In Term of Molecular Technique, Taxonomic and Diagnostic Aspects of Chronic Human Brucellosis in Ramadi City

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Abstract

Background: The diagnosis of chronic brucellosis is frequently difficult to establish. The disease may clinically mimic any infectious and noninfectious disease. This study has been laid down to evaluate PCR technique in the diagnosis of chronic brucellosis in comparison to conventional techniques.

Patients and methods:- One Hundred Forty Four peripheral blood samples obtained from tow group: one hundred twenty four samples from patients with highly suspicion brucellosis and twenty samples from healthy volunteers. The samples were tested by serology using Rose Bengal test (RBT), serum agglutination test (SAT) and 2- Mercapto-ethanol. Blood culture using monophasic blood culture technique, Castaneda biphasic blood culture technique and lysis centrifugation blood culture method. Also, the samples submitted to polymerase chain reaction using primer sets (B4 and B5) to amplify a 223- bp region coding for 31- kDa *Brucella* antigen was achieved. Furthermore all positive PCR samples were submitted to PCR cocktail to differentiate *Brucella* species.

Results:- Out of 124 (86.1%) blood samples from patients with chronic brucellosis, 36 (29.03%) showed strong positive for RBT. On the other hand, 61 (49.2%) cases were positive when SAT≥1/320, 104 (83.9%) cases revealed positive results when the SAT titer ≥1/160 and 118 (95.2%) represented positive cases when SAT titer ≥1/80. Also all blood samples submitted to mono and biphasic blood culture technique were 50 (40.3%) and 64 (51.6%) represented positive results respectively, while only 40 blood samples were submitted to lysis centrifugation blood culture technique, 35 (87.5%) revealed positive results. Also, 103 (83.1%) showed positive results for PCR. Out of these cases, 77 (74.8%) represented positive results (*B. melitensis*), while 26 (25.2%) showed negative cases. Finally, among the twenty (13.9%) controls, serological test, blood culture and PCR were negative.

Conclusions:- The study suggested that combination of Rose Bengal test and serum agglutination test ensured the diagnosis of brucellosis. Also Castaneda biphasic blood culture method had improved the rate of isolation and reduce the period of incubation. Further, lysis centrifugation blood culture technique showed increase in the rate of isolation especially in chronic stage. On the other hand, the current study suggested that PCR has several advantages over the conventional methods for the diagnosis of human brucellosis such as speed, safety, high sensitivity and specificity. Furthermore, PCR is very specific and highly sensitive technique that can be used not only for detection of *Brucella* antigen in any stage of the disease but also in differentiating *Brucella* species by using PCR cocktail which used different sets of primers.

Key words: chronic brucellosis, serology, blood culture, PCR, PCR cocktail.

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Introduction

Brucellosis is a zoonotic infection of domesticated and wild animals caused by organisms of the genus *Brucella*[1].The disease is endemic especially in countries of the Mediterranean basin, the Arabian Gulf, the Indian subcontinent and parts of Mexico and Central and South America. Human brucellosis is found to have significant presence in rural/nomadic communities where people live in close association with animals [2].

Brucellosis is caused by bacteria from genus *Brucellae*. It is Gram-negative bacteria, stained red using modified Ziehl-Neelsen technique and appearing as coccoid or short rod shaped cells from 0.5- 0.7x 0.6-1.5 microns in size[3].They are aerobic, non-motile, non-spore forming, non- toxigenic, non-fermenting, facultative, intracellular parasite[1].*Brucella* spp. are facultative intracellular bacteria that have the ability to avoid the killing mechanism and proliferate within the macrophages, similar to other intracellular pathogens[4].

The onset of human brucellosis may be acute or insidious. The disease is generalized and may involve any organ or system of the body [5].Clinically, human brucellosis divided into subclinical illness, acute or sub-acute disease, localized disease and complications, relapsing infection, and chronic disease[6].The diagnosis of human brucellosis cannot be made solely on clinical grounds due to the wide variety of clinical manifestations of this disease[7].

