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RESEARCH ARTICLE

Evaluation of molecular and serological methods for the diagnosis of brucellosis in human

Khaled A. Habeb[1], Inas Saad Mohammed[2] , Ashna Jamal Faik[3]

1. Department of Biology; College of Sciences for Women; Baghdad University.
2. Environmental Research Center; Microbiology and Toxicology Laboratory; University of Technology.
3. Central Public Health Laboratory/ Ministry of Health.

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Abstract

Objective: Diagnosis of brucellosis from human suspect to have brucellosis by serological methods include: Rose Bengal test and ELISA and comparison with PCR technique s in diagnosis of human brucellosis.

Duration and place of study: Samples were obtained from suspected brucellosis patients, referred to many hospitals in Baghdad city, which include: Al-Yarmook, Al-Karama, Al-Shaheed Al-Sadder, Al-Imam Ali (peace be upon him) and Al-Kadhmiya Hospitals, during the duration from (November 2009 to November 2010).

Methodology: A total 178 peripheral blood samples were from patients suspect to have brucellosis. The diagnosis of brucellosis was established by clinical findings confirmed by Rose Bengal test, ELISA, and molecular methods by PCR technique. DNA extraction was carried out using a commercial kit and a laboratory extraction procedure and examined by PCR involving specific primers for *B.melitensis* and *B.abortus* based on IS711 in the *Brucella* chromosome.

Results: We identified 108 samples were positive result by RBT and 10 samples were positive result by ELISA for the detection of IgM (as a result of acute stage) and 22 samples were positive result for the detection of IgG (as a result of chronic stage). When PCR technique was applied to patients blood, 13 patient blood samples were positive, which include: 3 patient blood samples were positive for *B.melitensis* and 10 patient blood samples were positive for *B.abortus.*

Conclusions: The results of present study showed that PCR assay is a rapid and sensitive technique for diagnosis of brucellosis compared to serological methods. However it is more valuable when coupled with serological methods.

Introduction

Brucellosis is a zoonosis that produces severe morbidity in humans, and, is still of both health and economic significance in many developing countries [1]. Brucellosis is a chronic infectious disease caused by bacteria of the genus *Brucella* that affects animals and humans. Each species of *Brucella* has its preferred host: *B. abortus* infects cattle, *B. melitensis* infects sheep and goats, *B. suis* infects swine, *B. canis* infects dogs, and *B. ovis* infects sheep, although they can also infect other animals [2]. The disease exists worldwide, especially in the Mediterranean basin, the Middle East, India, and Central and South America [3].

All *Brucella* species can cause infection in human exception of *B. ovis* and *B. neotomae.* New *Brucella* species pathogenic for humans – *B. cetaceans* and *B. pinnipedialis* – have recently been discovered in marine mammals [4]. Which is transmitted to humans either by direct contact with the infected animals or by consuming infected milk or fresh cheese [1].

Another major infection route is through occupational exposure to infected live stock, ie, inhalation of contaminated secretions of infected animals or contamination through skin cuts or abrasions [5, 6].

The diagnosis of brucellosis is based on clinical picture, epidemiological data, and different laboratory tests, such as bacterial culture, agglutination, and polymerase chain reaction [7, 8]. The serological tests that are used for the diagnosis of brucellosis, despite that they are easy to perform, lack the required specificity, especially in the endemic area of the disease, in patients with a suspected relapse and in patients with a recent history of brucellosis [9]. Moreover, most serological tests can produce cross-reaction with other bacteria [10]. The Rose Bengal test (RBT) or Slide Agglutination test is most commonly used serological test which detects antibodies against *B. abortus*,*B. suis*, *B. melitensis* and *B. canis*-specific antigens. Presently, enzyme linked immunosorbent assay (ELISA) is the most sensitive method for detection of immunoglobulin M (IgM), immunoglobulin A (IgA), and IgG anti-*Brucella* antibodies. *Brucella* enzyme linked immunosorbent assay (ELISA) test was introduced into clinical laboratory for the diagnosis of brucellosis [11]. The ELISA test has been reported to be rapid, highly sensitive, and specific by determining the *Brucella* specific IgG, IgM, and IgA antibodies in blood and CSF [12, 11].

