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Research Article

Seroprevalence of *Brucella canis* Infection of Dogs in Two Provinces in Turkey

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Abstract: The aim of this study was the determination and comparative evaluation of the seroprevalence of *Brucella canis* infection in dog sera obtained from different dog shelters located in İstanbul and İzmir provinces by tube agglutination test (TAT), 2-mercapto-ethanol tube agglutination test (2ME-TAT) and ELISA.

A total of 362 serum samples were obtained from 8 different dog shelters in İstanbul and İzmir provinces. The dogs were temporarily kept in these shelters. Of the 362 serum samples, 46 (12.7%), 28 (7.73%) and 27 (7.45%) were found to be positive by TAT, 2ME-TAT and ELISA, respectively. Dogs in 6 (75%) out of the 8 kennels were found to be positive for *B. canis*. The prevalence of seropositive animals were ranged between 5.26% and 31.57%, 5% and 17.54%, and 2.12 % and 15.78% by TAT, 2ME-TAT and ELISA tests, respectively. Amongst the 46 sera evaluated as positive by TAT, 18 (39.1%) and 19 (41.3%) were found to be negative by 2ME-TAT and ELISA tests, respectively. A 96.42% similarity was found between 2 ME-TAT and ELISA results. In conclusion, the tube agglutination test when used together with additional tests such as 2ME-TAT and ELISA would be useful in eliminating the false-positive results obtained in serological diagnosis of *B. canis* infection of dogs. Similar results were observed between 2ME-TAT and ELISA tests. Because *B. canis* antibodies were detected in sera of dogs from most of the shelters examined, it is concluded that preventive measures should be taken to eliminate *B. canis* from the entire dog population and carrier dogs should be kept separated from uninfected animals. The carrier dogs may also constitute a risk for human health.

Key Words: *Brucella canis*, dog, prevalence, serology

Türkiye'nin İki İlindeki Köpeklerde *Brucella canis* İnfeksiyonunun Seroprevalansı

Özet: Bu çalışmada, farklı köpek toplama ünitelerinden toplanan serum örneklerinde *Brucella canis* infeksiyonunun seroprevalansının tüp aglütinasyon testi (TAT), 2-mercapto-ethanol–TAT (2ME-TAT) ve ELISA testi ile belirlenmesi ve sonuçların karşılaştırılmalı olarak değerlendirilmesi amaçlanmıştır.

Çalışmada, İstanbul ve İzmir yöresinde, farklı yerlerden toplanan köpeklerin geçici olarak barındıkları, 8 farklı köpek toplama ünitesinden toplanan, toplam 362 adet serum örneği kullanıldı. İncelenen 362 serum örneğinin 46 (% 12,7)'sı TAT, 28 (% 7,73)'si 2ME-TAT ve 27 (% 7,45)'si ELISA ile pozitif bulundu. Serumların elde edildiği 8 toplama ünitesinden 6 (% 75)'sında *B. canis* seropozitifliği saptandı. *B. canis* antikorları saptanan toplama ünitelerinde, seroprevalansın TAT ile % 5,26 ile % 31,57; 2ME-TAT ile % 5 ile % 17,54 ve ELISA testinde % 2,12 ile % 15,78 arasında değiştiği saptandı. TAT ile pozitif değerlendirilen 46 serumun 18 (% 39,1)'i 2ME-TAT ve 19 (% 41,3)'u ELISA ile negatif bulundu. ME-TAT ile ELISA bulguları ise % 96,42 oranında uyumlu bulundu.

Sonuç olarak, köpeklerde *B. canis* infeksiyonlarının serolojik teşhisinde kullanılan tüp aglütinasyon testinin, 2ME-TAT ve ELISA gibi ilave bir teşhis yöntemiyle desteklenmesinin yanlış pozitifliğin ortadan kaldırılmasında yarar sağlayacağı ve 2ME-TAT ile ELISA bulgularının birbirleri ile uyumlu olduğu ortaya kondu. Ayrıca, *B. canis* antikorlarının incelenen toplama ünitelerinin çoğunda saptanması, köpeklerde *B. canis* infeksiyonlarına karşı koruma önlemlerinin alınması gerekliliğini ve bu infeksiyonun insan sağlığı için potansiyel bir tehlike olmaya devam ettiğini gösterdi.

