Short Communication

A Serological Diagnostic Survey for Brucella canis Infection in Turkish Patients with Brucellosis-Like Symptoms

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SUMMARY: The incidence of Brucella canis infection in humans is unknown in Turkey. In this study, we investigated the prevalence of B. canis infection in human sera obtained from six regions in Turkey and comparatively evaluated the results obtained by agglutination-based techniques using standardized antigens made from B. canis. The patients (n = 1,746) presented with clinical symptoms that were similar to those of brucellosis. All patients who tested negative in the Rose Bengal test for the smooth Brucella strains (abortus, melitensis, and suis) were screened for evidence of B. canis infection using the rapid slide agglutination test (RSAT), the microagglutination test (MAT), and the 2-mercaptoethanol RSAT test (2ME-RSAT). Of the samples tested, 157 (8.9%), 68 (3.8%), and 66 (3.7%) were positive for B. canis, as determined by RSAT, MAT, and 2ME-RSAT, respectively. The diagnostic sensitivity, specificity, positive predictive value, and negative predictive value of RSAT were 100%, 94.6%, 42%, and 100%, respectively, and of MAT were 100%, 99.9%, 97%, and 100%, respectively. We recommend the routine use of MAT and 2ME-RSAT to check the sera of all patients with symptoms of brucellosis who are negative for brucellosis using a smooth Brucella antigen.

The diagnosis of Brucella canis infection in humans is often delayed because of the nonspecific clinical presentation of the disease, the low index of suspicion on the part of physicians, and the lack of serological screening tests or the difficulties with diagnostic laboratory confirmation (1). The serological tests for brucellosis caused by Brucella abortus, Brucella suis, and Brucella melitensis all rely on the reaction of antibodies to s-lipopolysaccharide, which is located on the bacterial cell wall. B. canis has a rough cell-wall antigen; therefore, detection of antibodies requires the use of a specific antigen. The diagnostic serological tests available for B. canis are the rapid slide agglutination test (RSAT), the 2-mercaptoethanol RSAT test (2ME-RSAT), the tube agglutination test (TAT), the microagglutination test (MAT), the indirect fluorescent antibody test, the agar gel immunodiffusion test, and the enzyme-linked immunosorbent assay (2,3). The most commonly used are RSAT, 2ME-RSAT, TAT, and MAT. RSAT is considered highly sensitive but not specific. It is rare to have false negatives (4), but a rate as high as 50–60% of false positives occurs in dogs (5). A modified RSAT, 2ME-RSAT, combines 2-ME, which inactivates IgM in order to increase the specificity of the test (6). In Turkey, brucellosis due to B. melitensis has been detected in small ruminants and humans, either endemically or sporadically, in all regions (7–9). However, the incidence of B. canis infection in humans is unknown. There have been previous reports of the serological diagnosis of B. canis infection in dogs in Turkey (10,11), however, data on the current status of infection and the incidence of B. canis infection in humans are lacking. We report, for the first time, the use of agglutination tests to diagnose human brucellosis that is caused by B. canis in a population of patients in Turkey who were found negative for brucellosis by the Rose Bengal test. In this study, we aimed to detect antibodies to B. canis infection in hum-

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(Rceived May 16, 2011. Accepted August 30, 2011)
were not available for this study. The reference strain of *B. canis* (RM 6/66) was used to prepare the antigens (produced by the National Reference Laboratory for Brucellosis, Pendik Veterinary Control and Research Institute, Istanbul, Turkey) for RSAT, MAT, and 2ME-RSAT, according to the methods described by Alton et al. (12). The organism was cultured in *Brucella* broth at 37°C, shaken at 200–600 rpm for 96 h, and harvested during the stationary phase of growth. After inactivation of the harvested culture for 1 h at 80°C, the inactivated culture was centrifuged in order to remove debris. For RSAT antigen production, the deposited cells were washed three times in phosphate-buffered saline (PBS; pH 7.4). The cell suspensions were then stained by the addition of 5 mL of 2% aqueous solution of Rose Bengal dye per 100 mL of cell suspension. After staining overnight at 4°C, stained cell suspensions were centrifuged and adjusted to a 6% packed cell volume by the addition of Tris-maleate buffer (TMB; pH 9.0). The antigen was standardized using positive and negative control sera supplied by the Veterinary Laboratories Agency (OIE Brucellosis National Reference Laboratory, FAO/WHO Collaborating Centre for Reference and Research on Brucellosis, Addlestone, UK) in order to ensure similar levels of sensitivity. The MAT antigen was prepared without staining. For antigen production, deposited cells were suspended in formalinized Sorenson’s PBS (0.5%). This suspension was then centrifuged for 30 min at 10,000 rpm. Deposited cells were suspended in the same buffer and standardized to a density of 4.5% cells using the packed-cell volume method. The optical density of the antigen was determined (OD490 = 1.1) in a spectrophotometer.