Amplification of DNA by polymerase chain reaction (PCR) has been in use for decades to diagnose several infectious diseases caused by fastidious or slowly growing bacteria. Previous studies have detected the small amounts of *Brucella* DNA in pure cultures of human and animal samples by means of PCR [13, 14].

In this study, we investigated the potential role of the PCR technique in the detection of brucellosis from human using whole blood and compared its sensitivity with serological diagnostic tests.

Materials and Methods

A total of 178 peripheral blood specimens were collected from patients with high suspected of brucllosis, referred to Al-Yarmook, Al-Karama, Al-Shaheed Al-Sadder, Al-Imam Ali (peace be upon him) and Al-Kadhmiya Hospitals in Baghdad. The samples were taken from patient suspected to be with brucellosis after adequate antibiotic treatment, during the period from November 2009 till November 2010.

The diagnosis of brucellosis was established by the presence of a compatible clinical picture [15] including undulant fever, night sweat and serological diagnosis was carried by positive Rose Bengal test titer of ≥1:160 and ELISA test, moreover demographic, occupational, clinical, and risk factor details were recorded for each patient.

Serological tests:

A- Rose Bengal test (RBT):-

The RB test was performed, following the procedure described by Alton *et al*. [16]. The plates were shaken for 4 min and any agglutination that appeared within this time was recorded as a positive reaction.

B- ELISA (Enzyme-Linked Immunosorbent Assay):-

The ELISA was performed by (Axiom in vitro diagnosticum/ Made in Germany). The immunoglobulins of IgG and IgM classes specific to *Brucella* were detected in patients and control sera. The qualitative immunoenzymatic determination for these classes was based on the enzyme linked immunosorbent assay (ELISA) technique. Microtiter strip wells are precoated with *Brucella* antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti- human IgG or IgM conjugate is added. This immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of *Brucella*- specific IgG or IgM antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

DNA extraction from blood samples:

Genomic DNA was extracted from blood of *Brucella* spp. using a Wizard Genomic DNA Purification Kit / Promega – company (USA).

PCR for *Brucella* species:

Oligonucleotide primers specific for IS711 *B. melitensis* and *B. abortus* which were used in this study, were: 5'-AAA TCG CGT CCT TGC TGG TCT GA and 5'-TGCCGA TCA CTT AAG GGC CTT CAT for *B. melitensis* and 5'- GAC GAA CGG AAT TTT TCC AAT CCC and 5'-TGCCGA TCA CTT AAG GGC CTT CAT for *B. abortus*.[17]. PCR assay was performed in a final volume of 25 µl mixture containing 13.75µl~ 14µl H_2O, 5µl 10x PCR buffer, 0.5µl (dNTPs) mix (200 mM), 1.5µl $MgCl_2$, 1 µl for each oligonucleotides *B. melitensis* and *B.abortus* (0.5 µM each), 0.25µl of Taq polymerase, 2 µl of samples DNA. The amplifications were carried

out in a thermocycler USA, with the following steps: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 2 min, 55°C for 2 min and 72°C for 2 min with a final extension at 72°C for 4 min. The last step is (extention 2 or final extention) according to [18]. The products were analyzed by electrophoresis through a 2% (w/vol) agarose gel was performed at 70 V for 60 min, after which the gel was stained with 2 µl ethidium bromide, and DNA fragments were visualized by UV transilluminator at 320 nm and was photographed by polaroid system. Positive and negative controls of PCR were included in each experiment. Negative control, containing all the reagents but lacking template DNA was processed exactly as has been described to monitor for contamination with *Brucella* DNA. All were negative in all experiments. Positive controls with 100 ng of genomic DNA isolated from a suspension of *B. melitensis* and *B. abortus* were also included.

Results

A total of 178 peripheral blood specimens have been collected from suspected brucellosis patients. The diagnosis of brucellosis was established by clinical findings confirmed by Rose Bengal test, ELISA and molecular methods by PCR technique.

The main serological test used for diagnosis of brucellosis is the Rose Bengal test (RBT), total of 178 samples, 91 (51.12%) females which comprise: 61 (67.03%) samples were positive RBT and 30 (32.96%) samples were negative RBT, also 87 (48.87%) males of a total 178 samples, which comprise**:** 47 (54.02%) samples were positive RBT and 40 (45.97%) samples were negative by RBT. (Table 1). All 108 (60.67%) patients were positive by the RBT.

