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Prevalence and pathological characteristics of canine brucellosis in Korea

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Ji Youl Jung

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Ji-Youl Jung
(Supervised by professor Jae-Hoon Kim)

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This thesis has been examined and approved.

손 원 근
Thesis director, Won-Geun Son, Prof. of Veterinary Medicine, Jeju National University

Yoon-Kyu Lim, Prof. of Veterinary Medicine, Jeju National University

한 정 희
Jeong-Hee Han, Prof. of Veterinary Medicine, Kangwon National University

윤 순 석
Soon-Seek Yoon, Senior researcher, Animal and Plant Quarantine Agency

김 재 훈
Jae-Hoon Kim, Prof. of Veterinary Medicine, Jeju National University

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JEJU NATIONAL UNIVERSITY

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GENERAL INTRODUCTION

Etiology

Brucellosis, one of the major zoonoses in worldwide, is caused by a bacteria belonging to the genus *Brucella*. The *Brucella* (*B.*) genus is composed of six "classical" species based on host preference and genetic analysis: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* [12]. Although dogs can be infected by four (*B. canis, B. abortus, B. melitensis,* and *B. suis*) out of the six species of *Brucella*, only *B. canis* produces epidemiological importance [22]. *B. canis*, the etiologic agent of infectious abortion in dogs, was first isolated in 1966 [1]. *B. canis* is a small, rough, gram-negative coccobacillus intracellular bacterium [3, 5]. It grows in common culture media including tryptose agar and does not require CO_2 for culture.

Transmission

Transmission of *Brucella* occurs by several routes. The most common route is venereal transmission [12]. Dogs can also be infected when they are exposed to or ingest infected fetal membranes, aborted fetuses, vulvar discharge, or urine from infected dogs [10, 20]. Infected bitches transmit *B. canis* during estrus, at breeding, or after abortion through oronasal contact with vaginal discharges [10]. In males, the *Brucella* lives in the testicles and seminal fluids. Males excrete bacteria in their semen and urine. Although both sexes excrete bacteria in urine, the concentrations in male urine are higher, reaching 10^3-10^6 bacteria/ml of urine [10].

Transmission between dogs occurs via mucous membranes, so the bacteria may enter the body through the nose, mouth, conjunctiva of the eye, and vagina [22]. Bacteria may also be present in milk, saliva, nasal and ocular secretions, and feces. In addition, cages, equipment, and people in contact with infected dogs have been reported as sources of infection [14].

Pathogenesis

The routes of entry for the *B. canis* are genital, oronasal, or conjunctival mucosa [22]. After *Brucella* gain entry into the animal, they are phagocytized at contaminated mucosal sites by tissue macrophages and other phagocytic cells, and then transported to lymph nodes [10]. The bacteria also travel to the target reproductive tissues such as the prostate, testicles, and epididymides in the male, fetus, gravid uterus, and placenta in female. Bacteremia starts within 1-4 weeks after infection and persist for at least 6 months and then, intermittently, up to 64 months [4, 6, 10].

Clinical and pathological findings

B. canis affects the reproductive system both in female and male dogs, and canine brucellosis is characterized by reproductive failure or infertility. Some dogs remain asymptomatic despite active infection. Morbidity is high but mortality is low [12].

Litters are commonly aborted, usually in the last two weeks of gestation, or the puppies may born weak or die shortly after birth. A bitch usually aborts dead pups between 45 and 60 days of gestation [10]. Puppies are partially autolyzed and accompanied by a gray to green vaginal discharge. The bitch will continue to excrete vulvar discharge with high numbers of bacteria for several weeks after the abortion or parturition [5]. Brucellosis can also result in resorption or early embryonic death within the early weeks after breeding. In males, there are often no signs and they appear to be in good health, but may have epididymitis, orchitis, prostatitis, and testicular atrophy. In addition, enlarged scrotum and secondary ulcerative scrotal dermatitis can be observed [10, 19].

Nonreproductive abnormalities can also occur such as splenomegaly, lymphadenopathy, discospondylitis, and meningoencephalitis [11, 15]. *B canis* also can produce ocular lesions such as endophthalmitis and recurrent uveitis [9, 18].

Diagnosis

Diagnosis of canine brucellosis can be made by clinical laboratory findings, semen examination, serologic testing, bacterial isolation, and genetic detection [10].

Definitive diagnosis of brucellosis in the dog is the bacteriological isolation from a tissue, discharge, blood, semen, vertebra, or eye [22]. Presumptive diagnosis can be made by assessing specific cell-mediated or serological responses to *Brucella* antigens.

The most common history of canine brucellosis is infertility. A bitch that aborts after 45 days of gestation should be highly suspected of brucellosis [5]. In addition, abortion, enlarged lymph nodes, swollen scrotum or the tail of epididymis, abnormal sperm, testicular atrophy or no apparent clinical signs can be shown in canine brucellosis [9]. A differential diagnosis for infectious infertility include viral agent (canine herpesvirus), protozoan (*Neospora caninum*, and *Toxoplasma gondii*), and bacterial etiology (*Mycoplasma*, *Ureaplasma*, *Escherichia coli*, *Streptomyces*, *Salmonella*, and *Campylobacter*). Routine blood work and urinalysis may be performed but are often unremarkable or within reference ranges [10].

1. Serology

Serologic tests are the most frequently used diagnostic method to detect canine brucellosis. *B. canis* has a rough and not a smooth cell wall antigen as do *B. suis, B. abortus,* and *B. melitensis* [3].

Antibodies against *Brucella* can be detected at 2 weeks post-infection [23, 24]. The serologic tests include the rapid slide agglutination test (RSAT and ME-RSAT), tube agglutination test (TAT), indirect fluorescent antibody (IFA), cell wall agar gel immunodiffusion (AGIDcwa), cytoplasmic agar gel immunodiffusion (AGIDcpa), enzyme-linked immunosorbent assay (ELISA), and immunochromatographic test (ICT) [12]. These serologic tests show variable sensitivity and specificity. Among them, RSAT is the simplest microscopic method with high sensitivity to use in practice [22].

However, the ICT kit is broadly used as a national standard diagnostic method in all diagnostic laboratories for the canine brucellosis in Korea. The test is simple to perform and could be potentially used in routine clinical practice.

2. Bacterial isolation

Isolation of the bacteria from samples collected from a suspected case is the only way to confirm that the animal has been infected with *B. canis* [22]. The easiest sample to culture is blood, but the number of bacteria in the circulating leukocytes may be low, therefore, multiple samples of whole blood may be required [22]. Bacteremia starts between 2 and 4 weeks post-infection and persists for about 6 months, becoming intermittent over at least a year (generally 2-5 years) in untreated dogs [2, 6]. Organisms can be isolated from vaginal discharge, semen, lymph node, milk, placental and fetal tissue, prostatic fraction, bone marrow, and urine [12, 15].

3. Genetic detection

Polymerase chain reaction (PCR) is an alternative bacteriological method for direct diagnosis of brucellosis because it offers a rapid and sensitive technique. Semen, vaginal swabs, uterine swabs, and urine are appropriate samples to perform for PCR. Whole blood and serum can also be submitted for PCR, but serum PCR showed little value for the direct diagnosis of canine brucellosis as the assay had low sensitivity [7].

Treatment

B. canis is intracellular bacteria, and it is difficult for antibiotics to penetrate and eradicate this organism from a body [15, 22]. Although there are several reports about treatments that show relative success, treatment for *B. canis* is not encouraging.

1. Doxycycline (10 mg/kg PO q, twice daily), getamicin (5 mg/kg SC q, once daily for 7 days and repeated every 3 weeks), and rifampin (5 mg/kg PO q, once daily) for 3 months [21]

2. Tetracycline (30 mg/kg PO q, twice daily for 28 days) and streptomycin (20 mg/kg IV q, once daily for 14 days) [16]

3. Minocycline (10 mg/kg q, twice daily) and streptomycin (4.5 mg/kg IM for 7 days) [8]

It is recommended that neutering in combination with antibiotic therapy to reduce the risk of transmission to other animals and humans [8, 10, 13], but none have been 100% effective in eradicating the disease [12, 22].

Prevention

In canine brucellosis, no vaccine is available, and results of experimental studies have been unsatisfactory [10].

All new animals to a kennel should be quarantined at least 1 month and have two negative titers 1 month apart before being entered into a kennel [10, 17]. All females and males must be routinely tested serologically before mating. Intact positive dogs should not be bred. *B. canis* is susceptible to 1% sodium hypochlorite, 70% ethanol, iodine/alcohol solutions, glutaraldehyde, and formaldehyde, and the solutions may be used to clean facilities and equipment to decrease the spread of the disease [12, 15].

Public health consideration

Although transmission to humans is rare, this organism is also of zoonotic concern. The Center for Disease Control and Prevention has reported 30 human cases of canine brucellosis since this bacterium was first discovered in 1966 by Carmichael [10]. Clinical signs of humans are undulant fever, headache, and weakness. Unlike dogs, infected people respond well to antibiotic treatments including tetracyclines alone, or in combination with streptomycin or ampicillin [15, 19].

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CHAPTER Ⅰ

Prevalence of canine brucellosis in Korea

ABSTRACT

The aim of this study was to investigate the prevalence of canine brucellosis in Korea, and to determine the disease characteristics based on the geographic distribution and the sex and age of dogs.

We performed a large-scale survey based on serological and bacteriological test. Data were collected from 2,427 dogs including companion and stray dogs. Whole blood or serum samples were collected from dogs in each group, and serological test and bacterial isolation from blood cultures were performed. Of the 2,427 samples tested, 31 (1.3%) were positive for *B. canis* antibodies. Of these, 17 (0.9%) were from companion dogs and 14 (2.4%) were from stray dogs, respectively. Two (1.0%) of the 196 samples were positive in bacterial culture from stray dogs. The female dogs in two groups had significantly higher prevalence of brucellosis compared with the male dogs. The prevalence of canine brucellosis was significantly higher in old-aged stray dogs, over 6 years. However, there were no statistically significant differences of prevalence in both groups of dogs based on the geographic region in Korea. National control measures for canine brucellosis were not performed until today. Our findings suggest that appropriate screening tests and control measures are necessary to improve public health for dogs and human in Korea, particularly with the growth of the companion animal industry.

Key words: bacteriology, *Brucella canis*, canine brucellosis, prevalence, serology

I. INTRODUCTION

Brucellosis is an important zoonotic disease that poses serious public health risks and is associated with economic losses in worldwide [9]. The *Brucella* genus is composed of six "classical" species based on host preference and genetic analysis: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* [10]. Although dogs can be infected by *B. abortus*, *B. melitensis*, and *B. suis*, the infection of dogs with *B. canis* is the most common, and results in serious clinical events including spontaneous abortion [22].

The infection can be transmitted between dogs by venereal, oronasal, or conjunctival routes [22]. After *Brucella* enters the body, it is phagocytized by macrophages and travels to the lymph nodes and targeting reproductive (steroid-sensitive) tissue. The bacteria spread via the bloodstream to other tissues such as intervertebral discs, kidneys, and eyes. Bacteremia is evident 1-4 weeks after infection and can last for several years [10].

