucellosis is an important zoonosis, caused by several species of the genus *Brucella* that affect numerous domestic and wild mammals and humans (Whatmore *et al.*, 2006; Macedo *et al.*, 2008). *Brucella abortus* is a potentially pathogenic bacterium for the reproductive tract of cattle (Givens, 2006) that can be introduced into the uterus during natural breeding with the infected male (Crawford *et al.*, 1990) and artificial insemination with contaminated semen (Lim *et al.*, 2005). Localization of brucellae within reproductive tracts accounts for the principle clinical symptoms of abortion and/or reduced fertility in most natural host species (Robison *et al.*, 1998). The major clinical manifestation of brucellosis in cattle and wildlife is abortion, decreased fertility and retention of placenta (Enright, 1990; Yaeger and Holler, 1997; Rhyan *et al.*, 1994). Uterine discharge of aborted fetus is believed to be principle source of infection to other animals. Brucellosis in humans resulted from direct contact with the infected animals as well as consumption of contaminated milk and milk products (Pappas *et al.*, 2006). Undulant fever, arthritis and endocarditis are the clinical manifestations in human brucellosis (Young, 1995). Brucellosis accounts huge economic losses in livestock industries due to loss of offspring, increased calving interval, decreased fertility and culling of the infected animals (Radostits *et al.*, 2007).

Brucellosis has emerged as a serious animal and public health issue in many developing countries (Pappas *et al.*, 2005) including Korea (Park *et al.*, 2005) despite animal control and eradication program. *Brucella* infection in both cattle and people in Korea is commonly due to *B. abortus* biotype 1 (Park *et al.*, 2005; Wee *et al.*, 2008).

Rat model was used for the evaluation of the vertical transmission of *B. abortus* biotype 1 (Baek *et al.*, 2005). In the present study we investigated the effects of *B. abortus* biotype 1 infection on the reproductive performance in SD rat model with regards to the inoculation period. We infected sexually mature virgin female SD rats with *B. abortus* biotype 1 bovine pathogenic Korean isolate and noted infertility, stillbirth, losses of weight and number of offspring among infected rats after breeding.

MATERIALS AND METHODS

Ethics: The experimental protocols were approved by the Animal Care and Use Committee at the Chonbuk National

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University and were in accordance with the Chonbuk National University guide for the Care and Use of Laboratory Animals.

Experimental rats: Ten to twelve-week-old SD male (n = 12) and female virgin rats (n = 48) weighing 250-300 g were used in this study. All of these rats were obtained from a Specific Pathogen Free (SPF) laboratory animal company (Koatech, Pyungtaek city, Gyeonggido 451-864, Korea). The rats were housed in a stringently hygienic, climate-controlled environment and provided with commercial food (Koatech, Pyungtaek city, Gyeonggido 451-864, Korea) and water *ad libitum*. The animals were culture negative for *Brucella* infection and seronegative for *B. abortus* antibodies, prior to the experimental infection, ascertained by routine bacteriological and serological tests.

Bacterial strain and media: Pathogenic *B. abortus* biotype 1 originating from cattle in Korea was used for experimental infection in rats. The bacteria were maintained as frozen glycerol stocks and cultured on brucella agar media (Difco, Kansas City, Missouri, USA) for 5-7 days at 37°C with 5% CO_2. The grown bacteria were harvested in normal saline.

Experimental inoculations: The female rats were divided into an infected group (n = 24) and a control group (n = 24). All rats in infected group were injected intraperitoneally with 0.1 mL of normal saline containing 1×10^{11} CFU mL^{-1} of the *B. abortus* biotype 1. All control rats were injected intraperitoneally with 0.1 mL apyrogenic saline.

Clinical examinations: Clinical parameters, such as body temperature, appetite and thirst of the infected and control rats were recorded in the morning for a period of 7 days.

Breeding plan: To assess the effect of infection on the reproductive performance in relation to the inoculation period, the infected rats (n = 24) were divided into six subgroups for breeding. Four rats in each subgroup were separately bred at 3, 7, 14, 21, 28 and 60 days after infection with one uninfected healthy male rat (1 male for 4 females). Control rats (n = 24) were also divided into six subgroups and bred simultaneously with infected subgroups following the similar breeding protocol of infected rats. One male rat was kept with four female rats in a single cage for a period of one month. Pregnant rats were identified by abdominal distension and placed in individual cages 3 or 4 days before parturition. The pregnant rats were continuously monitored and after parturition number of live or stillborn offspring were counted and weight of the live offspring were measured.