Table 1: Relation between the Age Group and serum of patients determined using RBT (Positive & Negative).

Age Group (year)	Positive		Negative		Chi-square- χ^2 value
	Sample	(%)	Sample	(%)	
10-20	31	28.70	20	28.57	0.093 ns
21-30	50	46.29	25	35.71	2.722 ns
31-40	24	22.22	20	28.57	1.413 ns
> 40	3	2.77	5	7.14	1.330 ns
Total	108	100	70	100 %	--
Chi-square- χ^2 value	--	8.16 **	--	7.83 **	

**** (P<0.01) = highly significant, ns: non-significant.**

Out of 178 (89%) serum samples were detected by RBT revealed 108 (60.67%) positive, whereas 13 (7.30%) samples were positive using PCR. (Figure 1).

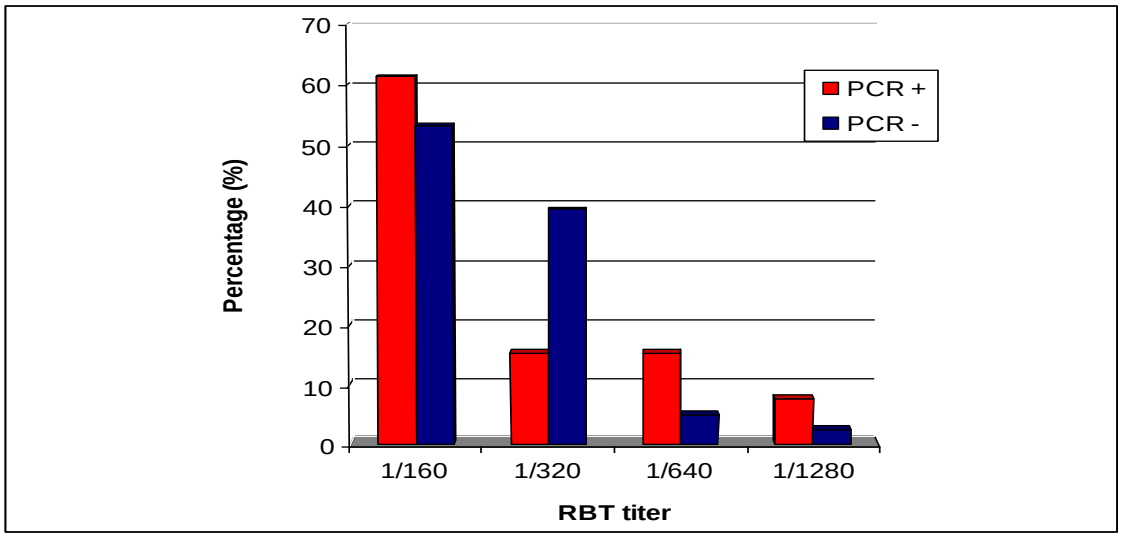

Figure1: The Comparison of *Brucella* antibody titer (RBT) and PCR Result.

Serodetection by ELISA for Anti-*Brucella* IgM and IgG, ELISA test used for diagnosis of brucellosis in human. They were included: acute cases with duration of symptoms less than 6 months and chronic cases with duration of symptoms more than 1 year. Thus, among these patients, 10 (5.61%) were acute cases (IgM) and 22 (12.35%) were chronic cases (IgG) of a total of 178 patients. (Table 2).

Table 2: Results of Acute and Chronic Cases for Different Age Group.

