In Iraq, First Documentation of Canine Brucellosis by Application of Three Techniques (Rapid test, Indirect ELISA and 16S rDNA Inter-spacer PCR)

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Abstract

Brucella canis is a Gram-negative organism infecting, mainly, the genital organs of both sexes and resulted in several reproductive problems. This bacterium is excreted in urine, milk, fetuses or semen of infected dogs, and transmitted to sensitive dogs through sexual, oral, nasal and conjunctival routes. In general, the routine detection of infection is done by serological tests and the confirmation through isolation of causative agent by the culture. Previously, many global studies documented the excellent efficacy of polymerase chain reaction in detecting the bacterial DNA, perfectly and high accuracy. The present study is the first Iraqi document that dealt with the diagnosis of *B. Canis*, serologically by Rapid test and Indirect ELISA test, and demonstration the infection, molecularly, in seropositive dogs by "16S rDNA inter-spacer PCR" technique. Serologically, the study revealed that 14 (5.76%) and 31 (12.76%) dogs were positive with Rapid test and Indirect ELISA, respectively, while only 5 (16.13%) dogs were positives molecularly. As well as, the received serologic data exhibited that all positive samples with rapid tests were, also, positives by indirect ELISA. At level of $P \leq 0.05$, the statistical differences were reported within the applied techniques, positive dogs with most common risk factors (sex and age). In relation to sex, the results appeared that the females had an infection rate more than males; while in association to age, the infection rates were similar in both first groups (<4 and 1-4 years) and increased apparently in the last aged group (> 4 years).

Keywords: *Brucella canis*, Iraq, Rapid test, Indirect ELISA test, 16S rDNA inter-spacer PCR

في العراق ، التوثيق الاول للبروسيلا الكلابية باستخدام ثلاث تقنيات (الاختبار السريع والاليزا

الغير مباشرة وتفاعل البلمرة المتسلسل 16S rDNA Inter-spacer)

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الخلاصة

البروسيلا الكلابية هي بكتيريا سالبه لصبغة كرام تصيب وبشكل رئيسي الاعضاء التناسلية لكلا الجنسين مسببة مشاكل تكاثرية عديدة . تطرح هذه البكتيريا مع بول وحليب واجنة او حيامن الكلاب المصابة لتنتقل بشكل مباشر او غير مباشر الى الكلاب الحساسة للاصابة عن طريق الجماع او من خلال الفم والانف وملتحمة العين . في العادة يتم التشخيص الروتيني للاصابة من خلال الفحوصات المصلية ويتم تأكيدها من خلال عزل المسبب

بواسطة الزرع . سابقا ، وثقت عدة دراسات عالمية كفاءة اختبار تفاعل البلمرة المتسلسل العالية في تحديد الحامض النووي الرايبوكسي للبكتيريا بشكل مثالي ودقة عالية . تعتبر الدراسة الحالية اول وثيقة عراقية تعاملت مع تشخيص البروسيلا الكلابية في الكلاب ، مصليا ، بواسطة الاختبار السريع واختبار الاليزا الغير مباشر وبرهنتها ، جزيئيا ، في الكلاب الموجبة مصليا باستعمال تقنية " تفاعل البلمرة المتسلسل 16S rDNA " . مصليا ، كشفت الدراسة الحالية ان 14 (5.76%) و 31 (12.76%) كلبا كانت موجبة للاصابة مع الاختبار السريع واختبار الاليزا الغير مباشر ، على التوالي ، في حين كانت فقط 5 (16.13%) من الكلاب موجبة جزيئيا . اضافة الى ذلك ، فقد اظهرت البيانات المصلية التي تم الحصول عليها ان كل العينات الموجبة مع الاختبار السريع كانت موجبة ايضا مع اختبار الاليزا الغير مباشر . عند مستوى P≤0.05 ، سجلت الاختلافات الاحصائية فيما بين الطرق المستعملة ، الكلاب الموجبة للاصابة مع عوامل الخطورة الاكثر اهمية (الجنس والعمر) . فيما يتعلق بالجنس ، اظهرت النتائج بان الاناث تمتلك معدل اصابة اعلى من الذكور ، اما فيما يخص العمر فقد كان معدل الاصابة متشابها في كلا الفئتين الاوليتين (4 and 1-4 years >4) ويزداد بشكل جلي في الفئة العمرية الاخيرة (4 years <) .