Reproductive performance: Reproductive performance in infected and control rats were assessed by counting or measuring the number of live or stillborn offspring/dam and weight of day-old live offspring.

Serological studies: After parturition, 3 mL of blood samples were collected from each of the infected rats by aseptic cardiac puncture under general anesthesia induced by intraperitoneal administration of 7-25 mg kg^{-1} of Tiletamine and Zolazepam (Zoletil 50®, Virbac Laboratories-06515 Carros-France). Blood samples were also collected from the non-pregnant infected female rats as well as male rats (n = 6) used for breeding with the infected female rats. Serum samples were collected and stored in small aliquots at -20°C until tested. Serum samples were screened for anti-*Brucella* antibodies by the Rose Bengal Plate Agglutination Test (RBPAT) as described previously (Alton *et al.*, 1988). Sera of the parturient infected rats bred at 3, 7, 14, 21, 28 and 60 days after infection were examined in the enzyme-linked immunosorbent assay (ELISA) against *Brucella* smooth-lipopolysaccharide (S-LPS) by using the anti-IgG1 and anti-IgG2a conjugates. The ELISA was performed as described previously (Lamb *et al.*, 1979) with little modification. Flat-bottomed 96-well polysterine microtiterplates (Nunc, Denmark) were coated with 100 µL of LPS (5 µg mL^{-1}) of *B. abortus* strain 1119-3 suspended in 0.05 mM sodium bicarbonate buffer (pH 9.6). Affinity purified rat IgG1 (Bethyl Laboratories, Inc, USA) and IgG2a (Bethyl Laboratories, Inc, USA) were used to coat the 96-well plate starting from 500 to 7.8 ng $well^{-1}$ for generation of standard curve. Each plate was incubated at 4°C overnight. Plates were washed three times with wash solution (PBST: PBS (pH 7.4) with 0.05% (v/v) Tween 20). Each well of the antigen-coated plates were blocked with 200 µL of blocking solution of 1% (w/v) bovine serum albumin (Sigma Aldrich Inc., St. Louis, MO, USA) in PBST and incubated at 37°C for 30 min. After three washes with PBST, 100 µL of sera samples, diluted 1:100 in sample diluent (50 mM tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) were added to each well in duplicate. The plates were sealed and incubated at 37°C for 1 h. After five washing cycles with PBST, each well was incubated with 100 µL of 1:100000 dilution of goat anti-rat IgG1 and IgG2a antibodies conjugated to horseradish peroxidase (Bethyl Laboratories Inc, USA) diluted in conjugate diluent (50 mM tris, 0.14 M NaCl, 1% BSA, 0.05% Tween

20, pH 8.0) and the plates incubated at 37°C for 1 h. After five washings as described above, the color reaction was developed by adding 200 µL well^{-1} of a solution containing 1.0 mg mL^{-1} of O-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis, USA) in 0.05 M citrate buffer (pH 4.0) with 0.04% (v/v) H_2O_2. The plates were incubated in the dark for 30 min at room temperature. The colorimetric reaction was stopped by the addition of 50 µL well^{-1} of 3 M H_2SO_4. The absorbance measurements were made at 492 nm, using an automatic ELISA plate reader.

Bacteriological studies: After parturition all infected and live offspring born from them were euthanized. Non-pregnant infected as well as male rats (n = 6) used for breeding with infected females were also euthanized. Specimens of uterus and spleen were collected from all of the infected rats. Liver and spleen were collected from all of the live and stillborn offspring. Spleen and testes were collected from the six male rats. All samples were stored at -20°C until cultured. Thawed tissues were macerated in a stomacher (Costa Brava, Spain). All macerated samples were plated on brucella agar media (Difco, Kansas City, Missouri, USA) supplemented with antibiotics (cycloheximide, polymixin B and bacitracin that inhibit growth of bacteria other than *Brucella*) and incubated at 37°C with 5% CO_2 for 5-7 days. The identification of the isolates in the culture positive specimens was conducted by routine methods (Alton *et al*., 1988).