Human brucellosis is one of the most important worldwide zoonotic diseases and is re-emerging in some countries. Human brucellosis is caused by the infection of *B. abortus*, *B. suis*, *B. melitensis*, and *B. canis* [15]. Although no cases of *B. canis* infection have been reported in humans in Korea, more attention should warranted for this disease due to the increasing ownership for companion dogs.

Carmichael LE [6] first identified *B. canis* in 1966 as the cause of abortion among beagles in the USA. Since then, the disease has been reported in several countries including Argentina [19], Canada [4], Germany [23], Mexico [8], Nigeria [1, 5], and Zimbabwe [7]. In Asia, the disease has been reported in China [11], Japan [18], and Malaysia [12].

In Korea, *B. canis* was first isolated in outdoor dogs in 1984 [16]. After that, several studies for the seroprevalence of canine brucellosis have been conducted [3, 17, 20]. However, there was no available nationwide survey for the distribution and/or prevalence of canine brucellosis until today.

Thus, the aim of the present study was to investigate the prevalence of canine brucellosis in Korea, and to determine the disease characteristics based on the geographic distribution and the sex and age of dogs.

II. MATERIALS AND METHODS

Animals

Whole blood or serum samples from 2,427 dogs from 2 months to 23 years old (1,174 females and 1,253 males) were used in this study. We classified the dogs into 2 groups such as companion dogs and stray dogs based on their living conditions. Between March 2015 and December 2016, blood or serum samples of companion dogs (n=1,852) were collected from 17 animal hospitals and stray dogs (n=575) from 5 dog shelters located in different regions (Table 1-1). For statistical analysis, data were collected for sex, age, and three geographic region [northern (Gyeonggi and Gangwon Province, Seoul Special City, Incheon Metropolitan City), central (Chungnam, Chungbuk, Gyeongbuk Province and Daejeon Metropolitan City), and southern (Jeonnam, Gyeongnam Province, Gwangju, Busan, Ulsan Metropolitan City, and Jeju Special Self-Governing Province)] (Fig. 1-1).

Table 1-1. Number of dogs examined for the prevalence of *Brucella canis*

Age (yr)	Companion dogs		Stray dogs	
	Male (*n*)	Female (*n*)	Male (*n*)	Female (*n*)
2 <	246	174	103	54
2-6	231	234	163	97
6≥	444	522	66	92
Subtotal	921	931	332	243
Total	1,852		575	

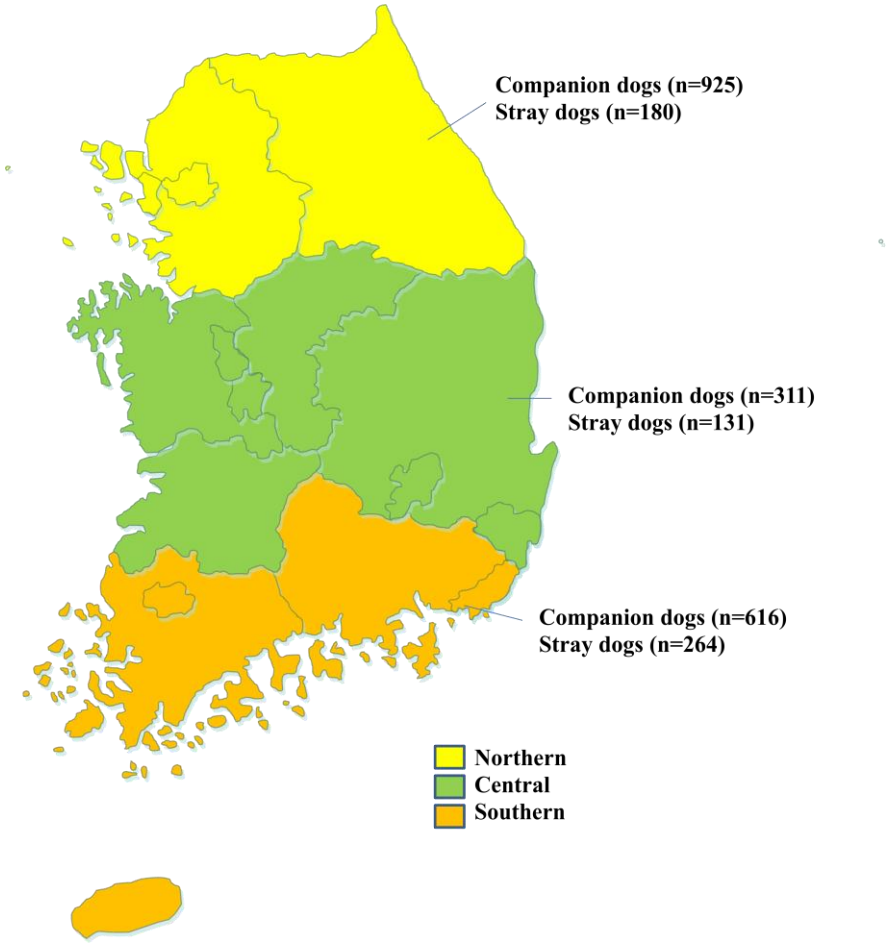

Fig. 1-1. Geographic distribution for the collected samples of companion and stray dogs in Korea.

For statistical analysis, the samples were assigned to the northern, central, and southern regions.

Sample preparation

Approximately 2-3 mL of whole blood was collected from stray dogs using heparin as an anticoagulant for serological test and bacterial isolation. For serological test, blood was centrifuged at 4℃, at 3,000 rpm for 30 minutes. The plasma was collected from the tube, inactivated at 56℃ for 30 minutes, and then stored at -20℃.

Because only serum samples were obtained from animal hospitals, bacterial isolation could not be performed in companion dogs. The serum samples were stored at -20℃ until required for further use.

Serological test

Serum or plasma samples were analyzed using an immunochromatographic test (ICT) for canine brucellosis (BioNote Inc., Hwaseong, Korea), according to the manufacturer's instructions. The samples of whole blood, plasma, and serum can be used in this kit. Serum from dogs previously infected with *B. canis* was used as the positive control.

Blood culture and identification

After separating plasma from whole blood, buffy coat was used for bacterial isolation. The buffy coat layer was inoculated in tryptose phosphate agar (TSA) (BD, Franklin Lakes, NJ) containing 5% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and incubated at 37℃ under aerobic conditions for 3-5 days. Suspected colonies were selected and purified by cultivation on 5% sheep blood agar for 2-4 days at 37℃. Because of the small volume of blood, the number of tested samples was variable for serology and bacterial isolation in this study.

For bacterial species identification, genomic DNA was extracted from the isolates using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The identification of the isolates was confirmed using the novel Bruce-ladder multiplex PCR assay [13].

Statistical analysis

A chi-square test was used to evaluate the association between the prevalence of *Brucella* and living condition, sex, age of dogs, and geographic region. All statistical analyses were performed using SPSS software, version 21.0 (IBM corp., Armonk, NY, USA). A *P*-value less than 0.05 was considered statistically significant.

III. RESULTS

In this study, *Brucella* infection was diagnosed with ICT and bacterial culture. *Brucella* infection was determined if one or both tests were positive. The detailed results of serology, bacterial culture, and overall prevalence of canine brucellosis is shown in Table 1-2. Of the 2,427 samples tested, 31 (1.3%) were positive for *B. canis* antibodies. Of these, 17 (0.9%) were from companion dogs and 14 (2.4%) were from stray dogs, respectively. Two samples (1.0%) of the 196 stray dogs were positive in bacterial culture. The status of *Brucella* infection associated with the sex and the age of dogs, and geographic regions is demonstrated in Tables 1-3. In companion dogs, female dogs had significantly higher positivity (1.4%) to *B. canis* than in the males (0.4%). Moreover, the prevalence of *B. canis* in female stray dogs (4.7%) was significantly higher than in males (0.9%).

According to the age of dogs under 2 years, 2-6 years old, and over 6 years old, the prevalence of *B. canis* was recorded as 0.5 % (2/421), 1.5% (7/465), and 0.8% (8/966), respectively ($P > 0.05$) in companion dogs. Meanwhile, the prevalence in stray dogs under 2 years old, 2-6 years old, and over 6 years old was 1.3 % (2/157), 1.2% (3/260), and 5.7% (9/158), respectively ($P < 0.05$).

There were no statistically significant differences of prevalence in both groups of dogs based on the geographic region.

Table 1-2. Results of ICT and bacterial culture for *Brucella canis* between two groups of dogs

Group	ICT[a]		Bacterial culture		ICT or bacterial culture	
	No. tested	No. positive (%)	No. tested	No. positive (%)	No. tested	No. positive (%)
Companion dogs	1,852	17 (0.9)	NT[b]	-	1,852	17 (0.9)
Stray dogs	575	14 (2.4)	196[c]	2[d] (1.0)	575	14 (2.4)
Total	2,427	31 (1.3)	196	2 (1.0)	2,427	31 (1.3)

[a] Immunochromatographic test

[b] Not tested

[c] Bacterial culture was examined in 196 out of 575, due to restricted volume of blood samples.

[d] ICT also positive

Table 1-3. Prevalence of *Brucella canis* infection according to the sex and the age of dogs and geographic region

Group		Companion dogs			Stray dogs		
		No. tested	No. positive (%)	*P*-value	No. tested	No. positive (%)	*P*-value
Sex	Male	921	4 (0.4)	0.030	332	3 (0.9)	0.005
	Female	931	13 (1.4)		243	11 (4.7)	
Age (yr)	< 2	421	2 (0.5)	0.252	157	2 (1.3)	0.008
	2-6	465	7 (1.5)		260	3 (1.2)	
	≥ 6	966	8 (0.8)		158	9 (5.7)	
Region	Northern	925	12 (1.3)	0.232	180	1 (0.6)	0.052
	Central	311	1 (0.3)		131	10 (7.6)	
	Southern	616	4 (0.6)		264	3 (1.1)	

IV. DISCUSSION

Although several reports for the occurrence of canine brucellosis had been conducted mainly in breeding kennel dogs [16, 17, 20], there is very limited information for canine brucellosis in Korea. In the present study, we surveyed the prevalence of canine brucellosis using 2,427 samples from dogs based on the three geographic region of Korea. This study includes nationwide survey for geographic distribution and prevalence of canine brucellosis in companion dogs as well as in stray dogs.

We used ICT for the serodiagnosis of canine brucellosis in this study. This test detects antibodies directed to the rough cell wall antigens of *Brucella*. The test is simple to perform and could be potentially used in routine clinical practice [22]. Previous reports evaluated the usefulness of ICT for the diagnosis of canine brucellosis by comparing with the RSAT, 2-mercaptoethanol RSAT (2ME-RSAT), AGID, and ELISA [22]. The ICT kit is broadly used as a national standard diagnostic method in all diagnostic laboratories for the canine brucellosis in Korea. Therefore, we used ICT for the sero-diagnosis of canine brucellosis to clarify the prevalence of *B. canis* in companion and stray dogs.

The prevalence of canine brucellosis may vary according to the test method and living condition of target animals. Previous studies using different methods demonstrated variable prevalence rates of canine brucellosis in other countries: 17.6% (57/324) in Zimbabwe using ELISA [7], 14.7% (33/224) and 10.7% (24/224) in Argentina using RSAT and IELISA method [19], 0.3% (6/2,000) in Canada using AGID [4], and 2.5% (12/485) in Japan using MAT [18]. Detected prevalence (1.3%) in this study is lower than the result of Japan, but higher than that of Canada.

