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

Hams Hussien Hashim Handool Alfattli
hamshussienhashim@gmail.com; hams.hashim@qu.edu.iq
Clinical Laboratory Sciences Dept. / College of Pharmacology / Al-Qadisiyah University

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 الأول للبروسيلا الكلابية باستخدام ثلاث تقنيات (الاختبار السريع والاليزا والبمركة المتسلسل (16S rDNA Inter-spacer)

همس حسين هاشم هندول الفتلي
فرع العلوم المختبرية السريرية / كلية الصيدلة / جامعة القادسية

الخلاصة

البروسيلا الكلابية هي بكتيريا سالبة لصبغة كرام تسبب مشاكل تكاثرية عديدة. تطرح هذه البكتيريا مع بول وحليب ووجبة أو حيام الكاب المصابة أو من خلال الاصابة. في العادة يتم التشخيص الروتيني للإصابة من خلال الفحوصات المصلية ويتم تأكيدها من خلال عزل المسبب.
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 countries 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 55810^®_, 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: ACATAAGATGCCAGGCCAGTCA) and (ITS279: AGATACGGACCGACGCTAC), and for 774 (BME11426: TCGTCGCTGGACTGGATGAC) and (BME11427: ATGGTCCGGC 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**

<table>
<thead>
<tr>
<th>Technique</th>
<th>No.</th>
<th>%</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid Test</td>
<td>14</td>
<td>5.76</td>
<td>229</td>
<td>94.24</td>
</tr>
<tr>
<td>Indirect ELISA</td>
<td>31</td>
<td>12.76</td>
<td>212</td>
<td>87.24</td>
</tr>
</tbody>
</table>

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

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**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sample’s Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rapid Test</strong></td>
<td>14, 20, 34, 39, 51, 97, 122, 145, 152, 153, 180, 199, 203, 206</td>
</tr>
<tr>
<td><strong>Indirect ELISA</strong></td>
<td>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</td>
</tr>
</tbody>
</table>

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).

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

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

<table>
<thead>
<tr>
<th>Technique</th>
<th>Total Tested No.</th>
<th>Positives</th>
<th>Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR</strong></td>
<td>31</td>
<td>5, 16.13</td>
<td>26, 83.87</td>
</tr>
</tbody>
</table>
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](image)

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>
<thead>
<tr>
<th>Factors</th>
<th>Rapid Test (14)</th>
<th>ELISA (31)</th>
<th>PCR (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>164</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>Males</td>
<td>79</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 year</td>
<td>83</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>1-4 years</td>
<td>56</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>&gt; 4 years</td>
<td>61</td>
<td>7</td>
<td>18</td>
</tr>
</tbody>
</table>

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

**Discussion**

Mainly, *Brucella canis* might attacks large number of mammals as well as humans, giving it special socio-economic effects (19). The affiliation 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 Interspacers). Also, this study showed that there was a correlation between infection with sex and age).

References


enterocolitica O: 9. Clinical and Diagnostic Laboratory Immunology, 12(1), 141-151.


Counterparts. Infection and immunity, 83(12), 4861-4870.