Anahtar Sözcükler: *Brucella canis*, köpek, prevalans, seroloji

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Introduction

Brucella canis causes canine brucellosis characterized by abortions in females and testicular atrophy, epididymitis and infertility in males and generalized lymphadenitis in both sexes. It is usually a clinically inapparent disease and many infected dogs may go unnoticed. Carmichael identified *Brucella canis* in 1966 as the cause of abortion among beagle dogs in the USA, and several countries have reported infection since then. Several researchers reported rates of seroprevalence ranging between 2% and 30% in stray dogs in various countries (1-5). Researchers have reported serological diagnosis of infection in Turkey (6,7), but there are insufficient data on the current status of infection.

Definitive diagnosis requires laboratory confirmation as clinical findings such as abortion in females and infertility in males are not specific to the disease. Diagnosis of *B. canis* infection in dogs depends on both bacteriological examination and serological methods. Commonly used serological methods are rapid slide agglutination test, 2-mercaptoethanol slide agglutination test, tube agglutination test and ELISA. Following the development of serological methods, the diagnosis of infection has become available in routine diagnostic laboratories (8-12). However, amongst these tests the ones using agglutination methods have lower specificities, and false-positive reactions are not uncommon with them. In order to eliminate this problem, researchers have developed ELISA techniques using purified antigens and/or monoclonal antibodies (13,14). The exact diagnosis of the disease requires isolation of the causative agent. Repeated blood cultures and serologic monitoring are required before a dog can be declared negative (2,11).

In this study, we aimed to investigate seroprevalence of *Brucella canis* infection with serum samples obtained from different dog shelters in two provinces in Turkey and comparatively evaluate the results obtained by agglutination-based and ELISA techniques.

Materials and Methods

Bacterial strain: *B. canis* RM 6/66 strain (Culture collection of Department of Microbiology, Faculty of Veterinary Medicine, University of Ankara) was used as antigen in TAT, 2ME-TAT and ELISA tests.

Serum samples: A total of 362 serum samples obtained from the temporarily kept dogs in 8 different dog rehabilitation shelters in İstanbul and İzmir provinces. The origins of dog serum samples are presented in Table 1. All the dogs in shelters were sampled once, and blood samples were brought to laboratories obeying appropriate transport measures. Once the sera were obtained they were separated in new tubes. The serum samples in vacuum blood tubes were separated by centrifugation at 1000 rpm for 10 minutes. The sera were heat inactivated at 56 °C for 30 minutes and kept at –20 °C until use.

Table 1. The origin and number of dog serum samples obtained.

Origin	Number of samples obtained
İstanbul-1	59
İstanbul-2	47
İstanbul-3	38
İstanbul-4	59
İstanbul-5	57
İzmir-1	80
İZmir-2	16
İzmir-3	6
Total	362

Preparation of hyperimmune serum: Hyperimmune serum was prepared in 2 sero-negative dogs after intravenous inoculation of 3 ml of formalin-inactivated *B. canis* antigen (3.7×10^8 bacteria/ml) that was given three times with weekly intervals. Hyperimmune sera were obtained from dogs 2 weeks post-inoculation. Serum samples obtained from 8 healthy sero-negative dogs were used as negative control serum.

Tube agglutination (TAT) and 2ME-TAT: The tube agglutination antigen used in this test was prepared according to method reported by Alton et al. (15). Following the first culture, *B. canis* RM 6/66 strain was subcultured onto 20 solid media (peptone 10 g, NaCl 5 g, agar 20 g, distilled water 1000 ml) and incubated for 24 hours at 37 °C. At the end of the incubation period, all colonies were collected with 10 ml of formalinized (0.06%) Sorensen's phosphate buffered saline. Having filtered through several layers of sterile gauze the

inoculum was inactivated by heating to 70 °C for one hour. After the inoculum cooled, it was centrifuged at 10,000 rpm for 30 min. The supernatant was discarded and the pellet was suspended with formalinized (0.5%) Sorenson's buffered saline and this suspension in graduated centrifuge tubes was re-centrifuged for 30 min at 10,000 rpm. Antigen was standardized to have a 4.5% cell concentration and its OD value was determined (OD=110; 490 nm) in a spectrophotometer (Photovolt Model 401). Briefly, serum samples were diluted by 1/12.5 and then twofold serial dilutions were made up to 1/1600 from this dilution to obtain a final volume of 1 ml in test tubes. One ml of tube agglutination antigens was added to all tubes and the tubes were incubated at 37 °C for 48 hours. After that, they were evaluated and 1/200 and higher dilutions showing ++ reactions were determined as positive. 2ME-TAT was performed by adding 0.6% formalin and 0.1 M 2-mercapthoethanol (Merck) to serum dilution buffer in TAT test. All the steps and evaluations were done as in TAT. Titers of positive serum samples were determined as 1:400 in tube agglutination test. The average titer of negative controls was ≤ 1:25.