In a previous study of 501 dogs in Korea, the seroprevalence rates of *B. canis* in 69 indoor dogs, 177 kennel dogs, and 225 stray dogs were 1.5%, 17.5%, and 8.2% using 2-ME RSAT, respectively [3]. However, the sero-positive rate of *B. canis* in breeding kennel dogs was 14.1% using ICT, whereas 0% was recorded in companion dogs and stray dogs [3]. In that study, higher positive rate of *B. canis* in breeding kennel dogs was closely associated with recent occurrence of brucellosis [3]. Overall sero-positivty for canine brucellosis in this study was lower than the previous study. And

positive rate for *B. canis* in stray dogs (2.4%) is about three times higher than companion dogs (0.9%). This result of serologic tendency is similar with previous study. This may be closely related with the condition of uncontrolled mating and/or inadequate veterinary care in stray dogs.

In this study, females of companion and stray dog show significantly higher prevalence of brucellosis than in males. The different sexual prevalence in this study is very similar with previous studies in other countries [5, 7]. Infected males can transmit *B. canis* to females through the seminal fluid and urine. Seminal fluid and urine have been implicated as sources of infection from males that harbor organisms in their prostate and epididymis. If a single male dog is infected with *B. canis* and mates with several females, bacteria can transmit to other female dogs through infected semen [5].

Brucella infection in dogs is reported age-dependent, due to the longer period of exposure in adult dogs [2]. In domestic dogs, sexual maturity occurs between the age of 6 to 12 months for both males and females, although this can be delayed until up to two years of age for some large breeds. In this study, older stray dogs ($\geq$ 6 years) have higher prevalence (5.7%) of brucellosis than younger ones (1.2-1.3%), meanwhile the results of companion dogs is not age-dependent. Therefore, old dogs may play an important role for transmission of *B. canis* to stray dogs in Korea, especially in central region.

For screening canine brucellosis, several serological methods are used in many countries. However, some researchers require direct laboratory tests, such as blood culture and nucleic acid amplification via PCR for the definitive diagnosis of *Brucella* infection [14]. Bacteremia starts between 2 and 4 weeks post-infection and persists for about 6 months, becoming intermittent over at least a year [10]. We tried to isolate the *B. canis* organism from canine blood samples. In this study, only serum samples were obtained from local animal hospital. In addition, bacterial culture was performed limited number of stray dogs due to lack of blood volume. The bacterial isolation rate was lower than ICT results in stray dogs. This might be associated with the lower number of circulating leukocytes in lack blood volume. Moreover, bacteria cannot be cultured if the animal has received antibiotic treatment previously [21].

Bovine brucellosis, caused by *B. abortus*, is one of the most common zoonoses in Korea. The enforcement of control measures for bovine brucellosis (test and slaughter) have led to a reduction of *B. abortus* incidence. However, there was no national control program for canine brucellosis in Korea. Although the prevalence of canine brucellosis is low, zoonotic *B. canis* infection is circulate in companion and stray dogs in Korea. Therefore, more stringent screening tests and effective control measures should be warranted in Korea with the particular aspects of public health and increasing companion animals.

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CHAPTER Ⅱ

Pathological, immunohistochemical, and bacteriological findings in dogs infected with *Brucella canis*

ABSTRACT

This study describes pathological, immunohistochemical, and bacteriological findings in adult dogs and fetuses naturally infected with *Brucella canis*.

A total of 87 dogs were included in this study. Of these animals, 49 dogs and 2 aborted fetuses were classified into group 1 (*B. canis*-infected dogs), and 21 dogs and 15 fetuses were classified into group 2 (non-infected dogs) based on the serological and bacteriological results.

The most common gross lesions in infected dogs were swelling of lymph nodes and spleen. The testes showed marked swelling with reddish discoloration. The most significant histopathological lesions were observed in placenta. Placental trophoblasts were markedly hypertrophied due to the accumulation of intra-cellular Gram-negative bacteria. Furthermore, lymphocytic inflammation of varying severity revealed in the reproductive organs such as male testis, epididymis, and prostate and female uterus.

Strong immunolabeling was observed in the cytoplasms of most trophoblasts in the placental tissues. However, immunohistochemistry did not demonstrate any organisms in other organs of dogs and fetuses.

B. canis isolates were most frequently obtained from the whole blood (67.3%) and frequent in superficial inguinal lymph node (63.3%) in both sexes. However, isolation rate was higher in male genital organs than in females. Hence, the management of the male dogs is important because infected dog can play a role as a carrier.

Key words: bacteriology, *Brucella canis*, dog, immunohistochemistry, pathology

I. INTRODUCTION

Canine brucellosis is considered one of the most common bacterial zoonotic infections in worldwide and has been recognized as a cause of great economic loss in kennels [2, 7, 13].

General symptoms of canine brucellosis are not readily evident [5]. *Brucella* infected dogs are usually febrile and some dogs remain asymptomatic; most infections will not be diagnosed by routine history taking or physical examinations.

The classical signs of canine brucellosis are spontaneous abortion in a supposedly healthy pregnant bitch or failure to conceive. Late abortion occurs between 30 and 57 days of gestation, and higher frequency of abortion was observed between 45 and 55 days [3].

Aborted fetuses are usually partially autolyzed, and edema, congestion, and hemorrhage were presented in the subcutaneous abdominal region [7]. Prolonged, viscous, and serosanguinous vaginal discharge can last for 1-6 weeks after abortion [3].

Target organs of *B. canis* are androgen-dependent tissues in the stud dog (i.e., the epididymis and prostate). Orchitis or epididymitis causes pain and swelling of the testis. Males may have scrotal dermatitis because of constant licking and the secondary infection with nonhemolytic staphylococci. Chronic or prolonged infection in the stud dog eventually leads to unilateral or bilateral testicular atrophy [5].

Nonreproductive abnormalities can also occur. Diffuse lymphadenomegaly and splenomegaly are commonly detected. Generalized lymphadenitis and lymphoreticular hyperplasia are observed microscopically in lymphoid organs [4]. *B. canis* can also produce discospondylitis, anterior uveitis, meningoencephalomyelitis, and pyogranulomatous dermatitis [7].

The diagnosis of brucellosis is based on the isolation of pathogen from whole blood, semen, vaginal secretions, urine, and lymphoid tissues [5, 12]. Moreover, several serological tests are available. Another method for the detection of *B. canis* in tissues is immunohistochemistry [7]. This

method is sensitive and specific, and capable of demonstrating the distributions of bacterial organisms in the tissues with characteristic lesions.

Despite the fact that several studies have been published on canine brucellosis, much information still remains to be clarified in the aspect of pathologic lesions and immunohistochemical findings of this disease. This study describes the pathological, immunohistochemical, and bacteriological findings in adult dogs and fetuses naturally infected with *B. canis*.

II. MATERIALS AND METHODS

Animals

Four outbreaks of canine brucellosis were observed in breeding kennels at different regions in Korea. All farms had histories of repeated abortion and decreased fertility. After making a diagnosis of canine brucellosis, all dogs in kennels were examined based on the screening test such as serological and bacteriological tests according to brucellosis control strategies in Korea. If the result of screening tests was positive, infected dogs were euthanized.

A total of 87 dogs were included in this study. Among these animals, 49 dogs aged 6 months to 10 years (35 females and 14 males) and 2 aborted fetuses were classified into group 1 (G1, *B. canis*-infected dogs), and 21 dogs aged 6 months to 12 years (15 females and 6 males) and 15 fetuses were classified into group 2 (G2, non-infected dogs) based on the serological and bacteriological results (Table 2-1).

Table 2-1. Number of dogs examined for pathological studies on canine brucellosis

Ages (yr)	G1			G2		
	Female	Male	Fetus	Female	Male	Fetus
< 2	9	0	-	2	3	-
2-6	13	8	-	9	2	-
≥ 6	13	6	-	4	1	-
Subtotal	35	14	2	15	6	15
Total		51			36	

G1: *Brucella* infected group

G2: *Brucella* non-infected group

Gross and histopathological findings

At necropsy, tissue samples were evaluated for the presence of gross lesions. Samples of lymph nodes (retropharyngeal, superficial inguinal), mammary gland, uterus, placenta, testis, epididymis, prostate gland, scrotum, lung, liver, spleen, and kidney were collected from dogs, whereas samples of the lung, liver, spleen, and kidney were collected from fetuses. All collected samples were fixed in 10% phosphate-buffered formalin for 24 h and routinely processed. The processed tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) for light microscopic examination. Gram staining was also performed to clarify the bacterial pathogens in tissue sections.

Immunohistochemistry (IHC)

To detect *B. canis*, IHC was performed on replicated sections. The major organs were stained with polyclonal hyperimmune rabbit antiserum at a dilution of 1:500.

The antiserum for IHC was produced by two rabbits after intravenous inoculation of 1×10^6 colony-forming units of live *B. canis* strain.

The tissue sections were stained with the Ventana Discovery XT research instrument and RedMap Detection System (Ventana Medical Systems, Inc., Tucson, AZ, USA), and counterstained with hematoxylin. Tissue sections from an uninfected dog were used as negative controls.

Bacterial culture and identification

During necropsy, parts of the genital organs (female: uterus and mammary gland; male: testis, epididymis, and prostate gland), lymph nodes (superficial inguinal and retropharyngeal lymph nodes), lung, liver, kidney, urine, and whole blood were collected aseptically for microbiologic analysis.

To isolate *B. canis* from tissues, specimens were macerated with sterile phosphate-buffered saline (PBS). Tissue homogenates were inoculated directly onto tryptic soy agar (TSA) (BD, Franklin Lakes, NJ) supplemented with 5% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and antibiotic mixtures (25 U/mL bacitracin, 20 µg/mL vancomycin, 5 µg/mL nalidixic acid, 5 U/mL

polymyxin B, 10 μg/mL cycloheximide, and 100 U/mL nystatin). Plates were incubated in air supplemented with 5% CO_2 at 37℃ for 5-10 days and examined daily for the presence of colonies. Colonies were selected, inoculated onto 5% sheep blood agar for 2-4 days at 37℃.

For bacterial species identification, genomic DNA was extracted from the isolates using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The identification of the isolates was confirmed using the novel Bruce-ladder multiplex PCR assay [10].

Statistical analysis

The statistical analysis was performed using SPSS software, version 21.0 (IBM corp., Armonk, NY, USA). A chi-square test was used to evaluate the association between the pathological lesions and bacterial isolates in G1. A *P*-value less than 0.05 was considered statistically significant.

III. RESULTS

Gross findings

The most common macroscopic lesions in G1 were observed in the lymph nodes and spleen. These organs showed variable degrees of swelling (Fig. 2-1). The testes showed marked swelling with reddish discoloration (Fig. 2-2). In some male dogs, epididymal swelling and scrotal necrosis were observed (Fig. 2-3). Female dogs in G1 had few specific gross lesions. However, an aborting bitch showed brownish vulvar discharge. Aborted fetuses were often partially autolyzed with a brown or greenish-gray placenta (Fig. 2-4). However, there were no specific gross findings in G2.

