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Investigation of *Brucella canis* and *Brucella abortus* Seropositivity by In-House Rapid Slide Agglutination Test and In-House ELISA in Northern Cyprus

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The incidence of *Brucella canis* (*B. canis*) in humans is unknown in Northern Cyprus. In this study, we investigated the prevalence of *B. canis* and *Brucella abortus* (*B. abortus*) infection in human sera and evaluated the results obtained by agglutination-based techniques using standardized antigens made from *B. canis* comparatively. All of the subjects were negative in terms of Rose-Bengal plate test. Undiluted serum samples were initially screened by rapid slide agglutination test (RSAT), and those which were found positive were retested in the dilution of 1/25-1/200. Confirmation of the positive results was performed by using 2-mercaptoethanol standard agglutination test (SAT). The test antigen was prepared from the less mucoid M (−) variant of *B. canis*, and 1/1,048 titered dog antiserum was used as positive control. In 225 serum samples, 3.6% (8/225) was positive by *B. canis* M (−) RSAT, 4.4 % (10/225) was positive by *B. canis* M (−) indirect enzyme-linked immunosorbent assay (iELISA). 5.3% (12/225) was positive by *B. abortus* S99 RSAT and 9.8% (22/225) was positive by *B. abortus* S99 iELISA. Nine samples were positive by both *B. abortus* S99 RSAT and *B. abortus* S99 iELISA. Seven samples were positive by both *B. canis* M (−) RSAT and *B. canis* M (−) iELISA. One patient was positive by all methods. It is important to evaluate patient samples with RSAT and iELISA. Until the notification system gives better results to the Ministry of Health, in order to reach the real data for Northern Cyprus, multicenter prevalence determination studies should be done for future.

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Introduction

Brucellosis, an infection source for both animals and humans, is among the most common zoonoses in the world. The prevalence of brucellosis is reduced in countries such as northern Europe and Australia which are included in the control program for brucellosis animal diseases. However, the Mediterranean region, the Middle East, the Arabian Peninsula, Africa, Latin America and Asia are considered endemics areas for brusellosis infection (CDC, Center of Disease Control and Preventation 2021; Hassan et al. 2022).

There are different types of of *Brucella* such as; *Brucella albis* (sheep, goats), *Brucella abortus* (cattle),

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Brucella suis (feral swine), *Brucella canis* (dogs), *Brucella neotomae* (desert wood rat), *Brucella ceti* (whale, dolphin, pig, seal), *Brucella pinnipedialis* (briar) and *Brucella mictori* (red fox, field rat). The first four species are known to cause a brucellosis in humans (Greene and Carmichael 2006). Human brucellosis is encountered as an atypical acute and chronic febrile illness and is often misdiagnosed as it is clinically similar to other febrile infections such as typhoid fever or malaria. The infection is rarely fatal, but chronic infection is often debilitating and serious complications may occur (Bodenham et al. 2020). In bovine, ovine and sheep, these bacterias are known to cause an infertility by placing in the genital organs such as testis, breast and uterus, as well as causing chronic, necrotic, infectious and inflammatory infections by engaging in mammary glands and placenta. Veterinary public health officials are often called to investigate zoonotic infections with *Brucella abortus* (*B. abortus*), *Brucella melitensis* (*B. melitensis*) and *Brucella suis* (*B. suis*). All of these species are relatively well defined in terms of their impact on human health, diagnosis and treatment. However, the impact of *Brucella canis* (*B. canis*) on public health is not clear yet (Greene and Carmichael 2006).

In humans, *B. canis* infection occurs through contact with contaminated secretions from infected dogs, or as result of bad laboratory handling (de Oliveira et al. 2011; Krueger et al. 2014). Also, it can be venereally or orally transmitted by contact with infected secretions. *B. canis* infection is initially diagnosed using a screening test of the rapid slide agglutination test with 2-Mercaptoethanol (2ME-RSAT). And also, studies recommended the indirect enzyme-linked immunosorbent assay (iELISA) test which detected the level of antigen-specific IgG or IgM antibodies for the confirmatory test. However, the gold standard method is blood culture, but this test has low sensitivity because of the bacteria can be isolated most often from acute infection (Lucero et al. 2005).

