NOTE   *Public Health*

Evaluation of a Microplate Agglutination Test (MAT) for Serological Diagnosis of Canine Brucellosis

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ABSTRACT. A microplate agglutination test (MAT) was compared with the tube agglutinin test (TAT), a standard test for the diagnosis of *Brucella canis*, in terms of the sensitivity and specificity. The results showed that MAT was more sensitive, simpler to perform and easier to read the results than TAT. On top of that the MAT allows us to handle a larger number of samples at once. Using this method we conducted sero-surveillance of the prevalence of *B. canis* in dogs kept in an Animal Shelter located in Kanagawa Prefecture. Twelve of 485 (2.5%) showed seropositive against *B. canis*. These results indicate that *B. canis* infection in dogs is still occurring in Japan.
KEY WORDS: *Brucella canis*, canine brucellosis, microplate agglutination test.

Brucellosis, one of the major zoonoses worldwide, is caused by a bacteria belonging to the genus *Brucella* [4]. Among many species of the genus *Brucella, B. melitensis, B. abortus, B. suis* and *B. canis* are known to result in human brucellosis. Although *Brucella* spp. with smooth-type lipopolysaccharides (LPS), such as *B. melitensis, B. abortus* and *B. suis*, are known to infect several domestic animals, such as cows, sheep, goats and pigs, *B. canis*, one of *Brucella* spp. with rough LPS, infects a limited host range, such as dogs and wild canidae. *B. canis* infection in dogs is usually asymptomatic but can sometimes cause contagious abortion, epididymitis, testicular atrophy and infertility [3]. Most canine infections occur by direct contact with lochia at the time of abortion or vaginal discharges in infected female dogs. Semen and urine from infected male dogs have also been implicated as sources of infection [7]. Drug therapy for *B. canis* infection requires an appropriate regimen of antibiotic combination, but relapse may ensue, because *B. canis* often persists within macrophages or other type of cells [3]. Humans are rarely infected with *B. canis*. Most human infections are asymptomatic; however, several clinical symptoms, which are milder than those observed with other *Brucella* spp., are sometimes noticed [12].

In Japan, *B. canis* infection was first reported in a breeding colony of beagles in 1972 [20]. Several epidemiological studies of canine brucellosis in Japan were conducted in the 1970s and 1980s [10, 11, 15–18], but there have only been a few reports since then. In 2003 and 2006, canine brucellosis emerged as outbreaks in large breeding colonies, suggesting that *B. canis* infection is still enzootic in Japan. To assess the possible risk of *B. canis* on human, determination of the prevalence of *B. canis* in the dog population in Japan seemed helpful.

Although tube agglutination test (TAT) is the most widely used laboratory test for the detection of *B. canis* anti-

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bodies in both humans and canines, it is time-consuming and cumbersome in terms of performance and measurement of results [2]. On the other hand, microplate agglutination test (MAT) described for *B. canis* [5] and *B. abortus* [1, 6] appeared advantageous, because a larger number of samples can be processed simultaneously by this method. In the present study, we attempted to evaluate whether the use of MAT with safranine-stained bacterial cells as antigens could serve as a substitute for TAT to conduct sero-epidemiologic investigations of canine brucellosis in Japan.

TAT was carried out by placing 0.5 ml of 2-fold serially diluted sera and an equal volume of *B. canis* antigen solution (OD_{600}=1) purchased from the Kitasato Institute (Tokyo, Japan) in glass tubes. After incubation at 50°C for 24 hr, the agglutination titer was determined and expressed as a reciprocal of final serum dilutions, which gave rise to agglutination as observed in the 50% control tube. Titers of 160 or higher were considered positive. Anti-*B. canis* antibody was prepared in our laboratory by immunizing a rabbit with inactivated *B. canis* whole antigen and was included as a reference.

MAT was performed as follows. First, serum samples, 2-fold serially diluted in phosphate-buffered saline, were prepared in a 96-well U-bottom microplate. Then, an equal volume (25 μl) of *B. canis* antigen solution (Kitasato Institute), which is same as used in TAT, containing 0.005% safranine solution (2% of Favor G®, Nissui Pharmaceutical Co., Tokyo, Japan) was added to each well. The sealed plates were mixed gently for 20 sec and incubated at 50°C for 24 hr in a humid atmosphere. The titers were expressed as a reciprocal of the highest dilution of sera showing agglutination. Safranine-stained antigens made it possible to judge the results more easily and objectively. An agglutination titer greater than 160 was considered positive.