Histopathological findings

Collected tissues from 49 dogs and 2 fetuses in G1 and 21 dogs and 15 fetuses in G2 were examined histologically under microscope.

We analyzed histopathological lesions according to three different aged dogs (< 2years, 2-6 years, and ≥ 6 years) and fetus groups. Furthermore, histopathological lesions of genital organs in male and female dogs also analyzed.

The prevalence of microscopic lesions in genital organs of female and male dogs is summarized in Table 2-2. Mild to severe lymphohistiocytic interstitial inflammation was presented in the 78.6% (11/14) prostate glands of male dogs in G1 (Fig. 2-5). Scrotal dermatitis was observed in 78.6% (11/14) male dogs in G1 and was characterized by the infiltration of lymphocytes and neutrophils with epidermal ulceration or crust formation (Fig. 2-6). Lymphocytic epididymitis (Fig. 2-7) and orchitis with testicular atrophy (Fig. 2-8) were observed in 57.1% (8/14) and in 21.4% (3/14) male dogs in G1.

The mammary gland showed multifocal interstitial lymphocytic infiltration (Fig. 2-9) in 30.6% (11/36) female dogs in G1. And multifocal-to-diffuse lymphocytic endometritis (Fig. 2-10) was

observed in 33.3% (12/36) female dogs in G1. The lesions of reproductive organs in both sexes were more frequently observed in G1 than in G2.

The most common microscopic lesion of non-reproductive organs was presented in the liver of both sexes. Multifocal neutrophilic or lymphocytic hepatitis (Fig. 2-11) was more frequently observed in 69.4% (35/49) dogs in G1 than 52.4% (11/21) dogs in G2.

Lymphoid tissues such as the lymph nodes and spleen revealed consistent follicular and white pulp hyperplasia with variable degree (Fig. 2-12). Follicular hyperplasia (46.9%, 23/49) of lymph nodes was more prevalent than white pulp hyperplasia (34.7, 17/49) of spleen in the dogs of G1. These lesions in lymphoid tissues were seldom observed in the dogs in G2. Lymphocytic interstitial nephritis lesions (Fig. 2-13) were more frequent in dogs of G1 than in G2. Minor lesions such as uveitis and pneumonia were less frequent in the dogs of both groups.

Most histopathologic lesions in genital or non-genital organs were more frequently observed in aged dogs (over 2 years) in G1 than G2. However, there was no close relation between non-genital lesions and dog's age in dogs of both groups.

Mild bronchopneumonia and suppurative hepatitis were existed in several fetuses in G1. Placental trophoblasts were markedly hypertrophied due to the accumulation of intra-cellular bacteria (Fig. 2-14). These bacteria were confirmed as Gram-negative coccobacilli using Gram staining (Fig. 2-15). However, no specific lesions were observed in G2 fetuses.

Table 2-2. Frequency of pathological lesions of female and male genital organs in G1 and G2 according to age

Ages (yr)	Male dogs								Female dogs			
	Orchitis (%)		Epididymitis (%)		Prostatitis (%)		Scrotal dermatitis (%)		Lymphocytic mastitis (%)		Lymphocytic endometritis (%)	
	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
< 2	0.0 (0/0)	0.0 (0/3)	0.0 (0/0)	0.0 (0/3)	0.0 (0/0)	0.0 (0/3)	0.0 (0/0)	0.0 (0/3)	33.3 (3/9)	0.0 (0/2)	22.2 (2/9)	0.0 (0/2)
2-6	25.0 (2/8)	0.0 (0/2)	50.0 (4/8)	50.0 (1/2)	87.5 (7/8)	0.0 (0/2)	75.0 (6/8)	50.0 (1/2)	38.5 (5/13)	11.1 (1/9)	46.2 (6/13)	22.2 (2/9)
≥ 6	16.7 (1/6)	0.0 (0/1)	66.7 (4/6)	0.0 (0/1)	66.7 (4/6)	0.0 (0/1)	83.3 (5/6)	100.0 (1/1)	15.4 (2/13)	0.0 (0/4)	23.1 (3/13)	25.0 (1/4)
Total	21.4 (3/14)	0.0 (0/6)	57.1 (8/14)	16.7 (1/6)	78.6 (11/14)	0.0 (0/6)	78.6 (11/14)	33.3 (2/6)	28.6 (10/35)	6.7 (1/15)	31.4 (11/35)	20.0 (3/15)

G1: *Brucella* infected group

G2: *Brucella* non-infected group

Table 2-3. Frequency of pathological lesions of non-genital organs in dogs in G1 and G2 according to age

Ages (yr)	Hepatitis		Nephritis		Follicular hyperplasia		White pulp hyperplasia		Uveitis		Pneumonia	
	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
< 2	66.7 (6/9)	100.0 (5/5)	33.3 (3/9)	0.0 (0/5)	55.6 (5/9)	0.0 (0/5)	66.7 (6/9)	0.0 (0/5)	0.0 (0/9)	0.0 (0/5)	0.0 (0/9)	0.0 (0/5)
2-6	76.2 (16/21)	36.4 (4/11)	57.1 (12/21)	9.1 (1/11)	52.4 (11/21)	18.2 (2/11)	19.0 (4/21)	0.0 (0/11)	14.3 (3/21)	18.2 (2/11)	4.8 (1/21)	9.1 (1/11)
≥ 6	63.2 (12/19)	40.0 (2/5)	47.4 (9/19)	20.0 (1/5)	36.8 (7/19)	0.0 (0/5)	36.8 (7/19)	0.0 (0/5)	0.0 (0/19)	0.0 (0/5)	5.3 (1/19)	40.0 (2/5)
Total	69.4 (35/49)	52.4 (11/21)	49.0 (24/49)	9.5 (2/21)	46.9 (23/49)	9.5 (2/21)	34.7 (17/49)	0.0 (0/21)	6.1 (3/49)	9.5 (2/21)	4.1 (2/49)	14.3 (3/21)

G1: *Brucella* infected group

G2: *Brucella* non-infected group

IHC

Strong red-colored immunolabeling was observed in the cytoplasms of most trophoblasts in the placental tissues of G1 (Fig. 2-16). However, IHC did not demonstrate any organisms in the lesions of other organs of dogs and fetuses.

Bacterial culture

To examine bacterial isolation rates in various organs, genital and internal organs were obtained aseptically at necropsy. Bacteriological results are summarized in Table 2-4.

Of the 49 dogs, 40 (81.6%) had positive results in blood culture at least one samples examined. *B. canis* isolates were recovered in all tissues of the two aborted fetuses in G1.

Bacterial isolates were most frequently obtained from the whole blood and frequent from the superficial inguinal lymph node and spleen in both sexes. In detail, bacterial isolates were frequently obtained from the blood (71.4%; 25/35), superficial inguinal lymph node (65.7%; 23/35), and spleen (57.1%; 20/35) in female dogs. In males, *B. canis* was isolated from whole blood and superficial inguinal lymph node with same level (57.1%; 8/14), and spleen (50.0%; 7/14). Moreover, genital organs such as prostate gland (42.9%; 6/14) and epididymis (38.5%; 5/13) had high frequency of bacterial isolates.

G1 cases were analyzed to find correlations between bacterial culture and pathological changes (Table 2-5). According to these results, pathological lesion of the uterus was closely associated with *Brucella* infection ($p=0.02$). However, in other organs, no association was observed between bacterial culture and pathological lesions.

Table 2-4. Bacterial isolation rates of various samples in *B. canis* infected dogs (except aborted fetuses)

Samples	Female		Male		Total	
	No. tested	No. isolates (%)	No. tested	No. isolates (%)	No. tested	No. isolates (%)
Blood	35	25 (71.4)	14	8 (57.1)	49	33 (67.3)
Superficial inguinal lymph node	35	23 (65.7)	14	8 (57.1)	49	31 (63.3)
Spleen	35	20 (57.1)	14	7 (50.0)	49	27 (55.1)
Retropharyngeal lymph node	35	17 (48.6)	14	5 (35.7)	49	22 (44.9)
Liver	35	14 (40.0)	14	5 (35.7)	49	19 (38.8)
Lung	35	13 (37.1)	14	4 (28.6)	49	17 (34.7)
Kidney	35	9 (25.7)	14	2 (14.3)	49	11 (22.4)
Urine	29[a]	4 (13.8)	14	3 (21.4)	43	7 (16.3)
Uterus	35	10 (28.6)			35	10 (28.6)
Prostate gland			14	6 (42.9)	14	6 (42.9)
Epididymis			13[b]	5 (38.5)	13	5 (38.5)
Testis			13[b]	2 (15.4)	13	2 (15.4)

[a] 29 out of 35 samples examined.

[b] 13 out of 14 samples examined

Table 2-5. Association between bacterial culture and pathological lesions in various organs of dogs

Organs	Pathological lesions		No pathological lesions		*p*-value
	No. tested	No. isolates (%)	No. tested	No. isolates (%)	
Testis	3	0 (0.0)	10	2 (20.0)	0.352
Epididymis	8	3 (37.5)	6	2 (33.3)	0.814
Prostate	11	5 (45.5)	3	1 (33.3)	0.342
Uterus	11	7 (63.6)	24	3 (12.5)	0.002
Superficial inguinal lymph node	23	16 (69.6)	26	15 (57.7)	0.390
Retropharyngeal lymph node	23	12 (52.2)	26	10 (38.5)	0.336
Spleen	17	10 (58.8)	32	17 (53.1)	0.703
Lung	2	1 (50.0)	47	16 (34.0)	0.642
Liver	35	15 (42.9)	14	4 (28.6)	0.354
Kidney	24	8 (33.3)	25	3 (12.0)	0.074

Legends for Figures

Fig. 2-1. Note severe swollen superficial inguinal lymph node.

Fig. 2-2. Testicular swelling with reddish discoloration (left). Normal testis (right).

Fig. 2-3. Dermal congestion and edema around scrotum.

Fig. 2-4. An aborted fetus in the brown or greenish-gray placenta.

Fig. 2-5. Interstitial lymphocytic infiltration and the irregular glandular structures in the prostate gland. H&E. Bar = 100 µm.

Fig. 2-6. Crust formation in epidermis and neutrophilic infiltration in the superficial dermis of scrotum. H&E. Bar = 200 µm.

Fig. 2-7. Interstitial lymphocytic infiltration in the epididymis. H&E. Bar = 200 µm.

Fig. 2-8. Severe lymphohistiocytic orchitis with fibrosis and atrophy of seminiferous tubules in testis. H&E. Bar = 200 µm.

Fig. 2-9. Interstitial lymphoplasmacytic infiltration in the mammary gland. H&E. Bar = 100 µm.

Fig. 2-10. Lymphoplasmacytic infiltration in the lamina propria of endometrium. H&E. Bar = 200 µm.

Fig. 2-11. Infiltration of neutrophils and lymphocytes in the portal triad of liver. H&E. Bar = 200 µm.

