Detection of canine brucellosis by a rapid agglutination test using *Rhizobium tropici* as antigen

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**SUMMARY**

The *Brucella canis* infection induces various reproductive disorders in dogs and also in humans. The aim of this study was to evaluate the feasibility to use antigens from related germs, such as *Rhizobium tropici* in serological methods for evidencing circulating anti-Brucella antibodies in dogs, because of the zoonotic risk. For that, brucellosis seroprevalence on 135 dogs stemming from pounds or from the Faculty of Veterinary Medicine of the Konya region was determined with specific serological tests (Modified Micro Plate Agglutination Test (MPAT), 2-Mercaptoethanol Tube Agglutination Test (2Me-TAT) an indirect ELISA (I-ELISA)) using *B. canis* NCTC 10854 strain antigens and with a non specific rapid slide agglutination test (RSAT) using *R. tropici* CIAT 899 strain as antigens. The canine brucellosis prevalence was 21.5% with I-ELISA and was weakly higher with the MPAT (25.2%) and the 2Me-TAT (22.2%), the relative performances of these 2 specific tests compared to I-ELISA remaining elevated (Agreements, sensitivity and specificity around 90% or more). Dogs from pounds and females were significantly the most infected. By contrast, the proportions of false positive and false negative sera with RSAT were markedly elevated (22.6% and 69.0% respectively) leading to low relative performances compared to the I-ELISA (agreement: 67.4%, sensitivity: 31.0% and specificity: 77.4%). Genetic similarity between *B. canis* and *R. tropici* was determined 30.8% by RAPD-PCR. These results demonstrated that canine brucellosis is a relatively common infection in the Central Anatolia and that the RSAT using *R. tropici* as antigens cannot be use for the serological diagnosis of the disease.

**Keywords:** *Brucella canis*, brucellosis, *Rhizobium tropici*, I-ELISA, agglutination tests, serology, dog.

**RÉSUMÉ**

Détection de la brucellose canine par un test d’agglutination rapide utilisant *Rhizobium tropici* comme antigène

L’infection par *Brucella canis* induit des affections variées de la fonction de reproduction chez le chien mais aussi chez l’homme. En raison du risque de zoonose, l’objectif de cette étude a été d’évaluer la faisabilité d’utiliser des antigènes issus de bactéries apparentées telles que *Rhizobium tropici* pour détecter les anticorps circulants anti-brucella chez le chien au cours d’un test sérologique. La séroprévalence de la brucellose a été déterminée à partir de 135 chiens issus des fourrières et de la Faculté de Médecine Vétérinaire de la région de Konya à l’aide de 3 tests spécifiques (test modifié d’agglutination en micro-plaque (MPAT), Agglutination en tube en présence de 2-mercaptoéthanol (2ME-TAT) et ELISA indirecte (I-ELISA)) utilisant les antigènes de la souche NCTC 10854 de *B. canis* et un test non spécifique d’agglutination rapide sur lame (RSAT) utilisant les antigènes de la souche CIAT 899 de *R. tropici* (RSAT). La prévalence de la brucellose canine mesurée par le test I-ELISA a été de 21.5 % et s’est avérée légèrement plus élevée avec les tests MPAT (25.2 %) et 2ME-TAT (22.2 %), les performances relatives de ces 2 tests spécifiques comparées au test restant élevées (agrément, sensibilité et spécificité étant d’environ 90 % ou plus). Les chiens des fourrières et les femelles ont été significativement les plus infectés. En revanche, les proportions de faux-positifs et de faux-négatifs obtenus avec le test RSAT ont été importantes (respectivement, 22.6 % et 69 %), conduisant à de faibles performances par rapport au test I-ELISA (agrément : 67.4 %, sensibilité : 31.0 % et spécificité : 77.4 %). Déterminée par RAPD-PCR, la similitude génétique entre *B. canis* et *R. tropici* n’a été que de 30.8 %. Ces résultats montrent que la brucellose canine est une infection relativement fréquente en Anatolie Centrale et que le test RSAT utilisant des antigènes de *R. tropici* ne peut pas être utilisé pour le diagnostic sérologique de cette maladie.

