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Smooth and Rough Brucella Infections in Dogs

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ABSTRACT

Background: *B. canis* infections have been reported in many countries of the world. *B. canis* can cause infections both in dogs and human beings. It can be transmitted to human *via* laboratory accidents during handling and contact with sick dogs. is an infectious agent for humans and it is transmitted to humans by various ways including laboratory accidents and contact with sick dogs.

Methods: In this study serum samples were collected by using stratified random sampling method from the Diyarbakır Metropolitan Municipality Animal Care and Rehabilitation Center, where approximately 600 dogs were housed.

Result: Out of 83 samples**,** 9.6% and 26.5% of serum samples were found as positive by *B.canis* RSAT and B.*canis* ELISA, respectively. While 6% and 9.6% of the samples were positive by *B. abortus* S99 RSAT and *B. abortus* ELISA, respectively. Sensitivity and specificity of *B. abortus* S99 ELISA was 98% and 95.5%, respectively. On the other hand, sensitivity and specificity of *B.canis M* (-) ELISA was 96.6% and 94%. As a result, it was concluded that *B. canis* infection is common in dogs and this should be considered both for dogs and public health. Unfortunately, there is no standardized commercial serological test for diagnosis of *B.canis* and other rough Brucella species currently. This is the main reason that rough brucella infections might not represent the real *B.canis* infection situation. Therefore, most of the cases have the possibility of being undetected. For this reason it is utmost important that *B.canis* RSAT and ELISA can be used as routine tests in the hospitals and veterinary clinics in the serological diagnosis of brucellosis.

Key words: *B. canis*, Dog, ELISA, Infection, RSAT.

INTRODUCTION

Dog brucellosis caused by *Brucella canis* was first described by Carmicheal in greyhound colonies in United States (USA) in 1966 (Carmichael and Joubert, 1987). It is an important cause of abortion and infertility in dogs worldwide. *B. canis* infections have been reported in many countries of the world (Carmichael *et al.,* 1970; Carmichael, 1990; Lucero *et al.,* 2002; Wanke, 2004). Dog brucellosis is also caused by other brucella species such as *B. melitensis* and *B. abortus*, which are both smooth strains. *B. canis,* which is a rough strain, is also an infectious agent for humans and it is transmitted to humans by various ways including laboratory accidents and contact with sick dogs. Smooth strains of Brucellae have lipopolysaccharide (LPS) in their cell wall while rough strains devoid of this molecule but rough LPS. Therefore, antibodies against *B. canis* do not react with antibodies produced against smooth strains. For this reason we aimed to detect seropositivity of dog brucellosis caused by smooth and rough species. The cause of under-reporting of *B.canis* incidence in human is due to absence of standardized tests (Currier *et al.,* 1982; Lopez *et al*., 2005; Lucero *et al.,* 2002; Lucero *et al*., 2005). Previous evidence of *B. canis* infections in Turkey was obtained from the couple of reports regarding to serological evidence of *B. canis* infections in dogs in Turkey. In a study carried out in 1983, 2-mercaptoethanol tube agglutination test (ME-TAT) was used to test 134 healthy dog serum samples in Ankara and a 6.7% seropositivity rate was detected (Diker *et al.*, 1983) and a similar study of 1987 on22 canine serum samples, researchers obtained similar results (Diker *et al.*, 1987). In another study, seropositivity rate of 12.7%, 7.73% and 7.45% was obtained by TAT, METAT and ELISA, respectively on 362 serum samples in İzmir and Istanbul provinces (Oncel *et al.,* 2005). However, studies on this subject are very few and there is not enough data to reveal the current status of the disease in dogs in our country. In addition, there is no report for dog brucellosis survey caused by smooth Brucella species in last decade. Diagnosis of the disease is based on bacteriological and serological tests. The isolation of the agent is time consuming and required skilled personel. Therefore, the diagnosis of canine brucellosis is largely based on serological tests (Badakhsh *et al.,* 1982; Carmichael, 1990; Carmichael and Joubert, 1987; Lopez *et al.,* 2005; Lucero *et al.,* 2002). Tests used in the serological diagnosis of the disease are rapid slide agglutination test (RSAT; RBPT) (George and Carmichael, 1974; George and Carmichael, 1978), Tube agglutination test (TAT), 2- mercaptoethanol TAT (2ME-TAT), agar gelimmunodiffusion test (AGID), microplate agglutination (MAT) (Kimura *et al.,* 2008) fluorescent antibody test (IFA) and ELISA (Alton *et al.,* 1988; Carmichael, 1990; Currier *et al.,* 1982). Although AGID test is highly sensitive, detection of

