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Efficacy of *Brucella abortus* strain RB51 vaccine in Korean mongrel dogs against virulent strains of *B. abortus* biotype 1 and *B. canis*

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Abstract

This study was performed to test the hypothesis that *Brucella abortus* strain RB51 (SRB51) might protect Korean indigenous mongrel dog against challenge with either virulent *B. abortus* biotype 1 or *B. canis*. A total of 12 Korean mongrel dogs were divided into four groups (Group A, B, C and D). Dogs belonging to Group A and C were inoculated subcutaneously with 1×10^9 CFU of SRB51 in 1ml of sterile phosphate buffered saline (PBS). Dogs of Group B and D were inoculated subcutaneously with 1ml of sterile PBS as control. At 12 weeks post vaccination, dogs of Group A and B were challenged by oral inoculation of virulent strain of *B. canis* (5.0×10^9 CFU) and dogs of Group C and D were challenged by oral inoculation of virulent strain of *B. abortus* biotype 1 (4.4×10^{10} CFU). The serum antibodies titers in all dogs were monitored at regular interval for eight weeks after challenge (AC) by standard tube agglutination test, plate agglutination test, rose bengal test, 2-mercaptoethanol rapid slide agglutination test and 2-mercaptoethanol tube agglutination test. No antibody titers in Group A and C was detected. Also, the challenge strains were not found from blood of all dogs of Group A and C from 1 week AC till the end of the experiment by culture and modified AMOS-PCR, whereas *B. canis* and *B. abortus* challenge strains were detected from blood of Group B and D, respectively. In addition, neither of two challenge bacteria was recovered from liver, spleen, kidneys, lymph nodes and reproductive tracts of Group A and C dogs after postmortem. However, *B. canis* and *B. abortus* challenge strains were isolated from these tissues of Group B and D, respectively. These data suggest that SRB51 could be a promising vaccine candidate for immunizing dogs to control canine brucellosis caused by *B. canis* or *B. abortus.*

Key words : Canine, Vaccination, *Brucella abortus* strain RB51, *B. canis*, *B. abortus* biotype 1

INTRODUCTION

Brucellosis in dogs is usually caused by *B. canis*. In some countries, particularly in USA and Canada, where dogs are kept in close association with cattle (Bicknell and Bell, 1979; Forbes, 1990; Bicknell and Halling, 1994; Palmer and Cheville, 1997) infection can also be caused by *B. abortus*. The most likely and efficient means of cattle to dog transmission resulted from ingestion of aborted fetus or placental membranes infected with *Brucella* (Barr et al, 1986; Forbes, 1990; Palmer and Cheville, 1997). Dog to cattle transmission of *B. abortus* has been reported in an experiment, using infected dogs confined with *Brucella* free cattle (Forbes, 1990; Palmer and Cheville, 1997). Circumstantial evidence of dog to cattle transmission of *B. abortus* under field conditions has also been reported (Forbes, 1990). Clinical signs of canine brucellosis are uncommon and variable (Carmichael, 1990) but abortion, epididymitis, and arthritis have been described (Clegg, 1968; Carmichael, 1990). The disease is insidious and many dogs are asymptomatic (Pikerill and Carmichael, 1972; Zoha

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and Carmichael, 1982; Carmichael, 1990; Forbes, 1990; Thanappa et al, 1990). DNA probe or polymerase chain reaction (PCR) methods are currently under development and can be used to demonstrate the agent in various biological samples (Bricker and Halling, 1994; Nielsen et al, 1996a; Ewalt and Bricker, 2000; MacMillan and Stack, 2000).

In Korea, increased outbreaks of human, bovine and canine brucellosis have been reported recently (Kim and Kim, 2000; Baek et al, 2003; Choi et al, 2007; Jung et al, 2007). Baek et al (2003) reported that a total of 84 dairy cattle in one farm were slaughtered under government brucellosis surveillance program during 2001. Three dogs in the farm were positive for *B. abortus*, *melitensis, ovis, suis* (AMOS) PCR and *B. abortus* in bacteriological and serologic tests (rose bengal test and plate agglutination test) and were negative in bacteriological and serologic tests (2-mercaptoethanol rapid slide agglutination test) for *B. canis*. Recently, 223 cases of human brucellosis have been reported to be associated with the livestock industry in Korea from 2002 to the present (Baek et al, 2003).