In this study, we investigated the prevalence of *B. canis* and *B. abortus* infection in human sera obtained from five regions in which people work with animals such as husbandry, veterian and butcher in Northern Cyprus, and comparatively evaluated the results obtained by agglutination-based techniques using standardized antigens made from *B. canis*.

Materials and Methods

Sample collection

North Cyprus was divided into five regions (Nicosia, Kyrenia, Famagusta, Omorphou and Trikomo), and sera of animal producers, veterinarians and butchers were collected. Study subjects included persons without signs of brucellosis such as fever, nausea, night sweats, headache and low back pain. Since most of the people who have contact with animals were animal breeders in Northern Cyprus population, the key population was designed as the 2% animal producers, 1% veterinarians and butchers.

B. canis and B. abortus strains

B. canis M strain and *B. abortus* S99 strain were obtained culture collections from Dr. Carmicheal and Harran University Faculty of Veterinary, retrospectively. Serum dextrose agar was preperad by adding 5-10% inactive fetal calf serum (Biochrom AG, Berlin, Germany) to tryptic soy agar (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA), basic fuchsin media, safranin media and thionin media were used for production of bacterial strains and biotype controls.

Positive and negative control sera

The positive and negative control sera for *B. abortus* S99 RSAT and ELISA test were obtained from the OIE *Brucella* reference laboratory of the Animal and Plant Health Agency (APHA) in England. The positive and negative dog sera for *B. canis* M-ELISA and RSAT were obtained from Prof. Dr. Silvia Marcela Estein MV Facultad de Ciencias Veterinarias Buenos Aires (Argentina).

Preperation of the B. canis and B. abortus antigen for iELISA

Development of the antigen for the iELISA was performed as previously described (Barrouin-Melo et al. 2007). First of all, the cultures were filtered through steril gauze and centrifuged at minimum 3,500 × *g* for 10 minutes. After the supernatant was discareded, the cells were suspended in phosphate-buffered saline (PBS) (pH 7.4). This step was performed twice. The last pellet was diluted with 10 ml of PBS and autoclaved for 15 minutes at 120°C under 1.5 atmosphere. Then, the cells were centrifuged at 12,000 × *g* for 20 minutes at 4°C. The supernatant was collected and divided into small amounts of sterile cryovial tubes and stored at −20°C for using iELISA solid phase antigen.

B. canis and B. abortus iELISA development

Development of the iELISA was performed as previously described (Alton et al. 1988; Nielsen et al. 2007). A 96-well microtiter plate was coated with 50 μl of the diluted ELISA solid phase antigen which was prepared at optimum antigen concentration and 50 μl of the antigen which was diluted in 0.005 M sodium carbonate (pH 9.6) in antigen coating buffer solution. Antigen-coated plates were incubated at 4°C for 18 to 24 h. The plates were washed five times with PBS containing 0.05% Tween 20 (PBST). Diluted at 1:200 with PBST, positive and negative-control samples and serum samples were added in duplicate in microtiter plate, and incubated for 1 hour at room temperature. Between each reaction step, the plates were washed five times with PBST. The protein A/G conjugate labeled with horse radish peroxidase (HRPO) (ImmunoPure, Pierce Lab., Rockville, MD, USA) was diluted in PBS-T in the ratio indicated in the product's package insert and added in 100 μl to all wells. Incubated for 1 hour at room temperature, the plates were washed five times with PBST. 100 μl

of chromogenic substrate [4.0 mM H_2O_2 and 1.0 mM 2.2′-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) diammonium salt in 0.05 M citrate buffer pH 4.5] was added all plates. After incubating with the substrate, plates were kept at room temperature for 10-15 minutes, and the reaction was stopped with 100 μl of 1 mM sodium azide. The absorbance was determined at 414 nm using a plate reader (VERSA max 3.13/B2573).