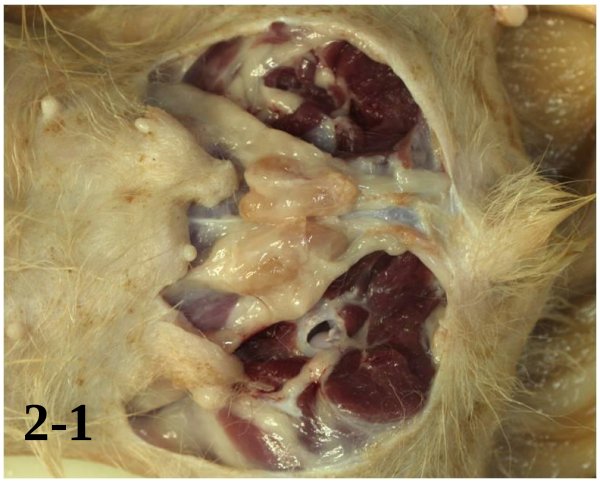
Fig. 2-12. Note germinal center with lymphohistiocytic proliferation in the lymphoid follicles of superficial inguinal lymph nodes. H&E. Bar = 200 µm.

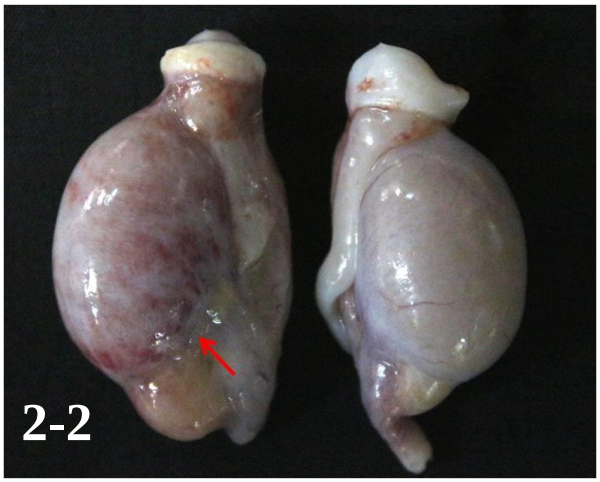
Fig. 2-13. Lymphoplasmacytic infiltration in the renal pelvis. H&E. Bar = 200 µm.

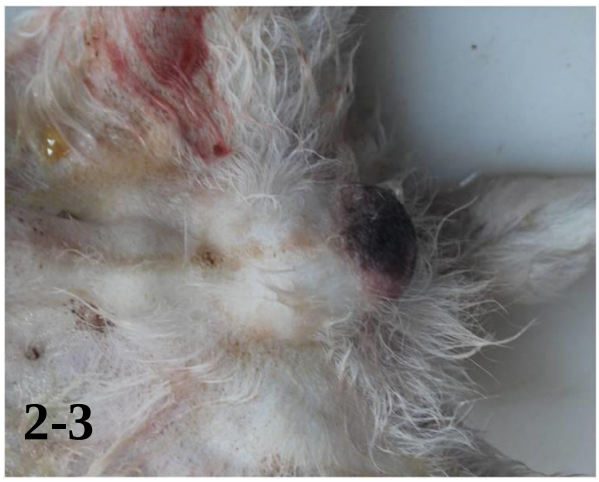
Fig. 2-14. The chorioallantoic membrane lined by bacteria-laden hypertrophied trophoblasts. H&E. Bar = 200 µm. (Insert: a higher magnification of the trophoblasts. H&E. Bar = 50 µm.)

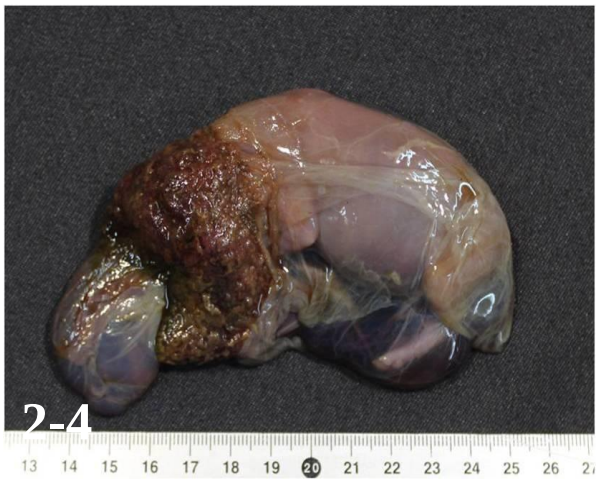
Fig. 2-15. Gram-negative coccobacilli within trophoblasts. Gram stain. Bar = 50 µm.

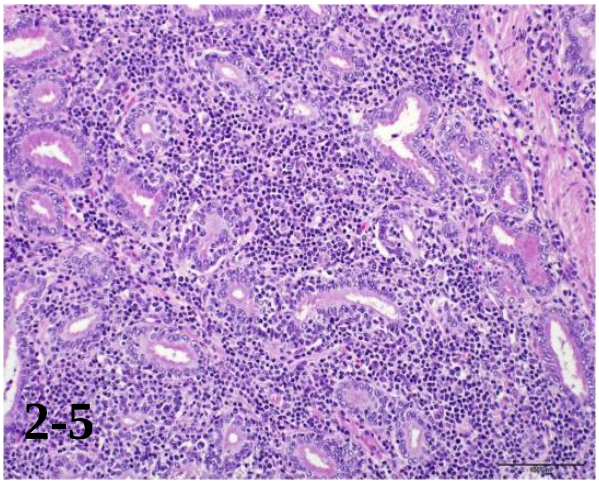
Fig. 2-16. A strong positive reactions within the cytoplasm of trophoblast cells. IHC. Bar = 100 µm.