*B. abortu*s strain RB51 (SRB51) vaccine was produced from a laboratory derived rough mutant of smooth *B. abortus* strain. SRB51 has been investigated as an alternative to strain 19 for vaccination of cattle and bison against brucellosis (Nielsen et al, 1995, 1996a, 1996b; MacMillan and Stack, 2000). Since 1996, SRB-51 has become the official USA vaccine for prevention of brucellosis in cattle (Nielsen and Ewalt, 2004), use of *B. abortus* S19 is now prohibited in the USA. Other countries where the vaccine is officially approved and used at this time are: Argentina, Chile, Colombia, Costa Rica, Mexico, Paraquay, and Venezuela (MacMillan and Stack, 2000). Each country is using slightly diffe-rent methods to administer the vaccine.

Several vaccines were deployed for protection of animals against brucellosis. Of these, SRB51 is a live rough bacterium that lacks the O'polysaccharide in the lipopolysaccharide. It is less virulent than S19 and, more importantly, does not result in serologic reactions that interfere with the traditional serodiagnostic tests used for cows and swine (Lord et al, 1998; Angus, 2000; Edmonds et al, 2001), 2-mercaptoethanol rapid slide agglutination test (2ME-RSAT) using a less mucoid variant strain (M-) of *B. canis* (*B. canis* M-) as antigens, and 2-mercaptoethanol tube agglutination test (2ME-TAT) using of *B. canis* RM6/66 as antigens in dogs (Hur et al, 2001). In addition, SRB51 provides cross-protection in vaccinated mice following challenge with virulent strains of *B. melitensis* or *B. ovis* (Winter et al, 1996; Edmonds et al, 2001). The study reported here was designed to determine the efficacy of SRB51 in protecting dogs against brucellosis by wild type *B. aborus* biotype 1 or *B. canis* isolated from Korean animals.

MATERIALS AND METHODS

Bacterial strains and growth condition

B. canis (isolated from Korean mongrel dog), *B. abortus* biotype 1 (isolated from Korean indigenous dairy cattle), *B. abortus* 1119-3, *B. canis* RM6/66 and *B. canis* M- were obtained from the National Veterinary and Quarantine Service (Anyang, Korea). SRB51 was purchased from the Colorado Serum Company, USA.

B. abortus biotype 1 and SRB51 were grown onto potato infusion agar (Becton Dickinson, Sparks, MD USA), and *B. canis* RM6/66 and *B. canis* M- were grown onto *Brucella* agar (Becton Dickinson, Sparks, MD USA) for 24 to 72 hours at 37℃.

Experimental animals

Twelve Korean mongrel dogs between 8~13kg body weig-hts and 8~10 months of age were used in this experi-ment. All dogs were healthy and seronegative for bru-cellosis by conventional serological tests such as STAT, RBPT, ELISA, and 2ME RSAT. All dogs were divided into four groups namely Group A, B, C and D. Each group consisting of three dogs (One male and two fem-ale dogs). They were housed in four separated concrete isolation rooms and fed with commercial dry food and supplied with adequate drinking water.

Immunization and challenge experiments

Dogs belonging to Group A and C were inoculated subcutaneously with 1×10^9 CFU of SRB51 in 1ml of sterile phosphate buffered saline (PBS). Dogs of Group B and D were inoculated subcutaneously with 1ml sterile PBS as control.

At twelve week post vaccination dogs belonging to Group A and B were challenged orally with virulent strain *B. canis* (1.0×10^{10} CFU). On the other hand dogs belonging to Group C and D were challenged orally with 1.0×10^{10} CFU virulent strain of *B. abortus* biotype 1. Oral inoculation was performed according to the method described by Palmer et al (1997).

Blood samples

Four milliliter blood was collected from the Jugular vein of all dogs at two days prior to challenge (0 week) and at 1, 2, 3, 4, 6 and 8 weeks after challenge (AC) for bacteriological and serological testing. For serologic test, 2ml of blood was collected in sterile tubes. They were allowed to clot by keeping in a refrigerator at 4℃ for 6 to 12 hours, after centrifugation clear serum was obtained. Serum was divided into 1.5ml aliquots and stored at −70℃ until use. The remaining 2ml blood sample without anticoagulant was used immediately for bacteriological culture as well as PCR screening.