الكلمات المفتاحية : البروسيلا الكلابية ، العراق ، الاختبار السريع ، اختبار الاليزا الغير مباشر ، تفاعل البلمرة المتسلسل 16S rDNA Inter-spacer

Introduction

Brucella canis is a significant intracellular facultative bacterium that infecting, mainly, dogs as well as other domestic and wild animals throughout the world, resulting in an incurable canine brucellosis (**1**). Worldwide, the isolation of *B. canis* was done, firstly, by (Carmichael, 1966) in United States, and then the organism identified in many continents and countries such as South and Central America, Europe and Asia (**2, 3**). In Asia, *B. canis* had been reported in India, Pakistan, Philippines, Taiwan, Korea, Japan, China, Malaysia, Turkey and Iran (**4, 5**). Although the actual incidence of canine *B. canis* isn't completely known, it becomes more detectable because of increasing the attention about infection, advancement and increasing the efficacy of the diagnostic techniques (**6**). Generally, *B. canis* has an extreme confusion in clinical signs and the majority of infected dogs seem, apparently, healthy resulting in misdiagnosed or under detectable infection (**7**). The definitive diagnosis of can be obtained through isolation of the organism (gold standard), with expecting the difficulties that comes from the fluctuant levels of bacteremia; with prolong period, risky adventurous and

insensitivity of cultural examinations (**8, 9**). Several serological methods are available for detection of canine brucellosis, which have, solely, limitations and variations in their sensitivities and specificities due to the cross-reactions that occur between *B. canis* with other gram negative bacteria (**10**). Globally, Rapid test is considered as one of the most an effective rapid field detectable method that practically due to its simplicity, rapidity, capability of performance it by veterinarians, and the high sensitivity and specificity in about 95.8 and 99.7%, respectively (**11**). As well as, the indirect ELISA has been developed to overcome most troubles due to the cross-reactions or vaccination and the high capability in detection of acute and chronic infections (**12**). The sensitivity and specificity of this technique is high, may reach 100 % especially with indirect ELISA, and consider an excellent remarkable serological test in diagnosis of brucellosis in most animals (**13**). The methods of DNA amplification as polymerase chain reaction (PCR) have proven to be a rapid confirmative tool, alone or with any of classical diagnostic and serological methods, in detection of brucellosis from clinical specimens such as blood, serum, milk,

urine, tissues and organs **(14, 15)**. PCR characterized by the high sensitivity and specificity with the speeding in performance, reducing the risk of exposure and simplifying necessary infrastructure requirements **(16)**.

The main goals of this study was to demonstrate an existence of IgG antibodies against *Brucella canis* with confirmation of infection by applying of molecular technique (16S rDNA Inter-spacer-PCR); and to evaluate the associations between positive infections with sex and age factors.

Material and Methods

 1- Samples

During about 14 months (August 2014-October 2015), 243 herder dogs of several rural areas in Wasit province, were submitted for this study. These dogs include both sexes (164 females and 79 males) and divided into three age groups; less than 1 year (83), 1-4 years (56) and more than 4 years (61) dogs. From each dog, 3-5 ml of blood samples were obtained from cephalic vein and packaged in sodium citrate tubes that submitted for centrifugation for serum isolation. The serum samples installed in special numbered 1 ml micro-tubes and kept at frozen **(17)**.

 2- Techniques

   2-A- Serological tests (Rapid test and Indirect ELISA test)

Every serum's sample was examined by, a commercially, rapid test kit (Anigen / Korea - Catalog Number: RB21-03), as well as an application of indirect ELISA (MyBioSource/Canada - Catalog Number: MBS748704). Both tests are licensed for detection of anti-*Brucella canis* IgG in dogs. The rapid test is qualitative immunochromatographic assay consists of lipopolysaccharide as a capture and monoclonal anti-canine IgG as detector, which gives the result in about 20 minutes with 93%

sensitivity and 100% specificity; whilst the indirect ELISA kit is a quantitative competitive immunoassay utilizing monoclonal anti-IgG antibody based on solid-phase technology, and intended for screening the specific IgG antibodies to *B. canis* in canine serum or plasma with 97% sensitivity and 100% specificity **(4, 13)**.

2-B- PCR based on 16S rDNA Inter-spacer

Polymerase chain reaction 16S rDNA Inter-spacer was used as a confirmatory tool for identifying and genotyping of *Brucella canis* in whole blood of naturally infected dogs. For this purpose, the commercial Genomic DNA Purification Kit (*Fermentas*) was applied to extract DNA from blood of seropositive samples; and a commercial (*quantit™ dsDNA HS Assay Kit, Lot 55810ᵃ, Invitrogen*) kit that used for DNA quantification and the results were read by the Fluorometer (*Fluorometer Qubit, Invitrogen*). Two sets of primers were used for 214bp (ITS66: ACATAGATGCCAGGCCAGTCA) and (ITS279: AGATACGGACCGAACGCTAC), and for 774 (BME11426: TCGTCGCTGGACTGGATGAC) and (BME11427: ATGGTCGGC AACGTGCTTTT). Reactions were considered positive for *B. canis* when they produced unique PCR products of 214 bp but products of 214 and 774 bp were considered positive for other *Brucella* species.