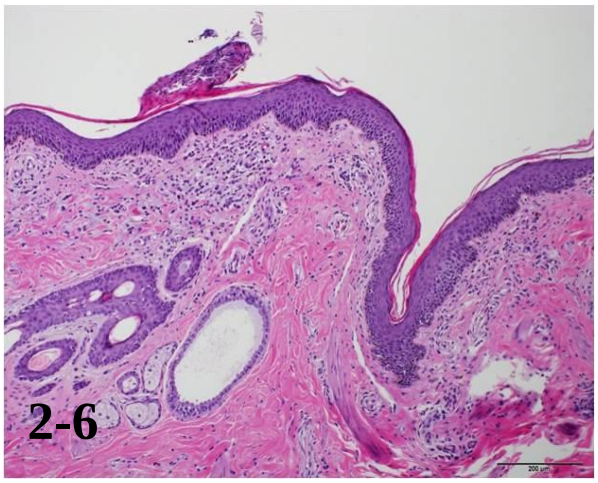


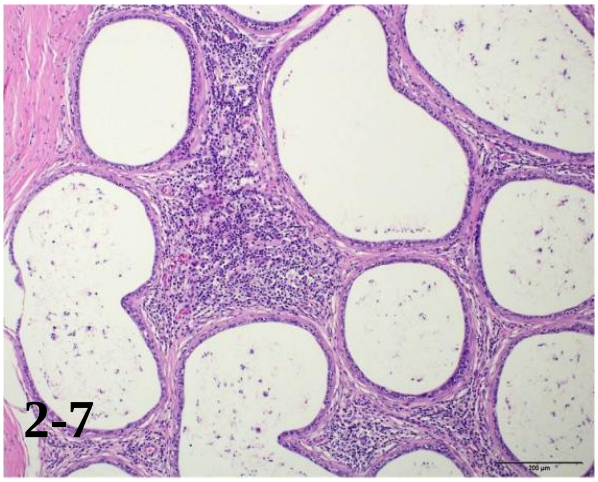


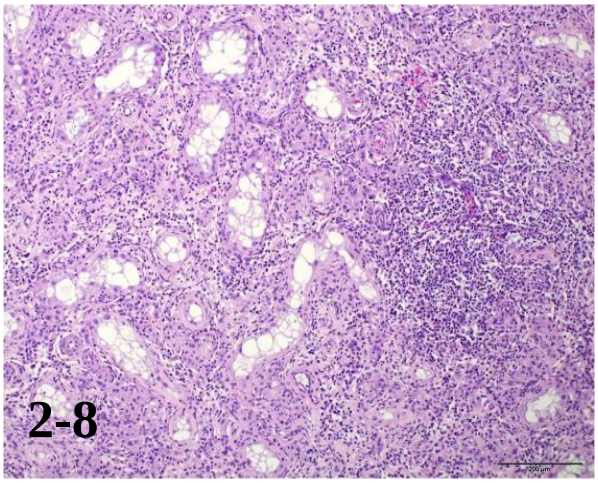


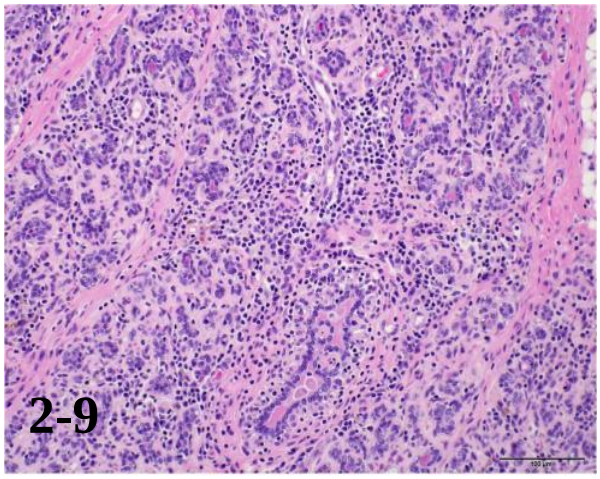


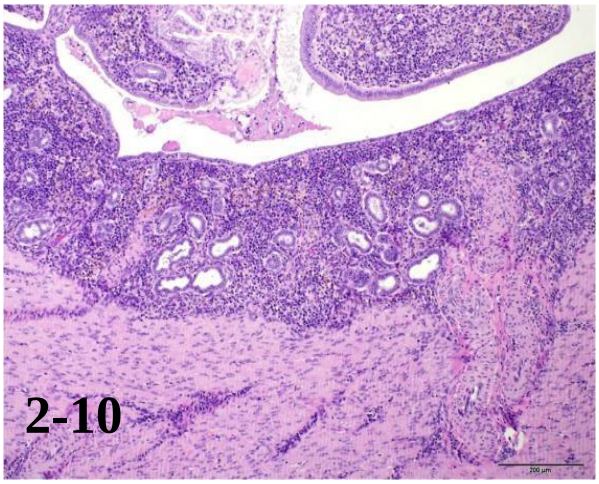


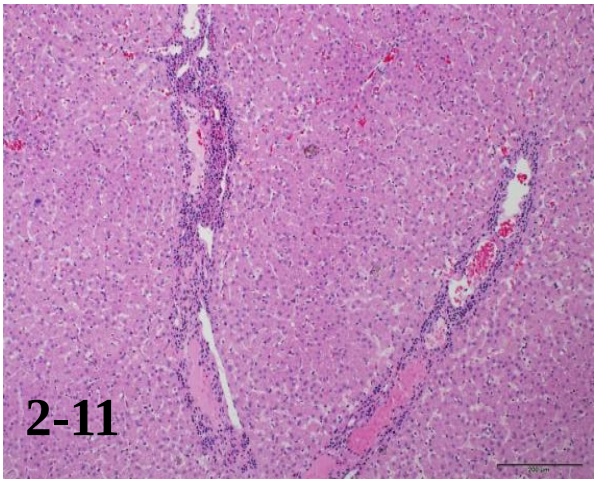


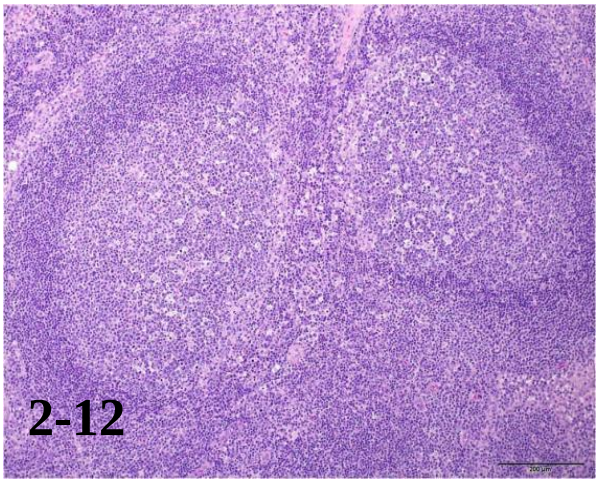


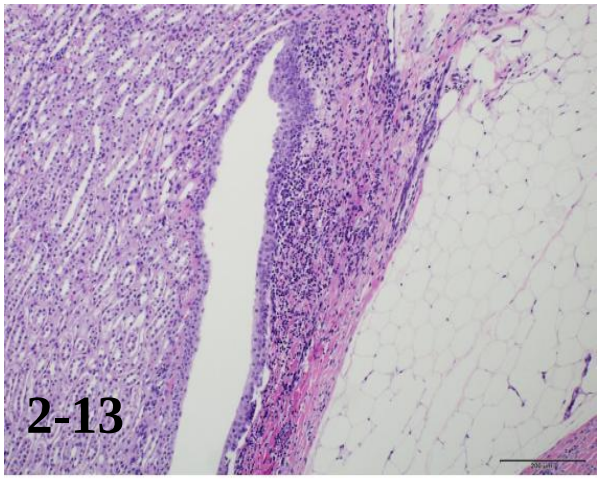


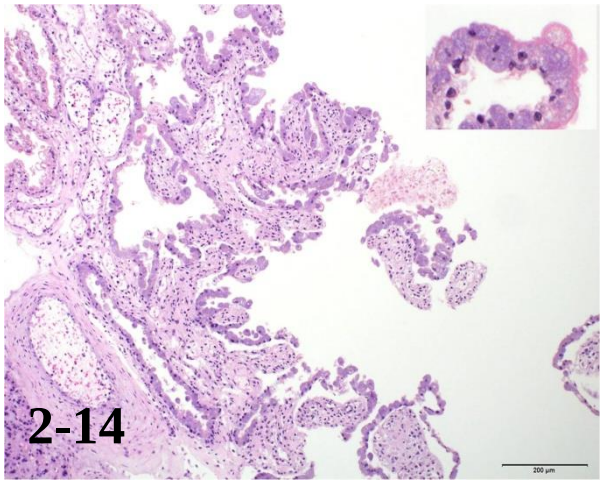


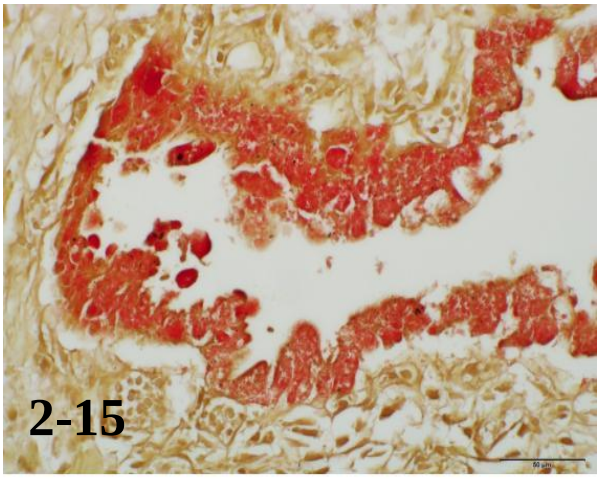


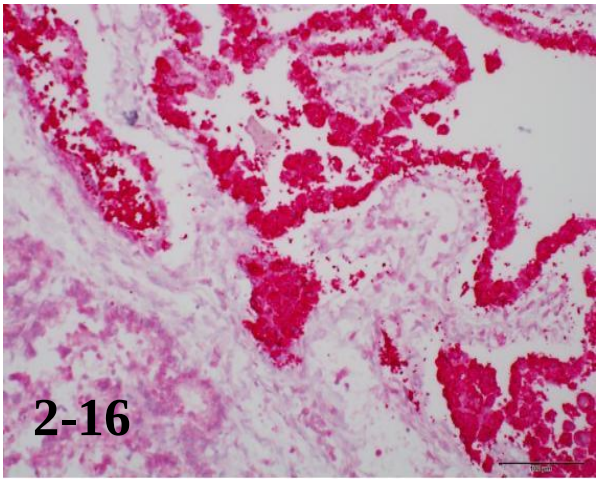












IV. DISCUSSION

This paper describes a comprehensive pathological, immunohistochemical, and bacteriological evaluation between dogs and fetuses naturally infected with *B. canis* and non-infected controls.

The specific clinical signs of canine brucellosis are reproductive failure and infertility. Aborted fetuses usually appear partially autolyzed with edema, congestion, and hemorrhage of the abdominal subcutaneous region and accumulation of serosanguinous peritoneal fluid [6]. Although several dogs were infected by *B. canis*, we could not find any typical clinical sings of canine brucellosis in this study. Most examined dogs were euthanized ones according to brucellosis control strategies in Korea. They might be euthanized before clinical signs were fully developed. We examined only two aborted fetuses in the present study. Most prominent lesions were focused in the placentas characterized by hypertrophic trophoblasts with numerous intra-cellular bacterial colonies.

In bovines, trophoblasts are thought to be the primary target cell for invasion and multiplication of *B. abortus* in the placenta because of the presence of erythritol, or hormone synthesis by trophoblast cells [14]. Erythritol is a preferred nutrient and growth stimulant for *Brucella* species [1]. *B. canis* is also sensitive to erythritol.

The most common gross lesions were lymphadenomegaly and splenomegaly in both sexes and testicular degeneration and ulcerative scrotal dermatitis in males, in accordance with previous reports [5, 9]. However, there were few abnormal features in females except one aborting bitch showing brownish vulvar discharge.

In the present study, lymphocytic or neutrophilic inflammations were observed in most internal organs of G1 group dogs. All genital organs such as testis, epididymis, prostate gland, and uterus showed multifocal to diffuse lymphocytic inflammation. Epididymitis and prostatitis in over 6 years old male dogs and lymphocytic endometritis between 2 to 6 years old female dogs are more prevalent in G1 than G2 dogs. Some dogs showed the swelling of lymph node and histopathologic lymphoid follicular hyperplasia were observed in 46.9% (23/49) dogs in G1 group. Swelling of lymph

node is the result of diffuse lymphoreticular hyperplasia [6]. In chronic cases, the spleen is filled with plasma cells and macrophages containing phagocytized bacteria. Mild lymphocytic interstitial nephritis also noted in dogs with *B. canis* infection [6]. About 49.0% (24/49) dogs in G1 group had lymphocytic interstitial nephritis lesions with variable degree. With the accordance of previous study [7], neutrophilic or lymphocytic hepatitis was more frequent in dogs of G1 than those in G2.

Comparing with young dogs, old dogs may have increasing chance to antigenic stimuli. Therefore, various inflammatory processes are more popular in old dogs than in young dogs. Most inflammation in animal organs can be induced by the injury or the infections including virus, bacteria, fungus, and others. We analyzed the pathological lesions in organs according to three different age groups (> 2 years, 2-6 years, ≤ 6 years). However, we could not find any relationships between dog's age and the severity of lesions in various organs. In addition, we do not perform other etiologic diagnosis except brucellosis. Although the pathological lesions of internal organs in G1 group dogs might be associated with *B. canis* infection, some lesions also presented in G2 group dogs. These mean that the more in depth further studies are needed to clarify the association between lymphocytic inflammation in various organs and *B. canis* infection.

We applied IHC to identify *B. canis* antigens in tissues using hyperimmune rabbit antiserum. There were several reports on the immunohistochemical results of *B. canis* infection using antiserum. Hofer *et al.* [8] demonstrated *B. canis* antigens in placenta and aborted fetal lung using IHC method [8]. Gyuranecz *et al.* [7] reported immunolabeling in an aborted placenta and in a few macrophages and giant cells in the tonsils and lymph nodes of adult dogs. In this study, the only but very strong positive result was obtained from an aborted placenta. The abortion of dogs in this study could be clearly attributed to *B. canis* infection, as large amounts antigens were detected in placenta. Unfortunately, we could not find any positive reactions for IHC in internal organs of dogs and fetuses.

B. canis isolates were recovered in tissues from 40 out of 49 adult dogs and 2 aborted fetuses. The bacterial isolation was most frequent in blood samples. Whole blood is considered as the best choice of sample for the isolation of *B. canis* because of the characteristic prolonged bacteremia. Moreover, blood is the easiest material for aseptic collection and easy handling without sacrifice of

dog [11]. In previous studies, the best organs for isolation, biopsies, or sampling at necropsy were the lymph nodes, prostate, spleen, and sometimes the liver and testes [5]. The positive culture results were similar to those in previous reports. In bitches, the uterus, placenta, and vaginal or uterine fluids were the most consistent tissues for bacterial isolation [7]. Isolation rates of *B. canis* are higher in lymphoid tissues such as lymph node and spleen than female and male genital organs in this study. *B. canis* were isolated more frequently in male genital organs including prostate gland and epididymis than in female uterus. But *B. canis* were also isolated from about 15.4% testis (2/13). Histopathologically, *B. canis* infected testis showed severe lymphohistiocytic orchitis with fibrosis and atrophy of seminiferous tubules. These pathologic findings may affect sperm abnormalities such as immature sperm, deformed acrosomes, and swollen midpieces [6].

In conclusion, although the clinical signs were not typical in the *Brucella*-infected group, the bacterial isolation rates were relatively high, and many dogs showed histopathological lesions in genital organs associated with *Brucella* infection. It is important that the periodic monitoring system in kennels should be confirmed for elimination of infected dogs and prevention of further disease transmission.

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CHAPTER Ⅲ

Long-term follow-up study of canine brucellosis

- Canine brucellosis positivity patterns at Korean breeding kennels

ABSTRACT

The first case of canine brucellosis in Korea was reported in 1984. Since then, several cases of the disease have been reported, mainly in breeding kennels. There was no any long-term follow-up investigation of canine brucellosis. The aim of this study was to analyze positivity patters of canine brucellosis at the breeding kennels after conducting control measures over one or two years tested period.

In the present study, a total of four kennels that were diagnosed as canine brucellosis by serological and/or bacteriological test were included. During the test periods, all *B. canis* infected dogs were euthanized, and other resident dogs were tested at 1 or 2 month interval according to brucellosis control strategies (test and stamping out policy) in Korea.