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false positives, the inability to detect early cases and the difficult interpretation of the formed precipitate lines are among the disadvantages of the test (Alton *et al.,* 1988). RSAT is the most widely used screening test among veterinary clinics in the USA (Carmichael, 1990). Although it is common and practical test, large number of false positive cases are detected by RSAT (Alton *et al.,* 1988; Carmichael, 1990; Mateu-De-Antonio *et al.,* 1994). In this study, an "in house" ELISA was used to detect anti brucellae antibodies in serum samples taken from the dogs in Diyarbakır Metropolitan Municipality Animal Care and Rehabilitation Center. Smooth and rough lipopolysaccharide antigens were used as antigens in ELISA.

MATERIALS AND METHODS

Bacterial strains

B. canis M (-) and *B. abortus* S99 strains and positive antisera for Smooth and rough strains were obtained from Harran University, Faculty of Veterinary Medicine, Department of Microbiology.

Reference and test sera

Serum samples were collected by using stratified random sampling method from the Diyarbakir Metropolitan Municipality Animal Care and Rehabilitation Center, where approximately 600 dogs were located. Positive and negative control sera for *B.abortus* and *B.canis* were obtained from OIE Brucellosis Reference Laboratory. Weybridge, England. The equation used to collect samples was:

$$n=\frac{Z2\times N\times P\times Q}{N\times D2+Z2\times P\times Q}$$

Here:

N= Sample size.

Z= Confidence coefficient (this coefficient is taken 1.96 for 95% confidence).

N= Main mass size.

P= The probability that the feature we want to measure is in the main mass (This ratio is taken as 50% because the study is multi-purpose).

Q= 1-P.

D= Accepted sampling error (a 10% sampling error was received for the study). According to this formula, the number of samples to be collected was determined as 83.

Antigen production

Raw LPS isolation was made from the test strains according to the methods described earlier (Yi and Hackett, 2000) with minor modifications. Briefly, *B. canis* M (-) and *B. abortus* S99 strains were cultured in tryptic soy agar supplemented with 5% foetal calf serum, harvested after 72 hours of incubation and inactivated by heat (one hour, 56°C). Inactivated bacterial suspensions were washed two times by PBS and centrifuged at 3500xg for 10 minutes. Resulted pellets were mixed with tri-reagent and it was left at room temperature for 10-15 minutes for a complete homogenization. Then chloroform was added to the mixture to create phase separation. The suspension was incubated for a further 10 minutes with rapid stirring in the vortex and then centrifuged at 12,000 g for 10 minutes. Thus, water and organic phases were separated. The water phases were collected and stored at -20°C for later use as the ELISA solid phase antigen.

Indirect ELISA

Each ELISA solid phase antigen to be used in the study was diluted in a ratio determined by checkerboard analysis in antigen-coated buffer solution (0.05 M sodium carbonate (pH 9.6) and distributed to 96 well flat bottom maxisorp polystyrene plates (NUNC 692620) as 100 µl except H11 and H12 wells, which are assigned as blanks. The coated plates were incubated at 4°C for 18-24 hours. The plates were then blocked for 2 hours with PBS solution containing 5% skim milk. The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST). Test and control sera were added at 1:100 dilution in PBS/T as primary antibody. The plates were shaken in the orbital shaker for 1 hour at room temperature. A/G recombinant protein conjugated with horseradishperoxidase (HRPO) was diluted in PBST containing 1% skim milk at the determined dilution and added to all wells as 100 µl after the washing steps. Following 1 hour incubation at room temperature, the plates were washed again 3 times with PBST and 100 µl of chromogenic substrate (2 µg ortho-phenylenediamine ve %0.03 H_2O_2 in 0.1 M citrate bufferpH 5.5) were added. After the plates were kept at room temperature for 15 minutes, 100 µl of 4 N H_2SO_4 was added to each well to stop the reaction and the absorbance values were read at 490 nm with the plates automatic ELISA reader (VERSAmax 3.13/ B2573). For each ELISA cut off value was calculated as the mean of the OD values of the negatives plus 2 standard deviations. Sensitivity and specificity of newly developed ELISAs were determined following formula:

$$\frac{\text{Number of true positives}}{\text{Number of true positives+number of false negatives}}$$

$$\frac{\text{Number of true negatives}}{\text{Number of true negatives+the number of false positive, respectively.}}$$