 3- Statistical analysis

All data introduced, arranged and tabled by using the Microsoft Office Word and the Microsoft Office Excel, (2013). The positive values of the applied tests had been compared between them, as well as, with the reliable risk factor's results (sex and age) and analysed by Chi-square test of

the IBM SPSS (version, 23) program at a level of P<0.05 **(18).**

Results

Serum samples of 243 dogs were tested by using two serological tests (Rapid test and indirect ELISA) and the results of positive *B. canis* dogs were 14 (5.76%) and 31 (12.76%), respectively, (Table 1).

Table (1): According to Diagnostic Techniques, Total Positives for (243) Dogs

Technique		Positives		Negatives	
		No.	%	No.	%
Serology	**Rapid Test**	14	5.76 [b]	229	94.24
	Indirect ELISA	31	12.76 [a]	212	87.24

Vertically, variation in small letters refers to a significant difference at level P ≤ 0.05

In (Table 2), all positive sample's numbers which obtained by the applied serological tests and yielded that all positive samples with Rapid test were positives with Indirect ELISA.

Table (2): Numbers of All positive samples by Serological Tests

Technique		Sample's Numbers
Rapid Test	14	13, 20, 34, 39, 51, 97, 122, 145, 152, 153, 180, 199, 203, 206
Indirect ELISA	31	4, 13, 18, 20, 34, 39, 51, 73, 92, 93, 97, 103, 122, 144, 145, 146, 152, 153, 178, 179, 180, 199, 203, 206, 217, 221, 222, 223, 224, 231, 239

PCR based on detection of 16S rDNA primers was performed, only, on the seropositive samples that included (31 positive samples) and the results were 5 (16.13%).

Figure (1): Positive Samples at 214 bp by 16S rDNA Inter-spacer PCR

Table (3): Total Results of PCR Test for All Seropositive Samples

Technique	Total Tested No.	Positives		Negatives	
		No.	%	No.	%
PCR	**31**	**5**	**16.13**	26	83.87

The overall positive dog's samples by all used tests which revealed on 14, 31 and 5 positive samples with Rapid, Indirect ELISA and 16S rDNA PCR, (Figure 2).
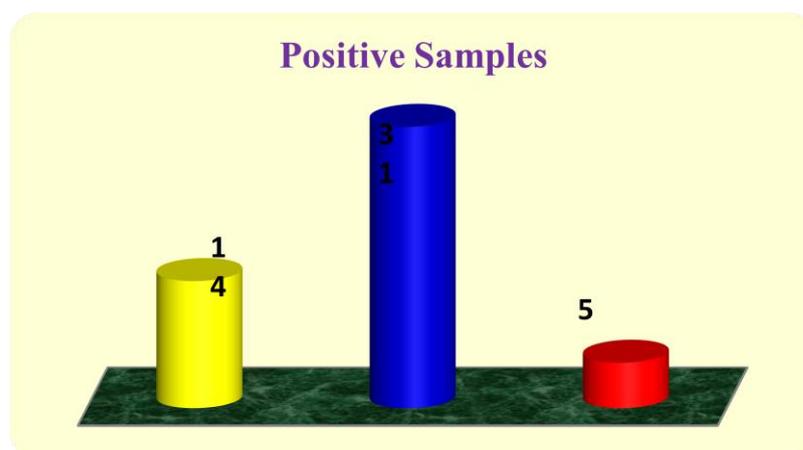

Figure (2): The Overall Positive Samples by the Used Tests

Table (4) was got the correlation between the most important risk factors (sex and age) with the positive *B. canis* infection by the applied techniques. In relation to sex factor, the results show that females had 10/164 (6.1%), 27/164 (16.46%) and 4/164 (3.66%), while the males had 4/79 (5.06%), 4/79 (5.06%) and (0) in Rapid test, Indirect ELISA and PCR, respectively. Whereas, in association to age factor, *B. canis* infection results were, in less than 1 year was 4/83 (4.82%), 8/83 (9.64%) and (0); in 1-4 years 3/56 (5.36%), 5/56 (8.93%) and 1/56 (1.79%); and in more than 4 years 7/61 (11.48%), 18/61 (29.51%) and 4/61 (6.56%), with Rapid test, Indirect ELISA and PCR, respectively.