Age Group (year)	**– –**	**– –**	**Clinical Status for Acute Cases**		**Clinical Status for Chronic Cases**	
	No. of Samples	**(%)**	**Sample**	**(%)**	**Sample**	**(%)**
10-20	**51**	**28.65**	**0**	**0.00**	**0**	**0.00**
21-30	**75**	**42.13**	**6**	**60**	**14**	**63.63**
31-40	**44**	**24.71**	**3**	**30**	**4**	**18.18**
> 40	**8**	**4.49**	**1**	**10**	**4**	**18.18**
Total	**178**	**100**	**10**	**100**	**22**	**100**
Chi-square- χ^2 value			**--**	**9.00 ****	**--**	**9.25 ****

**** (P<0.01) = highly significant.**

The comparison between ELISA and PCR indicated some differences in the results. (Figure 2) demonstrates the comparison results of PCR with ELISA. Of these 178 samples, 10 (5.61%) samples were suffering from acute brucellosis and 168 (94.38%) were negative for anti-*Brucella* IgM, whereas 9 (90%) were positive by PCR. While 22 (12.35%) from 178 samples were positive for anti- *Brucella* IgG and 156 (87.64%) were negative for anti-*Brucella* IgG, whereas 13 (59.09%) were positive by PCR.

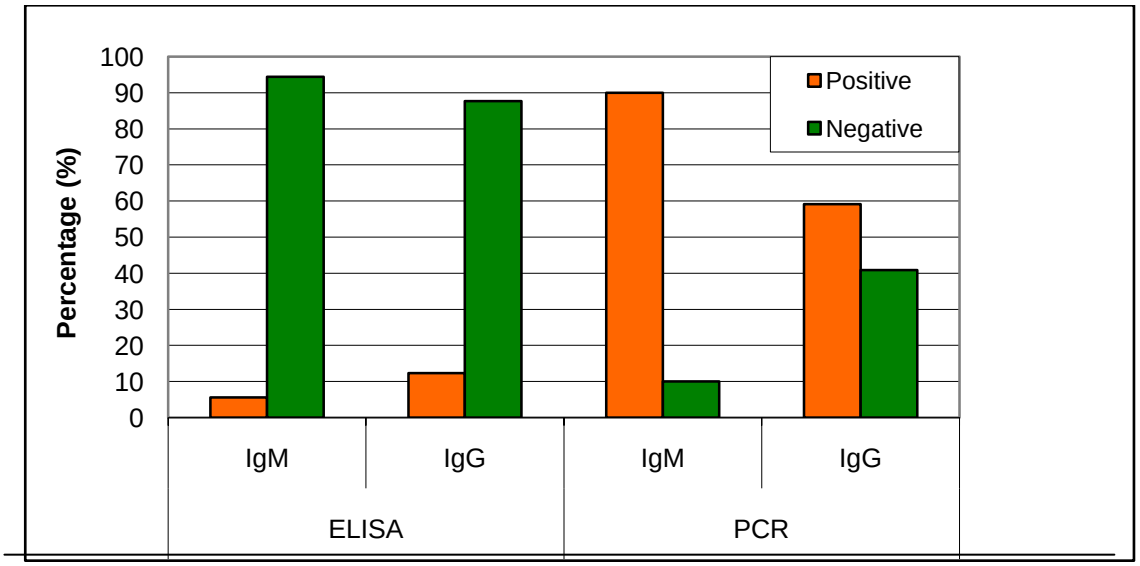

Figure 2: The Comparison between PCR & ELISA for both IgM& IgG.

In present study DNA extracted from 178 human blood samples, 178 (100%) samples were positive results by the Wizard Genomic DNA Purification Kit (Promega – company –USA) was used(Figure 3) and subjected to PCR using IS711 primer for *B.melitensis* and *B.abortus*.

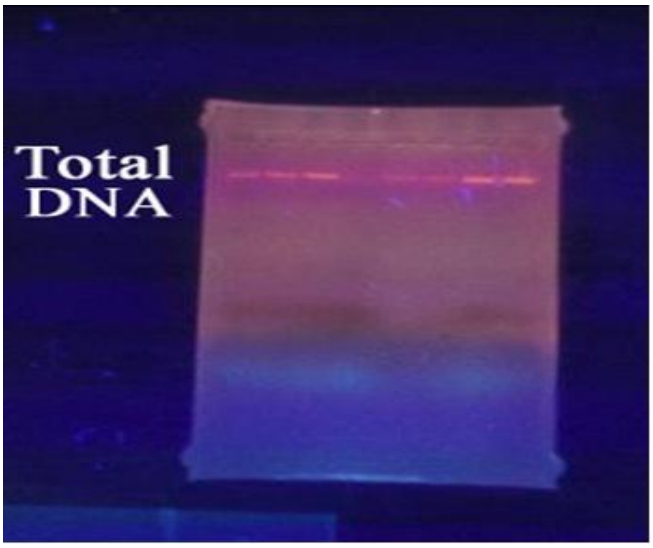

Figure 3: A total DNA extracted from human samples. The seven bands of isolated DNA from human blood. Separated by electrophoresis on 0.8% agarose gel stained with ethidium bromide at 70 volts for 90 min.