Preperation of the B. canis and B. abortus antigen for RSAT

Stock reagents were prepared by considering the Alton method (Alton et al. 1988). Accordingly, stock 1: formalin saline solution [10 ml formaldehyde (37-40%), 90 ml saline (0.85% NaCl)]; stock 2: saline solution (3.5%); stock 3: PBS buffer (pH 7.0) and stock 4: *B. canis* antigen (Alton antigen) were included. For the preparation of stock 4, after inactivation of the 96 hours culture of *B. canis* M (−) strain in the fermenter for 1 hour at 70°C, the culture was centrifuged at 10,000 × *g* for 30 minutes. The supernatant was discarded and the culture was washed three times with 0.5% formalin PBS (95 ml PBS + 5 ml stock 1). The density of the antigen was adjusted to 4.5% and stored at +4°C (Barrouin-Melo et al. 2007). Antigen suspension (3.5% NaCl, 0.06% formalin and 0.2% cell) was prepared by discarding 4.4 ml from 100 ml of main solution (0.6 ml stock 1 + 99.4 ml stock 2) and adding the same amount of stock 4.

Two different diluents were used for dilution of the samples in the study. Diluent 1 (3.5% NaCl, 0.06% formalin) included 100 ml prepared main solution (0.6 ml stock 1 + 99.4 ml stock diluent 2); diluent 2 included discarding 0.7 ml of the 100 ml of main solution (0.6 ml stock 1 + 99.4 ml stock 2) and adding the same amount of 2-mercaptoethanol (2-ME) for prepared the 2-ME added solution (3.5% NaCl, 0.06% formalin, 0.1M 2-ME).

The B. canis and B. abortus RSAT

All serum samples included in the study were tested with latex agglutination test (LAT) without dilution. For this purpose, a drop (0.05 ml) of serum sample was placed on the slide and the same volume of study antigen was added. The mixture is agitated gently for 3 minutes and the results are considered positive by observing agglutination. Samples results as complete and incomplete agglutination were diluated with both diluents 1 and 2 (with 2-ME addition) for prepared the 1/25, 1/50, 1/100 and 1/200 dilutions. In the evaluation of test results with 2-ME, complete and incomplete agglutinations below 1/50 are negative; with agglutination between 1/50-1/100 incomplete agglutinations are suspected; and full agglutination at 1/200 dilution was considered positive. Positive dog antiserum at 1/1,048 titer was used as positive control in the study. It was diluted in the same way (1/25, 1/50, 1/100 and 1/200) and tested in each run.

Statistical analysis

All statistical analyses were performed using SPSS® (Version 13.0 for Windows, SPSS Inc., Chicago, IL, USA). Chi-square test was used to determine the difference between the groups, and when sample size was small, Fisher's exact-test was used. Bivariate correlation was used to measure the strength of the relationship between the groups. Pearson's Correlation Coefficients was calculated to measure the strength and the direction of the relationship.

Ethical approval

Ethics committee approval was obtained for our study with the project number NEU/2015/28-179 at the meeting held by the NEU (Near East University) Scientific Research Ethics Committee on 26.03.2015.

Results

The distribution of the people included in the study by region is given in Table 1. 83.6% (n = 188) of the people included in the study were male, 16.4% (n = 37) were female and the mean age (standart deviation) was 39.44 (±12.80). When the experiences of the professional years of the groups were examined, it was determined that people with 1-10 years of experience constituted in 43.1% (n = 97) of the research group. When the ways/types of contact with the animal are examined, it is seen that husbandry workers

Table 1. Demographic data of the study group.

		n	%
Regions	Nicosia	65	28.9
	Kyrenia	40	17.8
	Famagusta	25	11.1
	Omorphou	49	21.8
	Trikomo	46	20.4
Sex	Female	37	16.4
	Male	188	83.6
Age (years)	18-20	9	4.0
	21-30	53	23.6
	31-40	60	26.7
	41-50	45	20.0
	51-60	49	21.7
	> 61	9	4.0
Work experience (years)	1-10	97	43.1
	11-20	55	24.4
	21-30	47	20.9
	31-40	18	8.0
	> 41	8	3.6
Occupations	Husbandry	112	49.8
	Veterinarian	49	21.8
	Butcher	64	28.4
Total		225	100

represent almost half of the key population (49.8%; 112/225). The remainder of the population consists of veterinarians at 21.8% (49/225) and butchers at 28.4% (64/225) (Table 1).