Serological tests

All sera samples were examined by STAT, PAT, and RBPT using whole cell antign of *B. abortus* strain 1119-3 (Alton et al, 1975). All sera were also evaluated by 2ME-RSAT (Carmichael and Joubert, 1987) and 2ME-TAT (Alton et al, 1975) using whole cell antigen of *B. canis* M- and *B. canis* RM6/66 antigens respectively. Results were tabulated as the arithmetical average of three dogs in each group.

Necropsy

At 8 weeks AC, all dogs were euthanatized by intravenous administration of sodium pentobarbital (Palmer and Cheville, 1997). Specimens of the lymph nodes (parotid, mandibular, medial, lateral retropharyngeal, superficial and mesenteric), spleen, liver, kidney, urinary bladder, testis, epididymis, and prostate gland or uterus and ovary were collected for bacteriological culture.

Bacteriological culture

Isolation and identification of *Brucella* spp. from blood and tissue samples were performed according to the protocol described by Alton et al (1975).

PCR and modified AMOS-PCR

In order to detect *Brucella* DNA in blood samples of vaccinated and unvaccinated dogs, PCR assay was performed according to the methods described by Jung et al (1998) (Table 1). In order to detect *B. canis*, *B. abortus* biotype 1 and SRB51 DNA in blood and tissue samples of vaccinated dogs modified AMOS-PCR assay was performed according to the methods described by Bricker and Halling (1994, 1995) (Table 1). Genomic DNA for modified AMOS-PCR was extracted from blood and tissue samples by using QIAamp DNA Blood Mini Kit and QIA DNA Mini Kit, respectively (QIAGEN Company, Germany).

Table 1. Nucleotide sequences of primers for PCR and modified AMOS-PCR

Primer (specific)	Nucleotide sequence (5′→3′)	Size of products (bp)	Reference
IS711	TGC CGA TCA CTT AAG GGC CTT CAT		11, 32
B. abortus	GAC GAA CGG AAT TTT TCC AAT CCC	498	
B. melitensis	AAA TCG CGT CCT TGC TGG TCT GA	731	
B. ovis	CGG GTT CTG GCA CCA TCG TCG	976	
B. suis	GCG CGG TTT TCT GAA GGT TCA GG	285	
SRB51/2308	CCC CGG AAG ATA TGC TTC GAT CC	364	
OMPB (F)	ACT GGA GGT CAG AAA TGA AC	981	18
OMPB (R)	GAT TAG AAC GAA CGC TGG AA		

RESULTS

Serological tests

In Group A, antibody titers against *B. canis* were not detected by 2ME-RSAT and 2ME-TAT. In Group B, however, antibody titers to *B. canis* were detected from 1 and 2 weeks AC by 2ME-TAT and 2ME-RSAT, respectively. These antibody titers against *B. canis* were gradually increased to the end of the experiment. In Group C, antibody titers against *B. abortus* biotype 1 were not detected by SAT, PAT and RBT. In Group D, however, antibody titers to *B. abortus* biotype 1 were detected from 2, 3 and 2 weeks AC by SAT, PAT and RBT, respectively. These antibody titers against *B. abortus* were gradually increased until the end of the study by SAT and RBT. These, however, were gradually increased to at 42 days AC by PAT (Table 2).

Bacteriological culture and modified AMOS-PCR

All dogs of Group A were negative for 2ME-RSAT and 2ME-TAT from the first week AC till the end of this study, whereas dogs of Group B were positive for 2ME-RSAT and 2ME-TAT from 2 and 1 weeks AC, respectively. The dogs of Group C were negative in STAT, PAT and RBT using whole cells of *B. abortus* 1119-3 as an antigen during the entire experimental period AC, whereas dogs of Group D were positive from 2, 3 and 2 weeks AC to the end of this study, respectively, by STAT, PAT and RBT (Table 2).