ELISA: *B. canis* RM 6/66 strain was propagated on Brucella agar and after being subcultured on 20 petri dishes containing Brucella agar, incubated at 37 °C for 24 hours. Growing colonies were collected from petri dishes with Phosphate Buffered Saline (PBS) containing 0.6% formalin. The collected cultures were pooled (approximately 100 ml) in an Erlenmeyer flask and then incubated at 60 °C for 30 minutes. The suspension was centrifuged for 10 min at 10,000 rpm in a centrifuge tube and this step was repeated three times with PBS. The precipitate was sonicated for 15 min and then centrifuged for 15 min at 10,000 rpm. The supernatant was collected and dialyzed against distilled water at 4 °C for 48 hours and used as ELISA antigen after this final step. ELISA was performed in high affinity microplates as reported by Mateu-de-Antoni et al. (16). For the determination of optimal antigen and the anti-dog peroxidase conjugate (A-6792; Sigma) the checkerboard method was used. While antigen was diluted twofold starting at 1/5 and up to 1/80 concentrations, the conjugate was diluted twofold from 1/500 to 1/16,000. The optimal dilutions of antigen and conjugate used in ELISA were determined as 1/10 and 1/8000, respectively. Optimal antigen dilution (1/10) was made in 0.05 M carbonate buffer (pH 9.6) and added to microplates in a volume of 100 µl for each well. Microplates were incubated at 4 °C overnight. After the incubation, plates were washed with 0.1 M PBS-Tween-20 solution (0.05%, pH 7.2) three times. Serum samples were diluted up to 1:200 with PBS-Tween-20 containing 1% bovine serum albumin (BSA) and 100 µl of this dilution was added to each well. Following serum addition, all plates were incubated at 37 °C for 1 hour. All test serum samples were repeated three times. Plates were washed as previously explained and 100 µl of optimal conjugate dilution (1/8000) prepared in PBS containing Tween-20 was added to each well. Microplates were incubated for 1 hour at 37 °C and following the washing step 50 µl of substrate solution [0.04% H_2O_2 (30%)+ o-phenylendiamine] was added to each well. Microplates were left in darkness for 30 min and the reaction was stopped with 2.5 N HCl. Optical densities (ODs) of serum samples were determined at 490 nm in an ELISA reader (Titertek Multiscan Plus) and at least 3 units higher than standard deviation of average OD of negative controls were determined to be positive.

Results

Of the 362 serum samples tested, 46 (12.7%), 28 (7.73%) and 27 (7.45%) were found to be positive for *B. canis* by TAT, 2ME-TAT and ELISA, respectively (Table 2). Six (75%) out of the 8 dog kennels examined were found to be positive for *B. canis*. Prevalence of seropositive dogs was 5.26%-31.57%, 5 %-17.54%, and 2.12%-15.78% by TAT, 2ME-TAT and ELISA, respectively. The average seroprevalences were 12.7%, 7.73%, 7.45%, respectively. Positive serum antibody titers were between 1:200 and 1:800 in TAT, while they were between 1:200 and 1:400 in ME-TAT. Eighteen (39.1%) and 19 (41.3%) out of 46 sera evaluated as positive by TAT were found to be negative by 2ME-TAT and ELISA, respectively. A 96.42% similarity was found between 2 ME-TAT and ELISA test results.

Discussion

Diagnosis of brucellosis in dogs is based on clinical findings, serological tests and bacteriological isolation. Due to possible false-positive results that may be obtained in serological tests, blood cultures should be attempted to confirm the diagnosis. On the other hand, serological

Table 2. Results obtained from TAT, ME-TAT and ELISA tests.