In serum samples taken from 225 subjects, 3.6% (8/225) was positive by *B. canis* M (−) RSAT, 5.3% (12/225) was positive by *B. canis* M (−) iELISA. 4.4% (10/225) was positive by *B. abortus* S99 RSAT and 9.8% (22/225) was positive by *B. abortus* S99 iELISA. Nine samples were positive by both *B. abortus* S99 RSAT and *B. abortus* S99 iELISA. Seven samples were positive by both *B. canis* M (−) RSAT and *B. canis* M (−) iELISA. One patient was positive by all methods (*B. abortus* S99 RSAT/*B. abortus* S99 iELISA and *B. canis* M (−) RSAT/*B. canis* M (−) iELISA) (Table 2).

B. canis M (−) seropositivity was determined in Kyrenia with the highest rate of 12.5% (5/40) by both RSAT and iELISA. While the seropositivity of *B. canis* M

Table 2. Positive samples with *B. canis* M (−) RSAT, *B. abortus* S99 RSAT, *B. canis* M (−) iELISA and *B. abortus* S99 iELISA.

	RSAT		iELISA	
Samples number	*B. canis* M (−)	*B. abortus* S99	*B. canis* M (−)	*B. abortus* S99
Positive control	+	+	+	+
7				+
12				+
23			+	
30		+		+
31		+		+
63			+	
73		+		+
75				+
88		+		+
96				+
108				+
109				+
119				+
124		+		+
136		+		+
139				+
142			+	
146	+			
148			+	
153			+	
158	+	+	+	+
160		+		+
161				+
163	+		+	
164				+
178				+
179	+		+	
183				+
191		+		
192	+		+	+
203	+		+	
205	+		+	
206	+		+	
224		+		+
Total n (%)	8 (3.6)	10 (4.4)	12 (5.3)	22 (9.8)

RSAT, rapid slide agglutination test; iELISA, indirect enzyme-linked immunosorbent assay.

(−) in the Trikomo region was 0% (0/46) by the RSAT, the seropositivity of *B. canis* was determined as 2.2% (1/46) with the iELISA. The highest and lowest seropositivity of *B. abortus* S99 was determined by iELISA in Famagusta 16% (n = 4) and Trikomo 4.4% (n = 2), respectively. However, the the highest and lowest seropositivity of *B. abortus* S99 by RSAT in Omorphou (6.12%, n = 3) and Famagusta (4%, n = 1), respectively (Table 3).

B. canis M (−) seropositivity rate was 5.4% by both RSAT and iELISA in females. However, seropositivity rate was 3.19% with RSAT and 5.32% with iELISA in males. While the *B. abortus* S99 seropositivity rate was 2.7% by RSAT and 5.4% by iELISA in females, 4.78% was by RSAT and 10.64% by iELISA in males. *B. canis* seropositivity between 31-40 age was found to be the highest age range according to the both RSAT and iELISA. The age between 18-20 were the *B. canis* M (−) seronegative with both methods. Also, people aged 61 and over, and between 18-20 were *B. abortus* seronegative with both methods. The highest seropositivity rate for *B. abortus* strain was found between 31-40 ages, and the rates determined by RSAT and iELISA were 8.3% (5/60) and 15% (9/60), respectively. *B. canis* seropositivity was the highest in veterinarians (6.12%, 3/49) with both methods. And also, the highest rate of *B. abortus* was detected in veterinarians by both RSAT (6.12%) and iELISA (12.24%).

The comparison of RSAT and iELISA methods according to the age and occupations was shown in Table 4. RSAT *B. canis* and RSAT *B. abortus* positivity were compared according to the age groups and occupations, and no significant relationship was found in either. Likewise, no statistically significant results were obtained when iELISA *B. canis* and iELISA *B. abortus* positivity were compared with the age and occupations.