B. canis challenge strain was neither detected from selective agar nor amplified on PCR from whole blood of Group A during the entire experimental period AC, while the challenge strain was isolated on selective and confirmed from whole blood of Group B from 1 week AC to the end of the study by PCR and modified AMOS-PCR. *B. abortus* biotype 1 challenge strain was not detected on selective agar and not amplified by PCR from whole blood of Group C from 1 week AC till the end of this study, whereas the challenge strain was detected from whole blood of dogs of Group D from 1 week AC to 4 weeks AC by PCR and modified AMOS-PCR (Table 3).

B. canis challenge strain was also not detected on selective agar and not amplified from lymph nodes (parotid, mandibular, medial and lateral retropharyn-

Table 2. Reciprocal antibody titer of sera in dogs orally challenged with *B. canis* and *B. abortus* biotype 1

Methods of diagnosis*	Group	Weeks after challenge						
		0	1	2	3	4	6	8
2ME-RSAT	A B C D			1:50	1:160	1:660	1:800	1:800
2ME-TAT	A B C D		1:50	1:100	1:260	1:530	1:660	1:1,330
STAT	A B C D			1:60	1:100	1:130	1:160	1:1,330
PAT	A B C D				1:60	1:160	1:330	1:130
RBT	A B C D			1:130	1:130	1:160	1:260	1:1,330

*STAT, Standard tube agglutination test; PAT, Plate agglutination test; RBT, Rose Bengal test; 2ME-RSAT, 2-mercaptoethanol rapid slide agglutination test; 2ME-TAT, 2-mercaptoethanol tube agglutination test

Table 3. Culture on tryptose agar, and PCR and modified AMOS-PCR from peripheral whole blood of dogs challenged with *B. canis* or *B. abortus* biotype 1

Method	Group	Microorganism	Weeks after challenge						
			0	1	2	3	4	6	8
Culture	A	*B. canis*	0*/3**	0/3	0/3	0/3	0/3	0/3	0/3
	B	*B. canis*	0/3	3/3	3/3	3/3	3/3	3/3	3/3
	C	*B. abortus* biotype 1	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	D	*B. abortus* biotype 1	0/3	0/3	0/3	0/3	0/3	0/3	0/3
PCR	A	*B. canis*	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	B	*B. canis*	0/3	3/3	3/3	3/3	3/3	3/3	3/3
	C	*B. abortus* biotype 1	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	D	*B. abortus* biotype 1	0/3	3/3	3/3	3/3	2/3	0/3	0/3

*The number of dogs cultured or amplified from peripheral whole blood
**The number of dogs challenged with *B. canis* or *B. abortus* biotype 1

Table 4. Culture on tryptose agar and detection of PCR and modified AMOS-PCR product from various tissues of dogs euthanized at 8 weeks after challenge with *B. canis* or *B. abortus* biotype 1

Group	Organisms	Tissues									
		Lymph node	Spleen	Liver	Kidney	Urinary bladder	Testis	Epidi-dymis	Prostate gland	Uterus	Ovary
A	*B. canis*	0*/3**	0/3	0/3	0/3	0/3	0/1	0/1	0/1	0/2	0/2
B	*B. canis*	3/3	3/3	3/3	3/3	2/3	0/1	1/1	1/1	0/2	1/2
C	*B. abortus* biotype 1	0/3	0/3	0/3	0/3	0/3	0/3	0/1	0/1	0/2	0/2
D	*B. abortus* biotype 1	2/3	2/3	0/3	2/3	0/3	0/3	0/1	1/1	0/2	0/2

*The number of dogs cultured or amplified from each tissue
**The number of dogs challenged with *B. canis* or *B. abortus* biotype 1

geal, superficial, and mesenteric), spleen, liver, kidney, urinary bladder, and reproductive organs of Group A at 8 week AC by PCR, whereas, the strain was detected from these tissues of Group B by PCR and modified AMOS-PCR. *B. abortus* biotype 1 challenge strain was also not isolated on selective agar and not detected from lymph nodes, spleen, liver, kidney, urinary bladder, testis, epididymis, and prostate gland or uterus and ovary of Group C at 8 weeks AC, while the challenge strain was detected from these tissues of Group D by PCR and modified AMOS-PCR (Table 4).