Polymerase chain reaction: Bacteria harvested from culture positive specimens of rats were also confirmed to be *B. abortus* biotype 1 by AMOS (abortus, melitensis, ovis, suis) Polymerase Chain Reaction (PCR) described previously (Bricker and Halling, 1994). For AMOS-PCR profiling, DNA was extracted from *Brucella* suspected colonies by a genomic DNA extraction kit (AccuPrep DNA Extraction Kit, Bioneer, Daejeon, Korea) using manufacture's protocol.

Histopathological analysis: Uteri collected from infected and control pregnant rats were fixed in 10% neutral buffered formalin. The fixed tissues were processed, sectioned and stained with Hematoxylin-Eosine (HE) and examined for histopathological changes under a light microscope (Meador *et al*., 1988).

Statistical analysis: All statistical analysis was conducted by the Student's t-test. Significance of all the analyses was established at p-value of <0.05.

RESULTS

Clinical findings: All rats infected with *B. abortus* biotype 1 became lethargic, anorexic and febrile within 24 h. The highest rectal temperature was 38.5°C at 72 h within 7 days observation period. No abnormal clinical signs were observed in control rats through out the experiment.

Reproductive performance: In six infected subgroups 25 to 75% rats became pregnant and the mean litter size and body weight of day-old offspring were ranged from 6.33±0.57 to 11.5±3.53 and 5.7±0.28 to 6.6±0.31 g, respectively. Stillbirth was recorded in all of the infected subgroups. Among six infected subgroups the lowest rate of pregnancy (25%) and highest rate of stillbirth (40%) were recorded in rats bred at 14 days after infection. In all control subgroups 100% rats became pregnant and the mean litter size and body weight of day-old offspring were ranged from 12±1.63 to 13.5±1.29 and 6.92±0.51 to 7.30±0.58 g, respectively. The reproductive performance of infected and control rats in six subgroups are shown in Table 1.

Table 1: Reproductive performance of the *B. abortus* biotype 1 infected and control rats bred at 3, 7, 14, 21, 28 and 60 days after infection

Breeding at days after infection	Group	Rat No.	No. of pregnant rat[a] (%)	Offspring data				
				No. of live offspring	No. of stillborn offspring (%)	Total No.	Mean litter size[a]	Mean weight[b] (g)
3	Control	4	4 (100)	52	0 (0)	52	13.00±0.81	7.22±0.22
	Infected	4	2 (50)	18	5 (21.73)	23	11.50±3.53	6.22±0.39
7	Control	4	4 (100)	54	0 (0)	54	13.50±1.00	7.15±0.35
	Infected	4	3 (75)	25	1 (3.84)	26	8.66±1.15	5.99±0.23
14	Control	4	4 (100)	48	0 (0)	48	12.00±1.63	7.30±0.58
	Infected	4	1 (25)	6	4 (40)	10	10.00	6.60±0.31
21	Control	4	4 (100)	54	0 (0)	54	13.50±1.29	6.92±0.51
	Infected	4	3 (75)	25	2 (7.40)	27	9.00±1.00	6.33±0.29
28	Control	4	4 (100)	50	0 (0)	50	12.50±0.57	7.15±0.23
	Infected	4	3 (75)	18	1 (5.26)	19	6.33±0.57	6.02±0.47
60	Control	4	4 (100)	51	0 (0)	51	12.75±2.62	7.17±0.55
	Infected	4	2 (50)	15	2 (11.76)	17	8.50±0.70	5.70±0.28

[a]Rat that delivered offspring, [b]Day-old live offspring only

Table 2: Summary of the reproductive performance of *B. abortus* biotype 1 infected and control rats

Parameters	Control rats (n = 24)	Infected rats (n = 24)	p-value
No. of parturient or pregnant female (%)	24 (100)	14 (58.33)	ND[b]
No. of non-pregnant or infertile female (%)	0 (0)	10 (41.67)	ND
Mean no. of offspring/litter[a] (range)	12.87±1.42	8.71±2.01	<0.001
	(10-15)	(6-14)	
Mean weight (g) of day-old live offspring	7.15±0.40	6.10±0.36	<0.001
No. of live offspring (%)	309 (100)	107 (87.70)	ND
No. of stillborn offspring (%)	0 (0)	15 (12.30)	ND