Discussion

The typical Mediterranean climate is dominant (30°C, 10°C for summer and winter months, respectively) in the Northern Cyprus (35°12' latitude and 33°43' longitude). In the Mediterranean where the disease is endemic, prevalence varies depending on such factors as moisture, climate and immunological response of the animal. Determining the seroprevalence of *B. canis* infection in healthy individuals in Northern Cyprus is important in determining the presence of infection in the community. For this purpose, it is aimed to compare the results based on agglutination (RSAT) and iELISA techniques in *B. canis* M (−) and *B. abortus* S99 strains for detection of antibodies to determine the prevalence of *B. canis* in healthy animal-related individuals. Although there is extensive information about *Brucella* infections in the literature, *B. canis* studies in which the study group was humans are extremely rare. Although the brucellosis of *B. melitensis* in Turkey is endemic and sporadic in all regions, the index of *B. canis* infection is unknown (Sayan et al. 2008; de Oliveira et al. 2011). In the Northern Cyprus, there is not enough data to present the current situation.

According to the studies in Turkey, it is highlighted that brucellosis is one of the major community health problems and there is no adequate information about the epidemiology of the disease. According to the study in Turkey between 1984-97, the results of 70,009 blood samples show that the seropositivity was 1.8% in normal population and 6% in the risk groups. According to the results of the same

Table 3. Demographic details of the positive samples with *B. canis* M (−) RSAT, *B. abortus* S99 RSAT, *B. canis* M (−) iELISA and *B. abortus* S99 iELISA.

		RSAT		iELISA	
		B. canis M (−)	*B. abortus* S99	*B. canis* M (−)	*B. abortus* S99
Region	Nicosia	1	2	2	6
	Kyrenia	5	2	5	5
	Famagusta	1	1	1	4
	Omorphou	1	3	3	5
	Trikomo	0	2	1	2
Sex	Female	2	1	2	2
	Male	6	9	10	20
Age	18-20	0	0	0	0
	21-30	2	0	2	1
	31-40	3	5	6	9
	41-50	2	1	2	6
	51-60	0	4	1	6
	> 61	1	0	1	0
Occupations	Husbandry	3	4	6	9
	Veterinarian	3	3	3	6
	Butcher	2	3	3	7

RSAT, rapid slide agglutination test; iELISA, indirect enzyme-linked immunosorbent assay.

Table 4. Comparison of RSAT and iELISA methods with the age and occupations.

		Age	p	Occupations	p
RSAT	*B. canis* M (−)	18-20 21-30 31-40 41-50 51-60 > 61	0.884	Husbandry Veterinarian Butcher	0.768
	B. abortus S99	18-20 21-30 31-40 41-50 51-60 > 61	0.246	Husbandry Veterinarian Butcher	0.671
iELISA	*B. canis* M (−)	18-20 21-30 31-40 41-50 51-60 > 61	0.948	Husbandry Veterinarian Butcher	0.88
	B. abortus S99	18-20 21-30 31-40 41-50 51-60 > 61	0.2	Husbandry Veterinarian Butcher	0.66

RSAT, rapid slide agglutination test; iELISA, indirect enzyme-linked immunosorbent assay.

study, researchers reported that 1,1750,000 people were exposed to *Brucella* (Yüce and Alp-Çavuş 2006). Therefore, researches thought that the data about brucellosis cases that are provided to Ministry of Health previously were very low, and as a result of this study, researchers strongly highlighted that the cases with brucellosis is higher than what is considered (Yüce and Alp-Çavuş 2006). According to the association of public health experts, Turkey Health Report stated that between 2015 and 2018, human brucellosis cases were 4,173 in 2015; 5,148 in 2016 and 6,457 in 2017. Also, the morbidity rate was reported 5.30% in 2015; 6.45% in 2016 and 7.99% in 2017 (Turkish Ministry of Health 2017).

Brucellosis in humans can occur in any age groups, but most of the cases are found in men between the ages of 20 and 40 years. This is generally associated with occupational hazards in young men. All people living in endemic areas have the same risk as healthy people of acquiring brucellosis (Yüce and Alp-Çavuş 2006). According to our study results, *B. canis* seropositivity was 2.3 times higher than women and the age range of the *B. canis* seropositivty was between 31-40.