**Mots clés :** *Brucella canis*, brucellose, *Rhizobium tropici*, I-ELISA, tests d’agglutination, sérologie, chien.

**Introduction**

*Brucella canis* (*B. canis*) can cause abortions and stillbirths in female pregnant dogs whereas males develop epididymitis and testicular atrophy. A generalized lymphadenitis in both sexes can occur and some dogs remain chronically infected for years [3]. Veterinary medicines, laboratory technicians and dog owners are the most exposed to this zoonotic infection [15, 17]. A worldwide distribution of the disease occurs but a limited number of studies on canine brucellosis prevalence have been conducted in Turkey [14, 20, 26]. Clinical diagnosis of the infection in dogs is difficult: the only definitive test for the diagnosis is the culture the *B. canis* bacterium from blood specimen [26]. Serological tests are routinely used to test large populations and a great deal of discussion for the serology has been made elsewhere [2]. The authors concluded that the most specific and sensitive test is ELISA. *Brucella spp.*, *Agrobacterium*, *Ochrobacterium* and *Rhizobium* which all belong to the α-2 subgroup of Proteobacteria [5, 7] exhibit various degrees of genetic similarities and cross-reactivity [4, 8, 13, 25]. On the other hand, working on a large scale culture of Brucella for antigen production requires bio-safety level-3 labs which are not readily available in developing countries where brucellosis is endemic, whereas the massive production of antigens from a non pathogenic agent which can be used for detection of anti-*Brucella* antibodies because of cross-reactivity, does not require strict bio-safety measures. Since antigens from the non pathogenic *Rhizobium* spp.,
have never been checked to diagnose canine brucellosis to our best knowledge, we aimed to examine the use \textit{Rhizobium tropici} as an antigen to diagnose canine brucellosis.

**Materials and Methods**

**REFERENCE STRAINS**

The \textit{B. canis} NCTC 10854 was obtained by Institute of Refik Saydam Hfzzissha, Ministry of Health and the \textit{Rhizobium tropici} CIAT 899 strain was kindly supplied by Assoc. Prof. Refik Uyanöz (Department of Soil, Faculty of Agriculture, Selçuk University).

**ANIMALS AND SAMPLES**

A total of 135 dogs (97 males and 38 females) stemming from the city pound of Konya, clinics and dog research unit of the Veterinary Faculty, Selcuk University, Konya were included in this study. All the dogs were clinically healthy except those (n = 9) stemmed from the clinics. They showed various clinical signs like fever, weakness, anorexia and malaise. In addition, lymphadenopathy was detected in two female dogs. No \textit{Brucella} isolations were accomplished from blood samples and vaginal swabs. Urine samples from males were not cultivated. The abortion history of the dogs stemmed from the city pound of Konya, clinics and dog research unit. All the dogs were clinically healthy except those (n = 9) stemmed from the clinics. They showed various clinical signs like fever, weakness, anorexia and malaise. In addition, lymphadenopathy was detected in two female dogs. No \textit{Brucella} isolations were accomplished from blood samples and vaginal swabs. Urine samples from males were not cultivated. The abortion history of the dogs stemmed from clinics was unknown.

Preparation of hyper-immune serum was made as described before [20]. Briefly, hyper-immune serum was prepared in 2 healthy dogs after 3 weekly intravenous injections of antigen obtained from formalin inactivated \textit{B. canis} (3.7 \times 10^8 bacteria/mL; 3 mL). Two weeks post inoculation hyper-immune sera were obtained from the dogs.

Blood samples were collected into sterile tubes without anticoagulant from the dog’s foreleg using a vacutainer vial. After clotting at room temperature for 2 hours, specimens were centrifuged at 500 g for 10 min. In addition, lymphadenopathy was detected in two female dogs. No \textit{Brucella} isolations were accomplished from blood samples and vaginal swabs. Urine samples from males were not cultivated. The abortion history of the dogs stemmed from clinics was unknown.

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Blood samples were collected into sterile tubes without anticoagulant from the dog’s foreleg using a vacutainer vial. After clotting at room temperature for 2 hours, specimens were centrifuged at 500 g for 10 minutes at room temperature and sera were then heat inactivated at 56°C for 30 min and kept at -20°C until analysis.

**SEROLOGICAL ANALYSIS**

Specific tests using \textit{Brucella} antigens, 2ME-TAT, MPAT and I-ELISA

For the 2ME-TAT (2-Mercaptoethanol Tube Agglutination Test) and the MPAT (Modified Micro Plate Agglutination Test) specific tests, \textit{B. canis} antigens were prepared as described before [1] from the NCTC 10854 strain and their concentrations were adjusted to 4.5% bacteria using the packet-cell volume method as described by ALTON et al. [1]. Thereafter, these 2 serological analyses were performed as previously described [1, 6]. The MPAT was performed as follows: briefly 25 μL of phosphate-buffered saline was added to each well of a round-bottomed microplate following by 25 μL of undiluted sera which was placed in the first well and diluted twofold serially, then 50 μL of antigen was added to each well. The plates were incubated at 37°C for 48 h. The test was considered as positive when a serum dilution in the test strictly superior to 1:48 has given a positive reaction [6]. For the 2ME-TAT, two fold dilutions of serum samples were obtained from 1/50 to 1/200 using as diluents 3.5% NaCl, 0.06% formalin and 0.1 M 2-mercaptoethanol (M6250, Sigma) giving a final volume of 1 mL in the tubes. The 2ME-TAT antigen (1 mL) was added to all tubes and then the tubes were incubated at 37°C for 48 hours. When dilutions superior to 1/100 have given agglutination, the test was considered as positive [1].