[a]Live and stillborn offspring only, [b]Not determined

Table 3: Results of isolation and identification of *B. abortus* biotype 1 from infected female rats and offspring born to them by the routine methods and AMOS-PCR assay

Group (n)	No. of culture positive rats (%)	No. of culture negative rats (%)	No. of isolates confirmed as *B. abortus* biotype 1 by AMOS-PCR
Pregnant or parturient female rats (14)	14 (100)	0 (0)	14
Non-pregnant female rats (10)	10 (100)	0 (0)	10
Live offspring (107)	85 (79.44)	22 (20.56)	85
Stillborn offspring (15)	15 (100)	0 (0)	15

A total of 14 rats out of 24 infected rats became pregnant and delivered 122 offspring. Out of 122 offspring, 107 were live and 15 were stillborn. All control rats were pregnant and delivered 309 live offspring. The overall reproductive performance of infected as well as control rats is summarized in Table 2.

Bacteriological findings: Characteristic colonies of *B. abortus* biotype 1 were cultured from uteri and spleen of all infected female rats. Out of 107 live offspring, 85 offspring born to infected dams were culture positive for *B. abortus* biotype 1. On the other hand, *B. abortus* biotype 1 was isolated from all of the stillborn offspring born to infected dam. No bacteria were isolated from any of the six male rats bred with infected females. The results of isolation and identification of *B. abortus* biotype 1 cultured from specimens of infected female rats and offspring born to them is summarized in Table 3.

Polymerase chain reaction: All culture positive bacterial colonies harvested from specimens of infected female rats as well as offspring born to them were confirmed as *B. abortus* biotype 1 by AMOS-PCR with the predicted 498-base pair PCR amplicons (Fig. 1).

Serological findings: Serum samples of the infected pregnant and non-pregnant rats were tested positive in the RBPAT screening test. Serum samples of breeding male rats were tested negative by RBPAT screening test. We determined the magnitude and Th-specificity of antibodies in sera of the infected pregnant rats generated against S-LPS of *B. abortus* by the indirect ELISA. In the sera of pregnant rats IgG2a indicative of Th1 response was significantly higher as compare to IgG1 indicative of Th2 response (p<0.001). The results of ELISA are presented in Fig. 2.
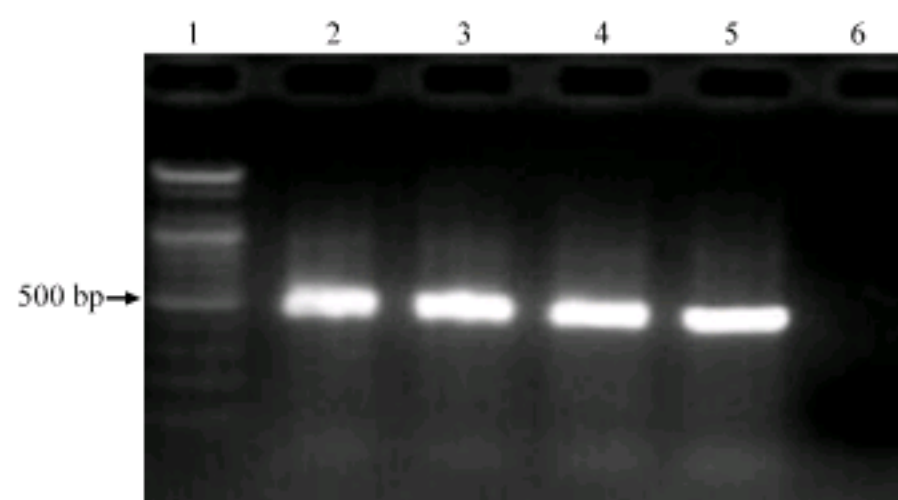