Sera were tested for evidence of *B. canis* infection by RSAT. Briefly, microtitration droppers were used to deliver 1 drop (0.05 mL) of stained antigen suspension to an equal volume of the serum sample. The mixtures were then mixed with individual wooden sticks, and plates were rocked gently for 3 min. Samples that tested positive by RSAT were retested by 2ME-RSAT. This test was used to inactivate IgM and, thereby, enhance the specificity. For 2ME-RSAT, 0.1 mL of a 0.2-M 2ME solution was added to the serum sample that was serially diluted (1/25 to 1/100) in TMB in an Eppendorf tube. After being mixed in a vortex for a brief period, 0.05 mL of the 2ME-treated serum samples were used in the same manner as in the RSAT procedure. The tests were read by observing any degree of agglutination (12,13).

MAT was performed following the method outlined by the National Reference Laboratory for Brucellosis, using prepared antigen as described by Alton et al. (12). A microplate agglutination test was used to test all RSAT-positive serum samples. Briefly, serum samples that were serially diluted 2-fold in TMB were prepared in a 96-well U-bottom microplate. Because of limited MAT antigen, 2-fold serial dilutions were used (1/25, 1/50, and 1/100). An equal volume (25 μL) of *B. canis* antigen solution was added to each well. The sealed plates were mixed gently for 20 s and incubated at 50°C for 24 h in a humid atmosphere. Positive and negative controls were used in each series of test runs. The plates were read by a microtiter mirror.

The control wells were expected to show agglutination between 1/50 and 1/100. An agglutination titer greater than 160 in sera from dogs is considered to be positive for canine brucellosis (13). However, the indicative titer for *B. canis* infection in human sera has not yet been validated, but Monroe et al. suggested that it can be as low as 1/12 (14). In our study, an antibody titer of 1/25 or greater was considered positive and was interpreted as indicating the presence of antibodies to *B. canis*. Titers in MAT were based on the final dilution of serum with round or folded-over circles in the well of the microplate. The 2ME-RSAT was used to calculate the diagnostic sensitivity, specificity, positive predictive value, and negative predictive value of the RSAT and MAT. The interregional significance of the *B. canis* infection ratio was evaluated with the 1-proportion significance *t* test.

Of the samples tested, 157 (8.9%), 68 (3.8%), and 66 (3.7%) were found positive by RSAT, MAT, and 2ME-RSAT, respectively. Table 1 summarizes the results of the analysis of sera for each test, according to the regions and provinces of Turkey. The 2ME-RSAT results showed prevalence rates ranging from 1.3% (Adiyaman in the Southeastern Anatolia region) to 7.8% (Manisa in the Aegean region), depending on the regions of Turkey. False-positive results from MAT were detected in patients from the cities of Kocaeli (3 cases), Adiyaman (1 case), and Van (2 cases), and false-negative results were obtained for patients from Manisa (2 cases), Aydin (1 case), and Antalya (1 case). The level of positivity in the MAT results ranged from 1/25 to 1/100. A 97% similarity was found in positivity between the MAT and 2ME-RSAT results. However, according to the results generated using serially diluted samples, MAT appeared to be more successful in detecting weak positives (Table 1). The diagnostic sensitivity, specificity, positive predictive value, and negative predictive value of RSAT were 100%, 94.6%, 42%, and 100%, respectively, and, for MAT, those values were 100%, 99.9%, 97%, and 100%, respectively. *B. canis* infection ratios were significantly lower in the Mediterranean and Southeastern Anatolia regions (*P* < 0.05 and *P* < 0.01, respectively) than in the rest of the tested regions (Table 1).

*B. canis* seroprevalence in dogs has been reported to range from 2% to 30% across different countries (3,15–17). Few serological surveys have been performed on dogs in Turkey. Diker et al. (10), Istambulluo glu and Diker (11), and Oncel et al. (6) reported seroprevalence rates of 6.3%, 6.7%, and 7.7%, respectively, with 2ME-TAT. Human *B. canis* infection, as diagnosed by ME-TAT, was reported in two patients in Turkey in 1984 (18). However, the seroprevalence rate of *B. canis* infection among humans in Turkey remains unknown. The seroprevalence rate determined by this study (3.7%) was slightly lower than what was observed for dogs in Turkey. However, the exact number of people who came into close contact with infected dogs was unknown. Unfortunately, reports of the seroprevalence rates in humans are extremely limited. Data from existing surveys include 13% in a group of hospital patients in Mexico, 0.3% in Germany, 2% in Argentina, 0.4% in US military populations, and 0.6% in Florida residents (19). A study from the US reported that *B. canis* was isolated from 4 of 331 confirmed infections (20). These
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The results of our study indicate that human infection with B. canis does occur in Turkey. However, B. canis has not been clinically isolated from patients, and therefore, routine brucellosis diagnosis should include serological tests for B. canis. Although symptomatic infections of B. canis in humans are rarely reported, Lucero et al. pointed out that B. canis infection in humans may be more widespread than has been speculated (19). Epidemiological studies may help increase the understanding of the prevalence of human B. canis infection, and they can infer preventive measures for reducing human exposure to the bacteria. We recommend the routine use of MAT and 2ME-RSAT in order to check sera from cases where the tests are negative for smooth Brucella antigens.