\textit{Brucella} antigens for the I-ELISA (Indirect Enzyme-linked Immunosorbent Assay) were prepared according to the method described by MYERS et al. [18] and the protein concentration was measured using a commercial kit (Bio-Rad lab., Cat No: 500-0116, USA). This ELISA test was performed according to the method described by MATEU-DE-ANTONIO et al. [16]. The optimum working dilutions of antigen, anti-dog IgG conjugated to the horse radish peroxidase (HRP) (A-9042, Sigma) and serum concentrations were determined following a checker-board titration method [20]. In brief, serum, antigen and conjugate were diluted starting from 1/50 to 1/400, from 0.5 μg to 8 μg and from 1/500 to 1/16000, respectively. Consequently, the concentrations of antigen, conjugate and serum used were 1.5 mg/L, 1:4000 and 1:300, respectively. Each serum dilution was tested in triplicate wells. The Immulon II microplate (Nunc C bottom Immunplate 96 well, 446612) was coated with 1.5 μg of antigen in 100 μL 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Microplates were then washed three times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8). To block non-specific binding a post-coat solution (200 μL) of 1% bovine serum albumin (A8531, Sigma) was dispensed to the wells, incubated at 37°C for 1 hour and then washed as above. Control and test sera were diluted 1:300 using 1% BSA and 0.05% Tween 20 PBS buffer and 100 μL of each diluted sample were added into the wells, incubated at 37°C for 1 hour then washed as above. The HRP conjugate anti-dog IgG (100 μL) was then added and incubated once more as above. Following another washing step, 100 μL of substrate (TMB, 3,3',5,5'-Tetramethylbenzidine; SIT-0440, Sigma) were added, followed by an incubation step at room temperature for 10 min and the reaction was stopped by addition of 100 μL H_2SO_4 2M. The absorbance at 450 nm was finally read by a spectrophotometer (MWG/Lambda Scan 200 Bio-Tek Inst. Inc. USA) and the result was considered as positive when the absorbance was superior to 1.754 (cut-off value).

Rapid slide agglutination test (RSAT) using \textit{Rhizobium tropici} as antigen

The method for antigen preparation from \textit{Rhizobium tropici} CIAT 899 strain was based on the protocol of LISLE and CARMICHAEL [14]. For optimizing the reaction, 20 μL of antigen were mixed with a same volume of serum on a slide and shaken gently for 2 minutes then formation of agglutination was considered as positive result.

RAPD-PCR GENOTYPING

The \textit{R. tropici} and \textit{B. canis} were grown in Yeast Mannitol
DETECTION OF CANINE BRUCELLOSIS BY RHIZOBIUM ANTIGEN 53 Agar (Y3252, Sigma) and Blood Agar Base (CM0271B, Oxoid), respectively. Bacteria were killed by addition of 0.5% formaldehyde then DNA was extracted using the protocol provided in Promega Wizard Genomic DNA purification Kit (Cat No: A1120, USA) and was used as template for RAPD-PCR. The extracted DNA was amplified in a total volume of 25 μL (5 μL 10x PCR buffer, 250 mM from each dNTPs (DNTP-100, Sigma), 1 U taq polymerase (EP0282, MBI Fermentas), 6.25 nM of primer (IDT, USA) (5’CGGCCCCTGT 3’), 1 mM MgCl2, and 2.5 μL extracted DNA). The cycling conditions with the Techne Progene (Cambridge Ltd., UK) were the initiation step at 94°C for 3 min, followed by 10 cycles coupling 94°C for 1 min., 35°C for 2 min. and 72°C for 1 min., and 30 further cycles coupling 94°C for 1 min., 50°C for 2 min. and 72°C for 1 min. and a final extension period at 72°C for 4 min. The amplification products (10 μL) were submitted to electrophoresis in 1.6% agarose gel in Tris-Borate EDTA buffer and the 100 bp ladder DNA marker (NL0403, Vivantis, New Zealand) was run concurrently. The ethidium bromide stained common DNA bands from the bacteria were visualised at 500 bp and the gel photographed [24]. The genetic diversity for the bacteria was calculated according to NEI and LI [19].