All kennels had clinical histories of continuous abortion and infertility after new entry of dogs from other kennels or auction house. Breeders did not perform any diagnostic test for *B. canis* to new additions to a kennel. Kennel 1 was tested six times and kennels 2, 3, and 4 were tested five times each for test periods. Initially, all kennels had positive rates for *B. canis* ranged from 19.91% to 34.21%. After conducting stamping out policy, *B. canis* positive rates gradually decreased and eventually recorded as 0 positive rates at kennels 2, 3, and 4. However, positive reactions were continuously observed for 38 weeks at kennel 1. Quarantine, test, and euthanasia of *B. canis* infected dogs are the best methods for the control and prevention of canine brucellosis. Because of zoonotic potential, dog breeders should be pay attention to handling *B. canis* infected dogs and diagnostic samples.

Key words: breeding kennel, canine brucellosis, control strategy, stamping out

I. INTRODUCTION

Infertility in dogs is a growing concern in breeding kennels [10]. There are many causes of infertility and abortion such as viral, bacterial, protozoal infection, and the environmental factors of the facility.

B. canis, a rough species of the genus *Brucella*, causes canine brucellosis characterized by abortions in females and testicular atrophy, epididymitis, prostatitis, and infertility in males [2]. Canine brucellosis is one of the most important bacterial disease associated with reproductive failure and infertility in breeding kennels. Serological test before entry and breeding of new dogs will reduce the incidence of canine brucellosis in many breeds [6].

Bovine brucellosis, caused by *B. abortus*, is one of the most common zoonotic diseases in Korea [13]. However, the enforcement of brucellosis control measures (test and slaughter) has led to a reduction in the incidence of *B. abortus* in cattle. In some countries, the control program relies on vaccination with attenuated live strains [12]. However, cattle have not been vaccinated against *B. abortus* in Korea. Unlike bovine brucellosis, there is no effective vaccine against *B. canis* in worldwide until today, and the results of experimental studies have been unsatisfactory [4].

The first case of *B. canis* infection in dogs was published in 1984 in Korea. Since then, many cases of canine brucellosis have been reported, mainly in breeding kennels [1, 9, 11].

Despite being endemic, little is known about the epidemiology of brucellosis at the breeding kennels in Korea. Furthermore, prevalence changes on kennels with canine brucellosis outbreaks remain underestimated.

The aim of this study was to analyze positivity patters of canine brucellosis at the breeding kennels after conducting control measures over 1 or 2 year tested period.

II. MATERIALS AND METHODS

Tested kennels

Four outbreaks of canine brucellosis were observed in breeding kennels at different regions in Korea. All kennels had histories of repeated abortion and decreased fertility. Therefore, the owners took diagnostic samples such as aborted fetuses and blood from aborting bitches to a local veterinary service to determine the cause of abortion. All kennels were diagnosed as canine brucellosis by serological and/or bacteriological test.

According to brucellosis control strategies (test and stamping out policy) in Korea, infected dogs are euthanized, and serological and/or bacteriological tests were performed to all resident dogs in the kennel. This step was repeated every 1 or 2 month. All kennels were tested for 1 or 2 year period.

In this study, the number of tested dogs has appeared differently due to the death associated with other disease and the new birth of puppies during the experimental period.

The following data were collected from each breeding kennel: the total number of dogs, and clinical histories. Information about movement to or from other kennels and contact with other animals was also included.

Sample preparation

Samples of whole blood were obtained from every resident animal at a 1 or 2 month interval and submitted to Animal Disease Diagnostic Division, Animal and Plant Quarantine Agency for laboratory examination.

Approximately 2-3 mL of whole blood was collected from dogs using heparin as an anticoagulant for serological test and bacterial culture. And then, blood was centrifuged at 4℃, at 3,000 rpm for 30 minutes. The plasma was collected from the tube, inactivated at 56℃ for 30 minutes, and stored at –20℃ before use.

Serological test

Plasma samples were analyzed using an immunochromatographic test (ICT) for canine brucellosis (BioNote Inc., Hwaseong, Korea), according to the manufacturer's instructions. For serological test, serum from dogs previously infected with *B. canis* was used as the positive control.

Blood culture and bacterial identification

After separating plasma from whole blood, buffy coat was used for bacterial isolation. The buffy coat layer was inoculated in tryptose phosphate agar (TSA) (BD, Franklin Lakes, NJ) containing 5% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and incubated at 37℃ under aerobic conditions for 3-5 days. Suspected colonies were selected and purified by cultivation on 5% sheep blood agar for 2-4 days at 37℃.

For bacterial species identification, genomic DNA was extracted from the isolates using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The identification of the isolates was confirmed using the novel Bruce-ladder multiplex PCR assay [8].

III. RESULTS

Collected information from four kennels with canine brucellosis is summarized in Table 3-1.

All kennels had clinical histories of continuous abortion and infertility after new entry of dogs from other kennels. The owners experienced economic losses for several years because of reproductive disorders.

Control or preventive program against canine brucellosis had not been conducted at these four kennels. Breeders did not perform any diagnostic test for *B. canis* to new additions to a kennel. Furthermore, female and male dogs were also not tested before mating.

After confirming canine brucellosis, all dogs in four kennels were tested serologically and/or bacteriologically every 1 or 2 months for 1-2 years periods according to Korean control strategies against brucellosis. In this study, *Brucella* infection was determined if the results of one or both tests were positive. The positivity pattern of four kennels is shown in Fig. 3-1.

Table 3-1. Collected information from four kennels with canine brucellosis

Kennel	Total number of dogs	Location	Clinical history	Time of occurrence of abortion	No. dogs with positive ICT or blood culture (%)
1	94	Chungnam	Abortion	After entry of a bitch from another kennel (2014)	18 (19.1)
2	158	Chungbuk	Abortion	After entry of a bitch from another kennel (2013)	41 (25.9)
3	167	Chungbuk	Abortion	After entry of dogs from another kennel/ or animal auction house (2016)	55 (32.9)
4	114	Gyeongbuk	Abortion	After abortion of recently imported pregnant female (2016)	39 (34.2)

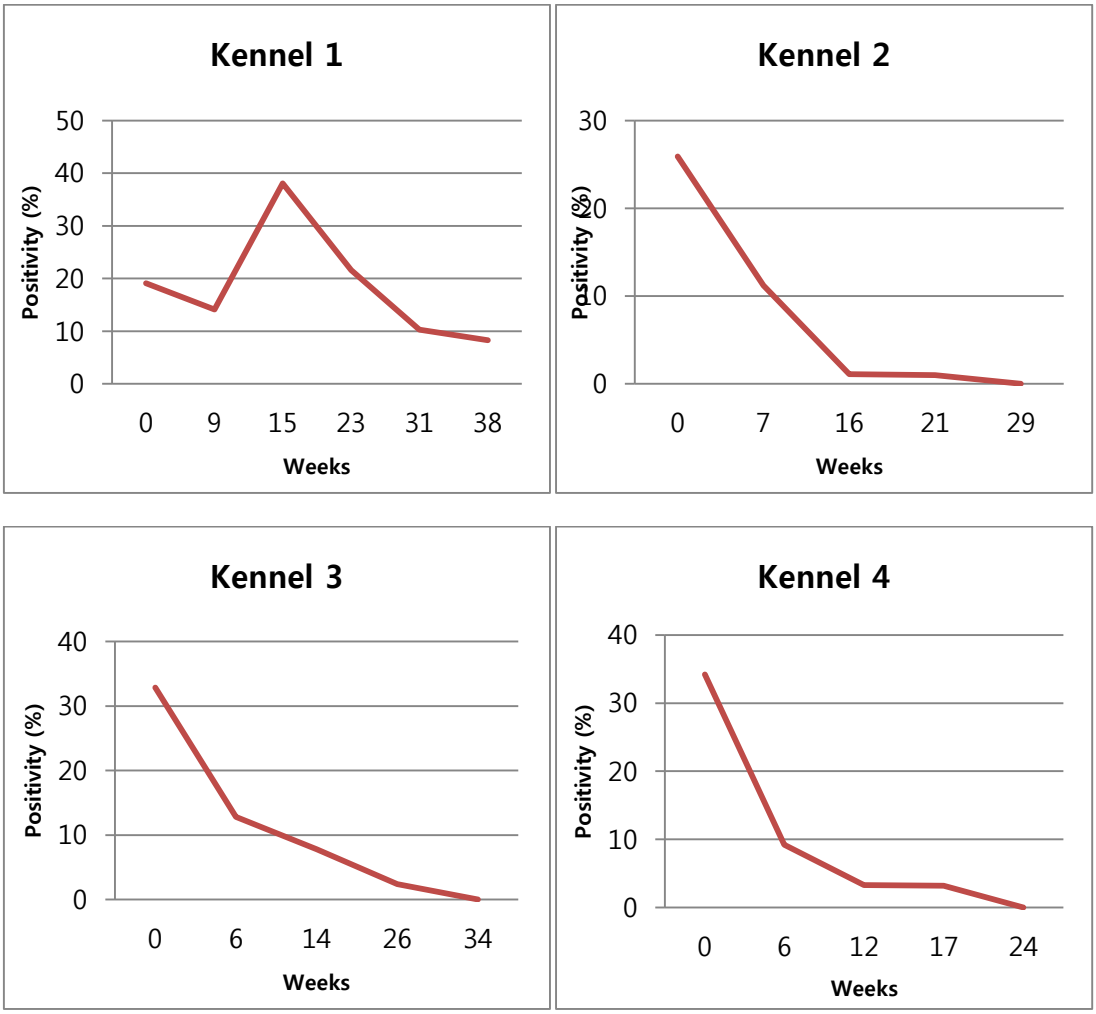

Fig. 3-1. Canine brucellosis positivity patterns of four kennels by serological and bacteriological tests

Kennel 1 was tested six times at 0, 9, 15, 23, 31, and 38 weeks (Table 3-2). At the beginning of the test, 18 out of 94 (19.1%) dogs were positive for *B. canis* and these 18 dogs were euthanized. Nine weeks later, 11 out of 78 (14.1%) dogs showed newly positive reactions. Additional euthanasia also carried out for 11 dogs. However, 32 out of 84 (38.1%) dogs also confirmed as positive and then euthanized. Although stamping out policy was performed in the kennel, positive reactions were continuously observed for 38 weeks periods. All dogs kept at kennel 1 were finally eliminated.

Table 3-2. Results of serological test and bacterial culture for *Brucella canis* at kennel 1

Weeks[a]	ICT[b]		Bacterial culture		Total (%)
	No. tested	No. positive	No. tested	No. positive	
0	94	18	NT[c]	-	18/94 (19.1)
9	78	11	NT	-	11/78 (14.1)
15	84	28	84	25 (4[d])	32/84 (38.1)
23	37	7	37	6 (1[d])	8/37 (21.6)
31	29	2	29	2 (1[d])	3/29 (10.3)
38	24	2	24	1	2/24 (8.3)

[a] Weeks after initiation of test

[b] Immunochromatographic test

[c] Not tested

[d] ICT negative and bacterial culture positive

Kennel 2 was tested five times at 0, 7, 16, 21, and 29 weeks (Table 3-3). Initially, 41 out of 158 (25.9%) dogs were positive for *B. canis* at kennel 2. Kennels 3 and 4 were also tested five times for 34 and 24 weeks, respectively (Table 3-4, 3-5). A total 55 dogs out of 167 (32.9%) dogs at kennel 3 and 39 out of 114 (34.2%) dogs were positive for *B. canis* at the beginning. After conducting stamping out policy, *B. canis* positive rates gradually decreased and eventually recorded as 0 positive rate at the fifth test at kennels 2, 3, and 4.