Although several different serological tests have been developed for the diagnosis of B. canis infection, there are no standardized serodiagnostic protocols or accepted methods of antigen standardization. Standardization by cell percentage alone may contain antigenic variations between batches or laboratories that prepare antigens, and, therefore, this does not represent an accurate representation of the antibody to antigen ratio; a standard serum must be used. In addition, there is no general agreement on the most appropriate test or the diagnostic threshold for TAT or MAT, and each laboratory determines its own criteria. This diversity of tests and the lack of a common protocol leads to difficulties in the interpretation of serological results that are obtained by different laboratories or investigators (22). TAT and MAT are the most widely-used laboratory tests for the detection of B. canis antibodies in both humans and carnivores. The 2ME-TAT procedure has technical disadvantages that limit its widespread use in the field evaluation of the disease; additionally, it is time-consuming and cumbersome in terms of the performance and measurement of results, and prozone phenomena might occur (23). However, MAT, as described here, is advantageous because a larger number of samples can be processed simultaneously (24). In this study, we used RSAT and MAT as screening tests for the diagnosis of B. canis infection with similar test results between MAT and 2ME-RSAT and limited false positivity (2 of 68 cases) with MAT. The positive predictive value of MAT (97%) was higher than that of RSAT (42%) (Table 1). The observation of the false-positive results obtained by RSAT in this study indicates that MAT or TAT, which both required serial dilutions of serum, may be more appropriate screening tests for human B. canis infection. RSAT is considered highly sensitive but not specific. It is rare to get false negatives, but as high as 50–60% of the results are false positives (5). According to Badakhsh et al., RSAT is the superior procedure for the identification of the early stages of B. canis infection (23). However, because of the provided semiquantitative results and the requirement for a small sample volume in the microplate, the MAT technique also has benefits. In the MAT technique, prominent results from weakly positive samples can be detected simultaneously with a range of dilutions. Lower titers may be found early B. canis infection, and the weak humoral response elicited in B. canis infection due to the fact that Brucella are facultative intracellular organisms could explain the lack of sensitivity of the serological tests.

The western part of Turkey represents a large urban population of people, many of whom are dog owners; additionally, stray dogs in the urban areas outnumber owned dogs. Human infections of B. canis commonly occur after contact with the blood, semen, urine, or placenta of infected dogs or with contaminated environments (2). Our findings show a high level of positive RSAT results from cities in the Aegean region. The high positivity was also observed in 2ME-RSAT. Mediterranea and Southeastern Anatolia regions showed the lowest antibody ratios (Table 1). The combined results for all regions suggest that B. canis infection is not an enzootic infection in Turkey. However, we have no documented information on the actual situation of B. canis infection in dogs because official routine examinations do not occur.

In conclusion, this study is the first documentation of B. canis antibodies that were detected by RSAT, MAT, and
and 2ME-RSAT in patients showing brucellosis-like symptoms. Our study showed that MAT and 2ME-RSAT can be used to screen human sera for \textit{B. canis}. We recommend the routine use of MAT and 2ME-RSAT in the screening of the sera of all patients with symptoms of brucellosis. It is also important to note that there is neither a standardized method for serological diagnosis of \textit{B. canis} nor a means to standardize the antigens in any of the tests. Therefore, the establishment of an international standard serum for \textit{B. canis} would assist in antigen standardization and be beneficial to international trade.

**Acknowledgments** We gratefully acknowledge the many colleagues, Dr. Tamer Sanlidağ, Manisa Celal Bayar University Hospital, Dr. Sevin Kirdar, Aydın Adnan Menderes University Hospital, Dr. Sibel Sasmaz, İzmir Bozyaka Hospital, Dr. Esvet Mutlu, Antalya Hospital, and Binnaz Gungor, Uludağ University Hospital to support this study with the sera samples.

**Conflict of interest** None to declare.

**REFERENCES**