STATISTICAL ANALYSIS

The statistical differences in different parameters between groups were determined by the X2-test [23] and the difference was considered as significant when P value was less than 0.05.

Results

Among the 135 sera tested, 34 (25.2%), 30 (22.2%) and 29 (21.5 %) were found to be positive for B. canis by MPAT, 2ME-TAT and I-ELISA, respectively (Table I). Five samples that were positive by both 2ME-TAT and MPAT were negative with I-ELISA. Consequently, the relative sensibilities (proportions of positive sera with the 2 compared methods) of the MPAT and the 2ME-TAT compared to the I-ELISA remained high (100% and 89.7% respectively) and the relative specificity (proportions of negative sera with the 2 compared methods) was identical (95.3%) (Table II). The respective positive predictive values (85.3% for the MPAT and 96.7% for the 2ME-TAT) were high allowing a marked positive gain diagnostic (0.638 and 0.752 respectively) whereas the negative diagnostic gains remained weak (0.215 for the MPAT with a negative predictive value of 100% and 0.177 for the 2ME-TAT with a negative predictive value of 96.2%). The agreements (frequency of identical results) between the MPAT and I-ELISA and between the 2ME-TAT and I-ELISA were elevated (96.3% and 93.3% respectively).

Whatever the specific method used, the brucellosis frequency was significantly higher in pounds than in animals from the Faculty of Veterinary Medicine, Konya (P < 0.05). With the MPAT method, the observed titres of the anti-Brucella antibodies in considered positive sera ranged from 1:96 to 1:768 (figure 1) whereas this one of the 2 positive dogs from the Faculty of Veterinary Medicine was 1: 96. Moreover, the serum anti-Brucella antibodies were significantly more frequently detected in females than in males according to the 3 specific methods (Table I).

On the other hand, 33 out of 135 sera agglutinated with Rhizobium antigen using the rapid slide agglutination test. However, only 9 of the RSAT positive sera gave also a positive reaction using the specific I-ELISA test (Table II). The agreement score between the 2 methods as well as the RSAT relative sensibility and specificity compared to the I-ELISA were low, 67.4%, 31.0% and 77.4% respectively. The RAPD-PCR analysis of B. canis and Rhizobium tropici strains produced only 2 common bands (540 bp and 1500 bp) for both species (figure 2). The genetic similarity between the two bacteria was only 30.8%.

Discussion

Although none of the serological methods are diagnostically conclusive, ELISA test is still accepted as the most specific

<table>
<thead>
<tr>
<th>Dog population (n = 135)</th>
<th>MPAT</th>
<th>2ME-TAT</th>
<th>I-ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Total</td>
<td>101 (74.8%)</td>
<td>34 (25.2%)</td>
<td>105 (77.8%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males (n = 38)</td>
<td>31 (81.6%)</td>
<td>7 (18.4%)</td>
<td>30 (79%)</td>
</tr>
<tr>
<td>Females (n = 97)</td>
<td>70 (72.2%)</td>
<td>27 (27.8%)</td>
<td>74 (76.3%)</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pound (n = 106)</td>
<td>74 (69.8%)</td>
<td>32 (30.2%)</td>
<td>78 (73.6%)</td>
</tr>
<tr>
<td>Clinics, FVM (n = 9)</td>
<td>8 (88.9%)</td>
<td>1 (11.1%)</td>
<td>8 (88.9%)</td>
</tr>
<tr>
<td>Research Unit, FVM (n = 20)</td>
<td>19 (95.0%)</td>
<td>1 (5.0%)</td>
<td>19 (95.0%)</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
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</table>

FVM: Faculty of Veterinary Medicine

TABLE I : Serological prevalence of B. canis infection using specific MPAT (Modified Micro Plate Agglutination Test), 2ME-TAT (2-Mercaptoethanol Tube Agglutination Test) and I-ELISA (Indirect Enzyme-linked Immunosorbent Assay) tests in 135 dogs.

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and sensitive [2]. MPAT is recommended as a screening test for B. canis infection in dogs and human [6, 17]. Furthermore, diagnostic performance of ELISA using crude cytosolic and membrane antigens from α-Proteobacteria for the diagnosis of human and animal brucellosis has been examined by DELPINO et al. [8]. Whereas the 2ME-TAT and MPAT were used as screening tests, the I-ELISA was considered as a confirmative test to measure the canine brucellosis prevalence. In agreement with that, the high positive diagnostic gains obtained for the MPAT and 2ME-TAT compared to the I-ELISA in the present study confirmed their utility as screening tests.