DISCUSSION

The purpose of our study was to test whether SRB51 can confer protection in Korea mongrel dogs challenged with *B. canis* or *B. abortus* biotype 1 isolated from Korean animals. All sera of dog belonging to Group A, challenged with wild type *B. canis* at 12 weeks post vaccination with SRB51, were negative in all traditional serodiagnostic tests used in this study. The organisms used for challenge were not isolated from whole blood and tissues such as reproductive organs after culturing then on selective agar and modified AMOS-PCR. However, all sera of dogs in Group B, challenged with wild type *B. canis* without vaccination with SRB51, were positive in traditional serodiagnostic tests using *B. canis* antigen. The organisms were isolated from whole blood and most tissues as well as reproductive organs after culturing them on selective agar and modified AMOS-PCR. Several studies have reported that antibodies against *B. canis* can be detected from 2 to 3 weeks post infection by taditional serodiagnostic tests (Carmichael, 1990; Wanke, 2004). A leukocyte associated bacteremia begins as early as 7 days after oronasal exposure, and it lasts for several months or years (Carmichael, 1990). Therefore *B. canis* can be isolated with ease from hemoculture positive dogs (Carmichael, 1990). The best organs for isolation, for biopsies or at post mortem examination, are lymph nodes, spleen, medulla and sometimes, liver and male reproductive organs (Wanke,

2004).

In several countries including South Korea, *B. abortus* infection in dogs has been reported under experimental and field conditions (Bicknell et al, 1976; Bicknell and Bell, 1979; Forbes, 1990; Baek et al, 2005). Seroconversion, based on immediate results, can occur as early as 4 to 14 days after exposure but is not necessarily coincident with positive culture. Seronegative culture positive dogs have also been described (Pidgeon et al, 1987; Scanlan et al, 1989; Forbes, 1990), similar to the situation in cattle (Forbes, 1990). Seropositivity may persist for up to 3 years (Zoha and Carmichael, 1982), but the maximum duration has not been demonstrated. The seroconversion of dogs containing Group D, challenged with wild type *B. abortus* biotype 1 without vaccination, was similar to these reports. In addition, *B. abortus* biotype 1 was isolated from blood and various tissues of dogs in Group D. However, seroconversions, and culture positive blood and tissues could not be found from dogs possessing Group C, challenged with wild type *B. abortus* biotype 1 at 12 weeks post vaccination with SRB51. Therefore these results indicate that SRB51 can be a good candidate vaccine to protect against brucellosis caused by *B. abortus* bioype 1 or *B. canis* in Korea dogs.

Brucellosis continues to be a major problem in Korea despite the existence of a 'test and slaughter' strategy program for eradication. There is a close contact between dogs and people, and dogs are obviously at high risk in brucellosis infected farms. Accordingly, it may be prudent to keep dogs away from farms known to be infected with *B. abortus* or to include them in all eradication programs (Baek et al, 2005). Both experimental challenge studies and field studies have confirmed the value of SRB51 for protecting cattle from brucellosis. The organism is attenuated in calves and adults. The vaccine is very stable and no reversion to smoothness has been described in vivo or in vitro. The organism behaves as an attenuated strain in a variety of animals including guinea pigs where it is rapidly cleared from the tissues and does not induce abortions.

It may have some virulence for humans, and infections may follow accidental inoculations should be treated with tetracyclines (MacMillan and Stack, 2000). SRB51 vaccine has also been shown to be effective in swine (Lord et al, 1998; Angus, 2000). Dogs orally inoculated with SRB51 can be necessarily infected via the oropharyngeal lymph nodes as well as spleen and liver. Infection of these sites, however, does not result in shedding of the organism in urine, feces, or estrual discharges. Moreover, SRB51 was not found in the prostate gland or kidney, which is common sites of infection in dogs with brucellosis attributable to *B. canis* or virulent *B. abortus* (Morse, 1951; Bicknell and Bell, 1979; Palmer and Cheville, 1997). In pregnant animals, oral inoculation with SRB51 can cause infection of the placenta and fetus, with resulting placentitis; however, placentitis does not lead to abortion. Moreover, intravenous inoculation with 7.5×10^7 CFU of SRB51 did not lead to abortion (Morse, 1951). Therefore our results may provide a valuable help for brucellosis eradication policy in cattle as well as dogs.

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