Serological studies for *B. canis* in patients with findings consistent with brucellosis in 2011 revealed a seropositivity of 8.9% with RSAT and 3.7% with 2-ME RSAT (Sayan et al. 2011). In the literature, the people who are under high risk for brucellosis are the people who have animal-related occupations like veterinarian, butcher and farmer. According to the seroepidemiologic studies, the seropositivity in these people who have higher risk was found to be 8.6-25% and in people who have no risk was found to be 0-8% (Kalkan et al. 1999; Kıyan et al. 1999; Altındiş 2001; Sayan et al. 2011). Brucellosis was observed less in some countries, and the main reason for this brucellosis was reported as occupational disease. For most of the cases, the main sources of brucellosis were *B. abortus* and *B. suis*, and for fewer cases the source of brucellosis was *B. melitensis* (Corbel 1997; Young 2005). Although the rate of *B. canis* infection is rarely reported in humans in the literature, it is stated by Lucero et al. (2010) that *Brucella* infection may have higher rates.

In a study using MAT test on 101 dogs in Northern Cyprus, the seropositivity rate of *B. canis* was determined as 0.0% (0/68) in female dogs, while the seropositivity rate of 1:160 titers in male dogs were determined as 3.03%. The selected people for this study were people who were

living in the countryside and 66.7% of these people were dog owners. The reason for the low rate of seropositivity we determined in our study may be due to the fact that Cyprus is an island country and therefore has controlled entry to the island (Ergene et al. 2019). As the results for bacterial zoonoses, island countries show a lower prevalance in general. In addition, our results are also compatible with those showing lower prevalence in bacterial zooneses for *B. canis* which seems to be ideal for Cyprus, as it is an island country.

While there are antibodies for routine serological tests for *B. abortus*, *B. suis* and *B. melitensis* to diagnose human infections, however, there are no antibodies used to detect *B. canis* in diagnostic laboratory routines. Antibodies used in other brucellosis types are "smooth phase" antigens ("flat" lipopolysaccharides in the cell wall). Since the cell wall of *B. canis* differs from other species where it has the "coarse" antigen, specific antigen must be used to detect the antibody (Wallach et al. 2004). *B. canis* M (+) has hydrophilic and mucoid properties. As M (−) variant is less mucoid and does not produce auto-agglutination, M (−) is used for serologic diagnosis for this study (Wallach et al. 2004; Lucero et al. 2005).

In this study, the seroprevalence was 3.6% with RSAT *B. canis* M (−), 4.4% with RSAT *B. abortus* S99, 5.3% with ELISA *B. canis* M (−) and 9.8% with ELISA *B. abortus* S99 in healthy subjects with no brucellosis. According to our results, it is possible to say that ELISA techniques are more sensitive than RSAT. Since our study results are not sufficient to show *B. canis*'s situation in Cyprus, we think that the seropositivity rates that we have obtained may help to provide an idea about the condition of the disease. Epidemiological studies are also important in helping to understand the prevalence of infection as well as in introducing preventive measures. Determining the prevalence of *B. canis* in healthy people who have contact with animals in Northern Cyprus is important in determining the prevalence of infection in those living in that country.

The most important reason why the relationship between infection rate due to *B. canis* in humans and the number of reported cases can not be correctly explained is that the antigen used in the routine serologic diagnosis of brucellosis can not detect *B. canis* infection. The divergence of the diagnostic ranges for the tests, the diversity in the tests, the lack of common protocol and having a different research group lead to difficulties in interpreting the results of the research. Given the possibility of continuing contamination by dogs for at least two more years, taking precautions to prevent infection from the clinically healthy dogs and getting early diagnosis is important. By examining the individuals who are under the risk groups, more detailed information on the condition of the disease in Cyprus will be obtained.

In brucellosis assay in humans, it is important to bear in mind that there is a possibility of infection with *B. canis*, which has a brucellosis-like symptoms and a negative reaction with Rose Bengal Plate Test (RBPT). Therefore, it is important to evaluate patient samples with RSAT and iELISA in strains containing rough lipopolysaccharides (R-LPS). Until the notification system gives better results to the Ministry of Health, in order to reach the real data for Northern Cyprus, multicenter prevalence determination studies should be done for future.

Conflict of Interest

The authors declare no conflict of interest.

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