RESULTS AND DISCUSSION

A newly developed indirect ELISAs for rough and smooth strains were used to test dog brucellosis caused by smooth strains and *B. canis*. Antibodies against *B. canis* were observed in 9.6% and 26.5% serum samples by RSAT and ELISA, respectively. While 6% of the samples were positive by *B. abortus* S99 RSAT, 9.6% of the serum samples were found as positive by *B. abortus* ELISA (Table 1). A wide variety of ELISA procedures have been described that use various types of antigens of *B. canis* (Barouin-Melo *et al.,* 2007; Lucero *et al.,* 2002; Nielsen *et al.,* 2004; Nielsen *et al.,* 2007;

Table 1: RSAT and ELISA results of dog sera.

Paddock number	*B. canis RSAT*	*B. canis* ELISA	*B. abortus* RSAT	*B. abortus* ELISA
A.24	3	9	1	2
B.27	3	10	1	2
C.32	2	3	3	4
Total 83	8 (9.6%)	22 (26.5%)	5 (6%)	8 (9.6%)

Oliviera *et al.,* 2011; Oncel *et al.,* 2005). Nielsen *et al.* (2007) used rough lipopolysaccharides (RLPS) isolated from *B. abortus* RB 51 strain as antigen when they tested *B. canis*-infected dog sera and they found the sensitivity and specificity of the test as 95.8% and 100%, respectively (Nielsen *et al.,* 2004). Researchers used horse radish peroxidase (HRPO) labeled A/G protein as conjugate in their test. In another study, HRP labeled protein A/G conjugate was reported to increase sensitivity and specificity of the test (Nielsen *et al.,* 2007). Barrouin-Melo *et al*., 2007) used heat-soluble bacterial extract (HSE) as antigen in their ELISA and they found sensitivity and specificity as 95% and 91%, respectively. In our study, sensitivity and specificity of *B.abortus* S99 ELISA were 98% and 95.5%, respectively. On the other hand, sensitivity and specificity of *B.canis M(-)* ELISA were found as 96.6% and 94%, respectively. Similarities and differences in specificity of ELISAs are largely depend on degree of purity of the antigen used while it seems that this factor do not play major role in sensitivity of the test. The humoral response to smooth strains and rough strains, except for only 2 serum samples, did not overlap with each other. Only 1 serum from paddock C and 1 serum from paddock B responded positively with both ELISA. The central (core) oligosaccharides in the LPS layer are similar in Rough and smooth LPS structures and therefore, it is considered usual to see such cross reactions in ELISA (Alton *et al.,* 1988; Carmichael, 1990). It has been determined that brucellosis caused by *B. canis* is more common than smooth brucella species. Dogs may be infected with smooth brucella species by being exposed to aborted cattle, sheep and goat fetuses in farms. Unfortunately, to feed dogs and cats with aborted fetuses in the farm is a common practice among farmers. Because the dogs in shelters are stray dogs living in the streets of Diyarbakır and its districts, it could be said that they have more *B. canis* infection compare to smooth species, which are more likely to be transmitted in rural areas. As a result, it was concluded that *B. canis* infection is common in dogs and this should be considered as concern for public health. Unfortunately, there is no standardised commercial serological test for diagnosis *B. canis* and other rough Brucella species currently. This is the main reason that rough brucella infections overlooked and undiagnosed most of the cases. For this reason it is utmost important that these tests be used routinely in the hospitals and veterinary clinics in the serologic diagnosis of brucellosis.

CONCLUSION

Activities for the control of disease should be followed strictly, in terms of testing quarantine and testing. Elimination of the infected by separating serological examinations at certain intervals and trying to prevent the transmission of the disease by taking the necessary hygienic measures are the main strategies in the control of the disease (Pickerill, 1970). In this regard, our newly developed in house ELISAs for both smooth and rough strains (mainly *B. canis*) showed relatively good sensitivity and specificity. It was concluded that ELISA together with RSAT can be used as easy and practical tests for serological diagnosis of dog brucellosis. Also dog owners should be awere of the possibility of transmission of the *B. canis* from infected dogs. For this reason owners should be told that their dogs should be tested regularly for this disease.

Conflict of interest

All authors declare that they have no conflict of interest.

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