We have experienced an outbreak of *B. canis* infection in 2003 [8]. Sera obtained from dogs involved in the outbreak were examined for the presence of anti-*Brucella* antibody by TAT. Fifty-one of 110 sera tested positive for antibody against *B. canis*. These sera were subjected to MAT for
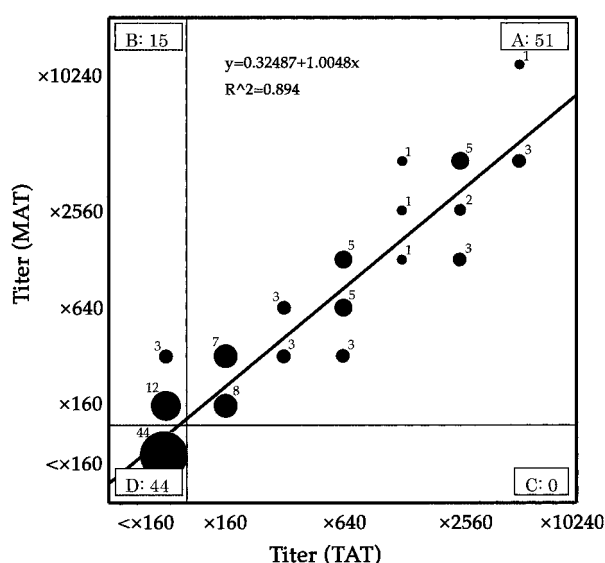
Fig. 1. Correlation of antibody titers determined by TAT and MAT. Sera were obtained from 110 dogs in the outbreak of *B. canis* infection in 2003. The titers are the reciprocals of the highest dilution of serum showing agglutination. Pearson's product-moment correlation coefficient (r) was determined to be 0.945, p<0.01 by regression analysis. A-D shows each area and the number of dogs of each area.

determining its specificity and sensitivity. As shown in Fig. 1, 15 sero-negative samples (1:80) by TAT became positive (1:160 and 1:320) when MAT was employed. Combinatorial polymerase chain reaction method [9] showed that *B. canis*-specific gene segments were present in the sera of those 15 dogs [8], which indicate that they were infected with *B. canis* (data not shown). Therefore, MAT appeared superior to TAT in terms of sensitivity as shown in the previous report by Dump *et al.* [5]. The titers determined by two methods correlated well ($R^2 = 0.894$) as shown in Fig. 1.

We have therefore decided to apply MAT in the sero-surveillance of *B. canis* in Japan. During the period from February 2003 to December 2006, 485 serum samples were obtained from dogs in an animal shelter in Kanagawa Prefecture (Table 1). The dogs were categorized into three groups according to their origin: pets, strays, and unknown.

The results are summarized in Table 1. Of 485 dogs, 12 (2.5%) tested positive for antibody against *B. canis*. Sero-

prevalence in this study (2.5%) seemed slightly lower than those of 1970's (0.8%–21.7%) [10], but there was no apparent difference. This indicated that the disease was enzootic but not epizootic in Japan. Of the 12 sero-positive dogs, 3 were pets and 9 were strays. The reason why the apparent prevalence of infection was higher in strays (5.7%) than in pets (0.9%) was unknown, but it was likely that stray dogs had more opportunities to encounter other dogs, fomites or environments contaminated by bacteria. No differences attributed to the sex (Table 1) or breed (data not shown) of dogs were observed in the prevalence of infection.

Although symptomatic infections of *B. canis* in humans are rarely reported, Lucero *et al.* [12] pointed out that the possibility of *B. canis* infection in humans may be more widespread than speculated. Recently, an unusually severe form of human brucellosis caused by *B. canis* was reported [13]. Because human infections commonly occur after contact with the blood, semen, or placenta of infected dogs [19], an understanding of the prevalence of *B. canis* infection in dogs may help in inferring preventive measures for reducing human exposure to the bacteria. The results of the present sero-epidemiologic study showed that *B. canis* infection is still enzootic in Japan. Moreover, human brucellosis cases reported in 2002, 2005 and 2006 were probably caused by *B. canis* [14]. It therefore seems, prudent that individuals at high risk of infection such as veterinarians, breeders and pet owners, be advised of possible *B. canis* infection in dogs.

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Table 1. Sero-prevalence of antibodies against *B. canis* in dogs determined by MAT

Year	Male		Female		Total	
	Negative	Positive	Negative	Positive	Negative	Positive
2003	67	0	24	1	91	1
2004	82	3	38	2	120	5
2005	103	3	74	1	175	4
2006	52	2	29	0	81	2
Total	304	8 (2.6%)	165	4 (2.4%)	467	12 (2.5%)

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