When PCR technique was applied to blood samples, 13 (7.30%) werw found positive, which include: 10 (5.61%) were found positive and gave (731bp) *Brucella abortus*, whereas 3 (1.68%) were found positive and gave (498bp) *Brucella melitensis* of total 178 patients. (Figure 4).

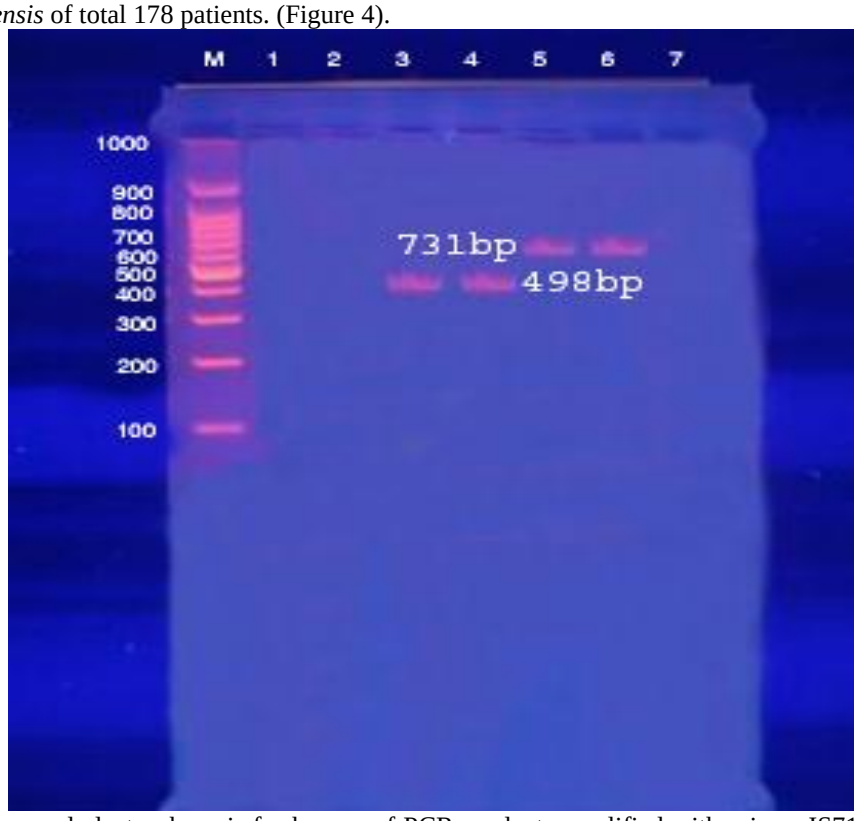

Figure 4: Agarose gel electrophoresis for human of PCR products amplified with primer IS711 *Brucella melitensis* (731bp) and *Brucella abortus* (498bp).

Lane M, molecular weight DNA ladder (100bp), lane 1: negative control. Lane 3: positive control for *Brucella aboruts*, lane 4: positive sample for *Brucella aboruts*, lane 5: positive control for *Brucell melitensis*, lane 6: positive sample for *Brucella melitensis*, lanes 2, 7: negative samples.

Discussion

Brucellosis has a worldwide distribution and remains a major problem in human. To prevent the transmission of *Brucella* infection to new born calf as well as in humans by ingestion of milk of infected animals it is utmost essential that milking animals must be free from brucellosis. The clinical symptoms of brucellosis are non specific and show high
variability. Diagnosis requires microbiological confirmation by means of germ isolation or detection of specific antibodies by serological methods and indirect enzyme linked immunosorbent assay in the diagnosis of acute and chronic human brucellosis. For diagnosis of brucellosis various serological tests are employed with varying degree of sensitivity and
specificity. The serological tests have important limitation due to the prevalence of antibodies in a healthy population from endemic areas and in persons professionally exposed to *Brucella*. The sensitivity and specificity of ELISA for diagnosis of human brucellosis in endemic areas can be high [19] and can generate false-positive results in regions of low endemicity. False-positive results can occur because of cross-reactions with antigens from other organisms, especially *Yersinia enterocolitica* O9 and to a lesser degree with other bacteria with LPS-rich outer membranes, such as *Escherichia coli* and *Vibrio cholerae* [20, 21].