Table 3-3. Results of serological test and bacterial culture for *Brucella canis* at kennel 2

Weeks[a]	ICT[b]		Bacterial culture		Total (%)
	No. tested	No. positive	No. tested	No. positive	
0	158	41	41	4	41/158 (25.9)
7	107	12	NT[c]	-	12/107 (11.2)
16	94	1	NT	-	1/94 (1.1)
21	98	1	NT	-	1/98 (1.0)
29	32	0	NT	-	0/32 (0.0)

[a] Weeks after initiation of testing

[b] Immunochromatographic test

[c] Not tested

Table 3-4. Results of serological test and bacterial culture for *Brucella canis* at kennel 3

Weeks[a]	ICT[b]		Bacterial culture		Total (%)
	No. tested	No. positive	No. tested	No. positive	
0	167	55	NT[c]	-	55/167 (32.9)
6	117	15	5	5	15/117 (12.8)
14	102	8	NT	-	8/102 (7.8)
26	82	2	4	0	2/82 (2.4)
34	71	0	NT	-	0/71 (0.0)

[a] Weeks after initiation of testing

[b] Immunochromatographic test

[c] Not tested

Table 3-5. Results of serological test and bacterial culture for *Brucella canis* at kennel 4

Weeks[a]	ICT[b]		Bacterial culture		Total (%)
	No. tested	No. positive	No. tested	No. positive	
0	114	39	NT[c]	-	39/114 (34.2)
6	76	7	NT	-	7/76 (9.2)
12	61	2	NT	-	2/61 (3.3)
17	63	2	5	2	2/63 (3.2)
24	62	0	1	0	0/62 (0.0)

[a] Weeks after initiation of testing

[b] Immunochromatographic test

[c] Not tested

IV. DISCUSSION

In Korea, outbreaks of canine brucellosis have been reported mostly in breeding kennels. Moon *et al.* reported that 33 out of 62 (53.2%) dogs were seropositive for *B. canis*, and 20 isolates from 33 dogs confirmed as *B. canis* in a large kennel in the Chonnam area [11]. More recently, Kim *et al.* reported that 45 out of 138 (32.6%) samples were seropositive for *B. canis*, and 30 *B. canis* were isolated in breeding kennels that were suffered from frequent outbreaks of abortion in the Gyeongbuk province [9]. However, there was no continuous follow-up study in the breeding kennels applied disease control program in Korea. In the present study, we examined four kennels with canine brucellosis outbreaks for one or two years after treating brucellosis control strategy. Test and stamping out policy were applied in these kennels.

All kennels tested in this study experienced a reproductive problem of abortion after arriving of new dogs from other kennels. Unfortunately, the breeders did not examine any diagnostic test for *B. canis* to new coming dogs. Hence, the sources of infection might be new additions from *B. canis* positive kennels or auction houses. Quarantine, test and removing are particularly important to prevent financial loss from *B. canis* infection in kennels [4]. It is recommended that all dogs for new entry in kennels should be examined and quarantined for 8 to 12 weeks [6].

To obtain positivity patterns, all dogs in four kennels were examined every 1 or 2 months over 1-2 years using ICT and bacterial culture test. In the middle of time, *B. canis* infected dogs were eliminated.

The positivity rates at kennel 2, 3 and 4 gradually declined after performing test and stamping out policy. It took very long time, 24 to 34 weeks, to establish brucellosis free status in kennel 2, 3 and 4. However, *Brucella*-positive dogs were still found during the test, even though infected dogs were removed from the kennels. Occasionally, several dogs with

previous *B. canis* negative showed positive reaction at next test. This might be related with the longstanding bacteremic condition of *B. canis* infected dogs. In general, bacteremia starts 4 to 6 weeks after oronasal exposure, and the dogs may remain bacteremic for 1 to 5 years [6]. Meanwhile, seroconversion starts 8 to 12 weeks after initial exposure [10].

Even though stamping out policy, *B. canis* positive rate was sharply increased at third test (15 weeks, 38.1%) and continued for 38 weeks in kennel 1. These abnormal serologic results imply that continuous exposure of *B. canis* would be occurred between dogs in the kennel. According to owner's description, some bitches with previous abortion were not handled properly. They had been kept in a separate space in kennel, and they were ruled out at the first round of test. Aborting bitches play a high risk factor of *B. canis* spreading in a breeding kennels. Infected bitches can transmit *B. canis* during estrus, breeding, or after abortion through oronasal contact with vaginal discharge. Furthermore, seminal fluid and urine have been implicated as sources of infection from males that harbor organisms in their prostate and epididymis. Cages, equipment, and people in contact with infected dogs might also be sources of infection. Given the ease of transmission by high density of animals in breeding kennels, appropriate disinfection procedures and preventive measures are particularly important [4].

In Korea, canine brucellosis is designated legally as a communicable disease. According to the "Act on the Prevention of Livestock Epidemics" in Korea, euthanasia is recommended for brucellosis outbreaks.

Some studies have reported on the treatment of canine brucellosis [3, 4, 6, 7]. However, *Brucella* spp. undergoes intracellular replication and is difficult to eliminate with antibiotic therapy. Moreover, several studies indicated that the use of a single antibiotic was not sufficient, and thus combined antibiotics and neutralization are recommended for treating brucellosis. However, with the possibility of relapse (even after neutering or spaying), the best treatment is removal dogs from the facility or euthanasia [6]. Quarantine, test, and

euthanasia of *B. canis* infected dogs are the primary methods necessary to eliminate the spread of disease in a commercial breeding facility [12].

The control of canine brucellosis in a kennel is very difficult and time consuming. Dog breeders should consider preventive measures against canine brucellosis and be careful to handle *B. canis* infected dog and diagnostic samples.

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국문초록

국내 개브루셀라병의 발생상황 조사 및 병리학적 연구

정지열

(지도교수 김재훈)

제주대학교 대학원 수의학과 수의병리학 전공

개브루셀라병은 *Brucella canis* 가 원인체로 번식장애가 주 증상인 제2종 가축전염병 및 인수공통전염병이다. 국내 반려견 사육규모가 커지면서 공중보건학상 개브루셀라병 관리에 대한 중요성이 대두되고 있으나 국내에서는 본 질병에 대한 조사가 미흡한 실정이다. 따라서 본 연구에서는 개브루셀라병에 대한 국내 발생상황 조사 및 병리학적 연구를 수행하였고, 발생농장에 대한 추적조사 연구를 실시하였다.

개브루셀라병의 발생상황 조사는 2015부터 2016년까지 2년에 걸쳐 반려견과 유기견 총 2,427두를 대상으로 혈액 또는 혈청을 수집하여 항체검사와 세균분리검사를 수행하였다. 반려견은 총 1,852두에 대해 항체검사를 실시한 결과 17(0.9%)두에서 양성을 보였으며, 유기견은 총 575두 중 14(2.4%)두에서 양성을 보였다. 또한, 유기견 192두 중 2두에서 세균이 분리되어 1.0%의 분리율을 보였다. 각 그룹의 결과에 대해 성별, 연령별, 지역별로 구분하여 분석한 결과 두 그룹 모두 암컷에서 유의성 있게 유병률이 높게 나타났으며

유기견의 경우 6세 이상에서 유의성 있게 높게 나타났다. 그러나 지역별로는 유의성있는 결과를 얻지 못했다.

본 연구를 수행하는 동안 4개의 번식견 농장에서 개브루셀라병이 발생하였다. 이 4개 농장은 모두 유산과 번식장애로 문제가 있었으며 항체 및 항원검사결과 개브루셀라병으로 진단된 이후 「가축전염병예방법」에 따라 양성인 개체들은 안락사를 실시하였다. 병리학적 검사를 위해 개브루셀라병 양성그룹 성견 49두 (암컷 35두, 수컷 14두), 유산태아 2두와, 음성그룹 성견 21두 (암컷 15두, 수컷 6두), 태아 15두로 구분하여 검사를 실시하였다.

개브루셀라병 양성인 그룹에서 가장 빈번히 관찰되는 육안병변은 림프절의 종대였으며 수컷에서는 고환 발적 및 종대, 유산한 모견에서는 태반의 황변화가 관찰되었다. 병리조직학적 검사결과결과 수컷 생식기 장기인 고환, 부고환, 전립선 등에서 림프구성 염증이 양성그룹에서 높게 나타났으며, 암컷 생식기 장기인 자궁에서도 림프구성 염증 병변이 양성그룹에서 다수 관찰되었다. 유산한 모견의 태반에 대한 검사결과 영양막세포의 세포질에서 다수의 세균이 관찰되었으며 이에 대한 면역조직화학염색 결과 *B. canis* 로 확인되었다.

또한, 장기별 세균분리율을 알아보기 위해 실질장기를 대상으로 세균분리를 시도한 결과 암컷과 수컷 모두 전혈 (67.3%), 서혜부 표층 림프절 (63.3%), 비장 (55.1%)에서 세균분리율이 높았고 수컷의 경우 전립선 (42.9%)과 부고환 (38.5%)에서도 분리율이 높아 수컷이 정액 또는 오줌을 통해 암컷으로 세균을 전파시킬 가능성이 높으며 병원체의 매개체로써 중요한 역할을 할 수 있기 때문에 보다 철저한 관리가 필요할 것으로 생각된다.

우리나라에서는 1984년 *B. canis* 가 처음 보고된 이후 번식농장을 중심으로 발생하고 있다. 앞서 기술한 개브루셀라병 발생농장 4곳에 대해

방역조치를 취하면서 주기적인 검사를 통해 양성률을 조사하였다.

최초 검사시 양성률은 19.91-34.21%로 나타났고 4개 중 3개 농장은 검사를 실시할 때마다 양성률이 감소하여 5회째 검사에서는 모두 음성으로 확인되었다. 그러나 1개 농장에서는 6회째 검사에서도 음성인 결과를 얻지 못하여 결국 전두수 안락사를 실시하였다.

본 연구를 통해 개브루셀라병의 유병률은 비교적 낮지만 국내 반려견과 유기견에서 개브루셀라병이 발생하고 있음이 확인되었다. 국내 반려견 산업이 성장하고 있으며 공중보건학적으로 중요성을 가지고 있기 때문에 지속적인 관심이 필요할 것으로 생각된다. 또한 번식견에서는 보다 높은 유병률이 확인되었고 번식농장에서 개브루셀라병이 한번 발생하면 경제적 손실이 심하고 청정화되기까지 시간이 오래 걸리기 때문에 주기적인 검사를 통해 양성인 개체를 색출하는 것이 중요하며 새로 입식하는 개체에 대해서도 입식 전 검사를 통해 질병의 전파를 막는 것이 필요할 것이다.

주요어: 개, 개브루셀라병, 면역조직화학염색, *Brucella canis*, 세균분리, 항체검사