Despite the worldwide distribution of this infection [2, 6, 12, 27], limited data about the current status of the infection in Turkey is available [9, 10, 20] and seroprevalence of the infection in some major provinces has never been checked before. By this study, a high brucellosis seroprevalence (21.5%) in dogs from Konya has been detected suggesting that canine brucellosis can be one of the major problems in this country. It has been suggested that the differences of the infection prevalence between the sexes were due to variations of exposure to Brucella rather than to the biological diversity [17]. A lower seroprevalence of B. canis antibodies in males than in females found in this study using specific serologies (MPAT, 2ME-TAT and I-ELISA) can be due to a decreased exposure to the agent. The decrease of male exposure to Brucella might be explained by a possible result of infection of the bitches [3]. By resorption of the foetuses, an infection of bones can rise to chronic osteomyelitis and spondylitis in mother dogs leading a relatively higher occurrence of the infection in females than males. A higher seroprevalence of the disease in the pound was also observed by this study. As two of the primary sources of the infectious agent, vaginal discharges of aborted bitches and the urine of

<table>
<thead>
<tr>
<th>Compared tests</th>
<th>I-ELISA results</th>
<th>Relative performances</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>MPAT</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>101</td>
</tr>
<tr>
<td>2ME-TAT</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>101</td>
</tr>
<tr>
<td>RSAT</td>
<td>Positive</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>82</td>
</tr>
</tbody>
</table>

Se: Sensibility (frequency of true positive); Sp: Specificity (frequency of true negative); Ag.S.: Agreement score (frequency of identical results between the 2 tests).

Table II: Contingency tables and relative performances of the MPAT (Modified Micro Plate Agglutination Test), 2ME-TAT (2-Mercaptoethanol Tube Agglutination Test) and of rapid slide agglutination test (RSAT) using Rhizobium antigen compared to the specific I-ELISA (Indirect Enzyme-linked Immunosorbent Assay) test for brucellosis.

Figure 1: Frequency of antibody titres of positive sera (n = 34) using the specific MPAT (Modified Micro Plate Agglutination Test) for brucella antigens according to the origins of the dogs (Pound, Research Unit and Clinics).

Figure 2: Comparative fingerprints obtained by RAPD-PCR from Brucella canis and Rhizobium tropici. Lane 1: Rhizobium tropici amplified product, lane 2: Brucella canis amplified product and lane 3: VC100bp Plus DNA Ladder (NL0403, Vivantis). Two common bands (540 bp and 1500 bp, arrows) were observed.
infected males are known [3]. The strong brucellosis seroprevalence seen in the ponds in Konya can result from the highly populated kennels in which this mode of transmission readily occurs, favouring by a low health management quality in the shelters.

Brucella spp. species belong to the α-Proteobacteria like other bacteria such as Agrobacterium, Ochrobacterium and Rhizobium and are the major pathogens for mammals including humans whereas the others are all not pathogenic for mammals. Some evidences about the close genetic (and antigenic) relationship between Brucella and other α-Proteobacteria have been reported [8, 21]. Furthermore canine infection by B. canis was detected with some degrees of specificity and sensitivity using antigens from Agrobacterium, Sinorhizobium and Ochrobacterium spp. [8]. Multilocus Enzyme Electrophoresis (MLEE) has been frequently used to determine the genetic relatedness in bacteria [11, 22]. GÁNDARA et al. [11] formed a cluster by MLEE-derived dendograms obtained by B. canis and B. suis and found them to be related to R. tropici. Their theory was based on the hypothesis that both Brucella and R. tropici have shared a common ancestor and conserved some inherited antigens.

To our knowledge, R. tropici has never been attempted as antigen to measure the antibodies to B. canis. The investigation of the potential usefulness of antigens from non pathogen bacteria (R. tropici) phylogenetically related to a pathogen specie (B. canis) could constitute a useful tool for the infection diagnosis principally when the use of the etiological agent is restricted by bio safety measures and internal policies. However, the low relative sensitivity (31.0%) and specificity (77.4%) of RSAT compared to the I-ELISA as documented between Brucella and other α-Proteobacteria like Ochrobacterium, Sinorhizobium and other α-Proteobacteria. The investigation extracts of a variant (M-) strain of B. canis on Systematic Bacteriology, Int. J. Syst. Evol. Microbiol., 2006, 56, 1169-1170.


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