Statistical analysis showed that the 108 (60.67%) patients revealed positive result by RBT and 70 (39.32%) patients negative result for RBT out of 178 patients. In this study occurs in the 10-58 year old age group. The lower prevalence found in children compared with adults may be a result of less exposure of children to the livestock due to their education. RBT of $\leq$ 1/160 is problematic in areas of enedmicity, since low RBT titers may be present in healthy people who previously suffered the disease [22], in patients during the first stage of the infection [10], and in patients suffering chronic brucellosis or a relapse [23], and also for presence of appropriate signs and symptoms, a presumptive diagnosis of brucellosis is usually defined serologically as a RBT titer of 1/160 or greater [24]. Hence statistic showed that seropositive of brucellosis by RBT 108 (60.67%) and it is increase comprised with PCR, thus 13 (7.30%) samples reported that PCR was positive. For analyses these results in this study as non of the available serological test (RBT) conferred unequivocal specificity, new means of diagnosis benefit from genetic tool, based on PCR assay were used but generally RBT is an excellent screening method, it detects infection especially in early stage [25]. Anti-*Brucella* IgG and anti-*Brucella* IgM antibodies were determined by enzyme linked immunosorbent assay. ELISA is a sensitive method for the detection of IgG and IgM anti-*Brucella* antibodies, if applied together [26]. The ELISA results showed that 10 (5.61%) IgM and 22 (12.35%) IgG out of 178 patients were positive, all these results based on ELISA as a confirmatory test after screening using RBT. Patients with active infection have both IgM and IgG agglutination in their serum [27]. But determination of IgG titer is important since low levels of IgM can remain in the serum for weeks to months after the infection has been treated [28]. When we compared between ELISA and PCR, ELISA results showed that (10) of the 178 suspected patients of brucellosis were positive to IgM which mean that the remaining (168) were negative, while PCR revealed (9) positive but remaining only (1) was negative to PCR out of (10) samples. While (22) of the 178 suspected patients of brucellosis were positive to IgG which mean that the remaining (156) were negative, while PCR found (13) positive but remaining (9) were negative to PCR out of (22) samples.

Recently many researchers tried to overcome some of the serological and PCR techniques limitations in diagnosis of brucellosis by the adoption of a combination between PCR and ELISA [29]. Finally ELISA is now widely used for serological diagnosis of the disease in human and other species.

Although most investigators prefer using commercial kits for extraction of *Brucella* DNA [30, 31, 32]. We successful to extract DNA by a commercial kit. We used a laboratory extraction procedure according to Wizard Genomic DNA Purification Kit / Promega – company –USA. Our results showed that the sensitivity of the PCR assay using blood samples for patients and using blood samples for animals was far superior 10 (5.61%) were found positive and gave (731bp) *Brucella abortus,* whereas 3 (1.68%) were found positive and gave (498bp) *Brucella melitensis* for patients. This very good sensitivity, confirm that the PCR assay could be a useful tool for the diagnosis of human brucellosis as other investigators showed by using whole blood [33, 29] serum sample [31].

Finally, in addition to the high yield of the PCR assay for the diagnosis of human brucellosis according to present study, and focal complications in such patients as previously reported [34], other important aspects are: 1) PCR is fast , providing results in 24 hour, which is much less than the time required for conventional methods to rescue a fastidious microorganism such as *Brucella* spp., 2) the technique almost completely obviates the necessity for direct handling of the pathogen , thus drastically reducing the risk of infection of laboratory personnel, and 3) the samples can be stored at -20°C until processing, thus enabling it to be collected by any physician and processed immediately, or else stored and safely sent to another laboratory if necessary. In this study molecular diagnosis of

brucellosis by conventional PCR which consider to newer and superior among other serological tests like: RBT and ELISA was evaluated.

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