Fig. 1: AMOS-PCR profile for amplification of DNA of *B. abortus*. Lanes: (1), 100 bp size DNA marker (Bioneer, Daejeon, Korea), (2) bacterial DNA of an infected female rat, (3) bacterial DNA of a stillborn offspring, (4) bacterial DNA of a live offspring, (5) positive control with DNA of *B. abortus* strain 1119-3, (6) negative control without DNA
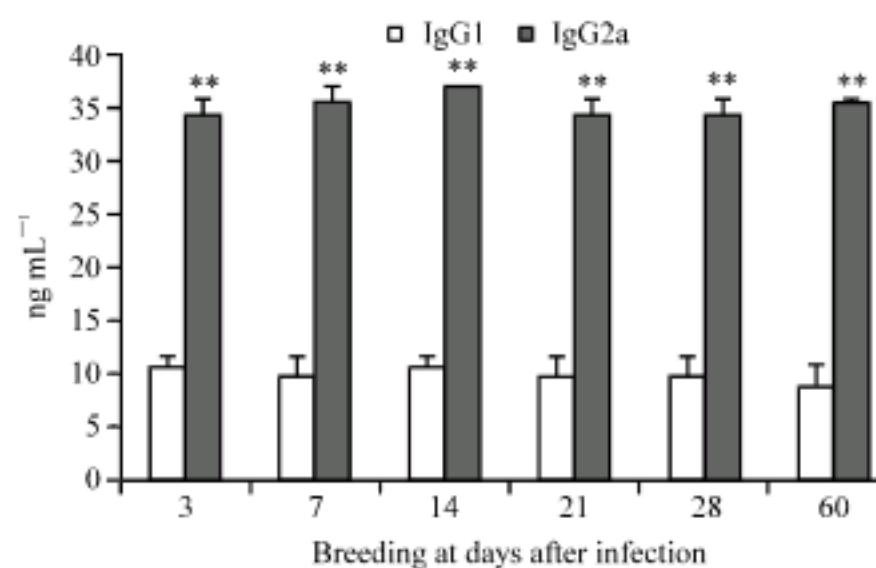

Fig. 2: Serum IgG1 and IgG2a responses measured by ELISA in *B. abortus* biotype 1 infected pregnant rats bred at 3, 7, 14, 21, 28 and 60 days after infection. The responses are presented as Mean±SD. Statistically significant difference between IgG1 and IgG2a are indicated by asterisks (**p<0.001)
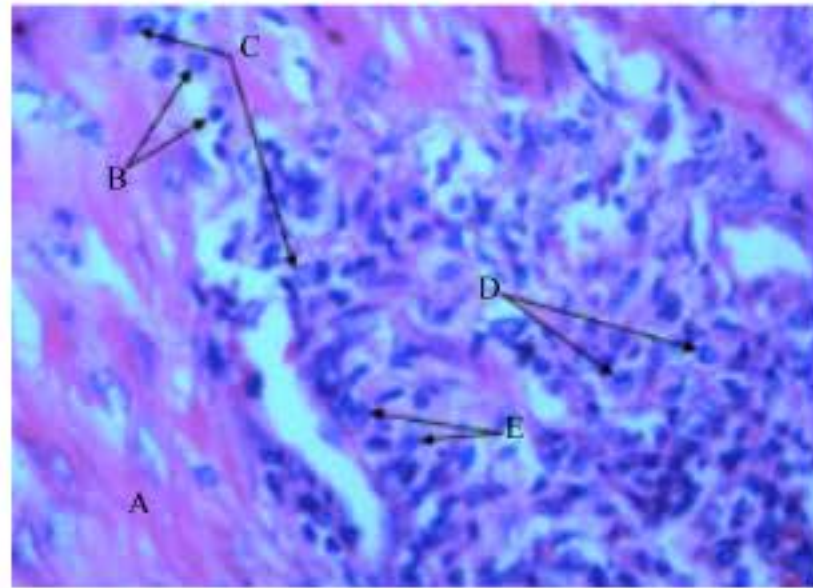
Fig. 3: Histopathological findings. The lesions in the endometrium of *B. abortus* infected uterus of a pregnant rat characterized by A: Muscular thickening, B: Accumulation of lymphocytes, C: Neutrophils, D: Monocytes and E: Macrophages, magnification 400x

Histopathological findings: Histopathological analysis of the uteri of the infected pregnant rats, compare to analysis of the control rat uteri, showed some lesions in the endometrium. Histopathological lesions of the endometrium of infected pregnant rats were characterized by thickened muscular wall with the moderate to diffuse infiltration of inflammatory cells such as lymphocytes, neutrophils, monocytes and macrophages (Fig. 3).