Table (4): Correlation between Risk factors (Age and Sex) with Positive *B. canis* Infections

Factors			Rapid Test (14)		ELISA (31)		PCR (5)	
		No.	No.	%	No.	%	No.	%
Sex	Females	164	10	6.1 [b]	27	16.46 [a]	5	3.05 [a]
	Males	79	4	5.06 [b]	4	5.06 [b]	0	0 [b]
Age	< 1 year	83	4	4.82 [b]	8	9.64 [b]	0	0 [b]
	1- 4 years	56	3	5.36 [b]	5	8.93 [b]	1	1.79 [b]
	> 4 years	61	7	11.48 [a]	18	29.51 [a]	4	6.56 [a]

Vertically, variation in small letters, for each factor, refers to a significant difference at level $P \leq 0.05$

Discussion

Mainly, *Brucella canis* might attacks large number of mammalians as well as humans, giving it special socio-economic effects (**19**). The affliction with this organism can result in a lifelong infection in dogs and the signs, which like for many diseases, required many months to appear (**20**).

Nonetheless, almost chronic dogs' infections could persist without any clinical signs of infection and act as a source for spreading of disease to other animals and humans (**21**). The direct detection of *B. canis* was remained complicated overwork, related to troubles, expensive and dangerous for

laboratorial workers and veterinarians **(22).** The indirect tests that depended on detection of specific antibodies or antigens in blood or other specimens had been developed as alternative methods for control-eradication programs and in epidemiological studies **(23, 24).** Although, serological techniques were cheap, rapid and high in sensitivity, but lack required specificity and need to supporting confirmatory tests characterized by both, sensitivity and specificity, thereby eliminating the false-positive reactions that common in certain other bacterial species due to the lipopolysaccharide antigens can cross-react with *B. canis* antigens **(10, 25).** The modified rapid and indirect ELISA tests, which used in this study, had been reported an efficacy, to some extent, in detection of specific antibodies against *B. canis* with presence of priority for indirect ELISA. The presence or absence of variations between serological tests might be related to degrees of sensitivity and specificity of each method, cross-reactions between *B. canis* with other species in *Brucella* genus or other negative bacteria, test's facilities and technician skills **(26).** To ensure and confirm the positive results received by both serological assays applied in this search, a molecular technique was employed. In recent years, several studies were carried out to evaluation of PCR in diagnosis a specific DNA for *B. canis* in depending on different samples **(27, 28). Aras and Uçan, (2010)** demonstrated that PCR technique had a detectable effectiveness equally for bacteriological culture in diagnosis of brucellosis with extra advantages including the fastness, speediness in performance, absence of riskiness with the highly sensitivity and specificity.

Also, he demonstrated that all negative culture samples were negative by PCR and suggested that the method can be applied "as a gold standard with sensitivity and specificity of 100%" **(29).** Various specific for Brucella were compared and demonstrated that the 16S rDNA was high in sensitivity than others **(30, 31).** The disagreement of 16S rDNA with the serology results might be due to the chronic and relapsing brucellosis, Intra-macrophage localization, nonviable or low numbers of *Brucella* during the late phase of infection, or could interpreted by a fact that" antibody titers remain elevated for a long time after infection, independent of circulating bacteria or DNA, and cross-reactions with the lipopolysaccharide of other bacteria" **(2, 32).** In concerning to sex, the present study showed that the females have an infection rate more than males, especially with indirect ELISA and PCR, and this could because either the ability of one adult male for matting many pitches and transmitting the infection until become infertile or localization and proliferation of *B. canis* organisms in placenta, especially during gestation, due to availability of erythritol **(33).** These results were agreement with **(17, 34, 35)**, and disagreement with **(4).** Also, this study showed that *B. canis* infections were found in all three age groups but increased significantly in (> 4 years) group and this could be due to increasing the exposure to organisms with advancing the age **(31).** Several studies were reported similar findings and showed that canine *B. canis* was an age-dependence **(33, 36, 37).**

In conclusion, the present study was the first one that performed for serodetection of *B. canis* infection in herder dogs of rural areas in Wasit province by using two serological tests

(Rapid test and Indirect ELISA) and confirmation the infection, in whole blood of naturally infected dogs, by using a molecular technique (PCR based on primers of 16S rDNA Inter-spacer). Also, this study showed that there was a correlation between infection with sex and age).

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