Serum origin	TAT	ME-TAT	ELISA
İst 1 n = 59	11 (18.64)	7 (11.86)	6 (10.16)
İst 2 n = 47	4 (8.51)	2 (4.25)	2 (2.12)
İst 3 n = 38	2 (5.26)	2 (5.26)	2 (5.26)
İst 4 n = 59	5 (8.47)	3 (5.08)	3 (5.08)
İst 5 n = 57	18 (31.57)	10 (17.54)	9 (15.78)
İzmir 1 n = 80	5 (6.25)	4 (5)	4 (5)
İzmir 2 n = 16	0	0	0
İzmir 3 n = 6	0	0	0
Total n = 362	46 (12.7)	28 (7.73)	27 (7.45)

tests are relatively easy to perform and provide a practical advantage in determining the prevalence of infection. This study's purpose was the determination and comparative evaluation of seroprevalence of *Brucella canis* infection with serum samples obtained from different dog shelters by agglutination tests and ELISA techniques.

The results indicated that the seroprevalence of *B. canis* antibodies in samples was between 5.26% and 31.57%, 5% and 17.54%, and 2.12 % and 15.78% by TAT, 2ME-TAT and ELISA, respectively. The average seroprevalences were 12.7%, 7.73% and 7.45% with the tests mentioned above, respectively. Eighteen (39.1%) and 19 (41.3%) out of 46 sera evaluated as positive by TAT were found to be negative by 2ME-TAT and ELISA, respectively. A 96.42% similarity was found between 2ME-TAT and ELISA results. These findings showed that false-positive results obtained with TAT were eliminated with 2ME-TAT and ELISA and the difference between the former and the latter two tests may have occurred because only *B. canis* specific IgGs were detected with 2ME-TAT and ELISA. Dogs can be declared negative when the agglutination tests are negative, especially with the rapid serum agglutination test (RSAT). However, only about 40-50% of the dogs whose sera were positive in agglutination tests are actually positive for canine brucellosis (2). Besides, 2-mercaptoethanol treatments in agglutination tests have been demonstrated to lower the false positive results by 50% (2,6,7). In this study, positive results obtained with TAT were lowered 40% with ME-TAT and these findings were confirmed with ELISA. The results obtained with this study are consistent with the previous studies. Baldi et al. (12) prepared and used two antigens, 18 kDa cytoplasmic protein and cytoplasmic protein lacked LPS, respectively, in two different ELISA procedures for the diagnosis of canine brucellosis. They compared results with the ELISA methods and found a 93.3% correlation between ELISA and 2ME-TAT results. They also found that capture ELISA using 18 kDa cytoplasmic protein as an antigen could be more useful than indirect ELISA for diagnosis. There is a correspondence between the findings of this study and those carried out by Baldi et al. (12).

In this study, amongst the 362 serum samples, 46 (12.7%), 28 (7.73%) and 27 (7.45%) were found to be positive by TAT, 2ME-TAT and ELISA, respectively. Two (75%) out of the 8 dog kennels examined were found to be negative for *B. canis*. Negative results obtained in the two shelters may be explained by the low number of dogs kept in the kennels. *B. canis* seroprevalence is reported to range between 2% and 30% in different countries (1-4). There are few studies performed on this subject in Turkey. Diker et al. (6) and İstanbulluoğlu and Diker (7) reported seroprevalence rates of 6.3% and 6.7% in 222 and 134 serum samples obtained from military, stray, and household dogs by the use of 2ME-TAT, respectively. The 2ME-TAT findings of this study (7.73%) are similar to those reported in previous studies (3,6,7). However, seropositiveness between shelters varies between 0% and 17.54%. Findings obtained from these studies show that there has been no serious increase in *B. canis* seroprevalence in last 20 years, but the infection still exists.

In conclusion, in this study it was observed that in order to eliminate false-positive results obtained in serological diagnosis of *B. canis* infections in dogs, tube agglutination test should be combined at least with one additional test such as 2ME-TAT or ELISA. A consistency between the results of 2ME-TAT and ELISA was also noted in the current study, which indicates that these tests are good supplementary and/or alternative tests for the serological diagnosis of *B. canis* infections. Since *B. canis* antibodies were detected in sera obtained from dogs in most of the dog shelters, it is concluded that protective measures should be implemented to eliminate *B. canis* infections in dogs and their residences.

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