DISCUSSION

In the present study we investigated the effects of *B. abortus* biotype 1 infection on the reproductive performance in the SD rat model. We noted infertility, stillbirth and loss of number and weight of the offspring among infected rats in this study. These data are discussed in relation to the bacteriological, serological and pathological findings.

In this study, we compare the reproductive performance of the infected rats with the control rats to figure out the effect of *B. abortus* biotype 1 infection on the reproductivity. We included control rats in all subgroups and simultaneously bred with the infected rats in order to match age during the time of breeding. In the present study, the highest infertility rate was seen in the infected rats bred at 14 days after infection (Table 1). The overall 41.67% infertility was recorded in the infected rats (Table 2) which were resulted from reproductive tract infection since *B. abortus* biotype 1 was isolated from the nongravid uteri of all of the infected female rats (Table 3). All of the non-pregnant infected rats elicited antibody responses against *B. abortus* biotype 1 in the RBPAT screening test also support the establishment of infection.

Uterine infection is a key risk factor for abnormal ovarian hormone profile and subfertility affecting up to 44% of animals (Royal *et al.*, 2000). Bacterial infection also affects the function of uterus. Endometrial and ovarian cell functions were modified by challenge with bacteria, their product such as LPS or proinflammatory cytokines (Herath *et al.*, 2006). LPS is the component of the cell wall of *B. abortus* that act as a virulent factor (Lapaque, 2005).

Infection causes damage to the uterine tissue and histological lesions of endometrium in the animal are associated with the poor reproductive performance (Bonnett, 1993). In this study, *B. abortus* biotype 1 was isolated from all of the pregnant rats after parturition which caused lesions of the uteri (Fig. 3). In cattle, the most destructive lesions of brucellosis occur in the gravid uterus consisted of changes in the interstitial tissue and accumulations of lymphocytes, macrophages and neutrophils (Deyoe, 1980).

Infectious agents can be responsible for reduced ovulation rates, fertilization rates and embryonic survival rates (Givens, 2006). Uterine infection precludes fertilization or embryo survival (Semambo *et al.*, 1991). In the present study, the mean number of offspring per litter was significantly lower in infected rats (8.71±2.01) compare to that of control rats (12.87±1.42) (p<0.001) (Table 2). Among infected subgroups the mean litter size was lower in rats bred at 28 days after infection (Table 1).

In mice, *B. abortus* colonization of the gravid reproductive tract leads to placental damage, fetal infection and fetal dead (Bosseray, 1982; Tobias *et al.*, 1993). In the present study, 12.30% of the offspring born to infected rats were stillborn which were found to be infected with *B. abortus* biotype 1 (Table 3). Stillbirths were reported in cattle infected with *B. abortus* (Kirkbride, 1993). The highest rate of stillbirth was recorded in rats bred at 14 days after infection (Table 1).

The mean weight of the day-old live offspring (6.10±0.36 g) born to the infected rats was significantly lower as compared to the offspring born to the control rats (7.15±0.40 g) (p<0.001) (Table 2). The lowest mean body weight of offspring was recorded in rats bred at 60 days after infection (Table 1). In *Bartonella birtless* infection, the weight of viable fetuses was significantly lower which were resulted from fetal suffering during gestation (Boulouis *et al.*, 2001). In this study, loss of weight of live offspring born to *B. abortus* infected rats might have resulted from fetal suffering during gestation.

There was no abortion recorded in any of the infected rats in our study. In cattle when they are infected before pregnancy seldom abort during subsequent pregnancy (Deyoe, 1980).

There is a relationship between immune response and outcome of pregnancy. In *Brucella* infections, cellular immune response plays a predominant role compared with the humoral response (Golding *et al.*, 2001). Cellular immune response against *B. abortus* infection is primarily mediated by Th1 type of immune response which adversely affect pregnancy outcome (Krishnan *et al.*, 1996). In this study, among infected pregnant rats Th1 type of immune response was significantly dominant (p<0.001) as compared to Th2 response against *B. abortus* biotype 1 infection (Fig. 2). Cellular immune response against *B. abortus* infection might affect the reproductive performance of the infected pregnant rats in this study.

Present study demonstrated that infection of SD rats with the *B. abortus* biotype 1 Korean bovine isolate resulted low fertility, stillbirth and loss of number and weight of the offspring. Intervention measures against reproductive tract infection caused by *B. abortus* need to be addressed.

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