The diagnosis of patient with possible brucellosis is often missed despite modern and suitable technologies especially in chronic stage of the disease which may be misleading where the conventional diagnostic techniques have serious limitations in chronic stage of this disease [8, 9].Diagnosis of brucellosis requires the combination of several approaches, including medical history, clinical examination, and should always be validated by serological and bacteriological tests and confirmed by molecular methods[10]. The diagnosis of brucellosis traditionally relies on serological test such as Rose Bengal test and serum agglutination test. In general, Rose Bengal test is used as a screening test and the positive results are confirmed by serum agglutination test [11].

The Definitive diagnosis of human brucellosis requires the isolation of the etiologic agent. The bacterial isolation rates are variable depending on the stage of disease, previous use of antibiotics, the clinical specimen, total volume of the sample and last but not least, the culture methods [12].

Molecular methodology offers an alternative way of diagnosing brucellosis. The use of the Polymerase Chain Reaction (PCR) to identify *Brucella* DNA at genus, species and even biovars levels has becoming extended to improve diagnostic tests and a diversity of methods have been developed[13, 14]. Molecular biology as a diagnostic tool is advancing and will soon be at the point of replacing actual bacterial isolation[7].

Patients and Methods

A total of 144 peripheral blood samples were obtained from 124 patients with highly suspicion brucellosis and twenty samples from healthy volunteers over a five months period. Out of 124 patients, 52 were males and 72 were females, aged between 14 and 80 years with mean 36.3 +13.57 years.

All the patients were submitted to serological test (RBT and STA), blood culture techniques (monophasic and biphasic Castaneda blood culture technique) and molecular method (PCR and PCR cocktail). Selected patients 40 (32,3%) were submitted to lysis centrifugation blood culture technique.

Serological test

For serology, blood samples were centrifuged (3000xg for 10 min) and serum stored at –20C°. All sera were evaluated using Rose Bengal test (RBT) and serum agglutination test (SAT). The RBT was done according to the method of [15]. Regarding to SAT, heat killed *Brucella abortus* strain 99 was used as a *Brucella* antigen . the test was performed according to the technique mentioned by Alton and associates,(1988) [16].

In order to confirm the stage of the disease, 2-Mercaptoethanol test (2ME) destroys disulfide bonds of IgM antibodies so only IgG remains as agglutinable antibodies (if present). This test was performed according to the methods mentioned by Young, (2006)[17].

Bacteriological test

Three blood culture techniques were used, monophasic, biphasic Castaneda blood culture technique, and lyses centrifugation blood culture method were used under aseptic condition as mentioned by Brooks and associates, (2004)[18].A loop full was taken from monophasic blood culture bottle and the deposited yield of lysis centrifugation tubes and cultured in to ordinary culture media (*Brucella* agar plates, blood agar plates and MacConkey agar plates) for 24- 48h at 37C° with and without 5- 10% CO_2. The steps of lysis centrifugation blood culture technique were performed according to the method described by Eltemadi and co- workers (1984)[19] and modified by Mantur and Mangalgi, (2004)[20] .

Regarding to Castaneda blood culture technique, the colony appearance of suspected *Brucella* was investigated on the solid phase of Castaneda bottle. After that by bacteriological and confirmatory tests were performed according to Baron and associates (1994)[21].

Molecular methods

All peripheral blood samples were examined for DNA extraction by Promega kit (TM050) which were assayed by PCR amplification process. Different PCR assays and different primer sets were used in the present study. The reagents required for PCR in this study were; PCR pre mix and primers which supplied by Bioneer Company, Korea. PCR pre mix was consisted of DNA polymerase (1U), each dNTPs (250 µM, Tris-HCl (10µM), KCl (30 µM), $MgCl_2$ (1.5µM, and stabilizer and tracking dye. Different sets of primers and different PCR programs were used in this study.

The first PCR assay used primer sets B4 and B5.B4 5`TGG CTC GGT TGC CAA TAT CAA3`and B55` CGC GCT TGC CTT TCA GGT CTG 3`. The second PCR assay used primer sets Ba, Bmel and IS711. Ba 5` GAC

GAA CGG AAT TTT TCC AAT CCC 3`, Bmel 5` TCG CGT CCT TGC TGG TCT GA 3` and IS711 5` TGC CGA TCA CTT AAG GGC CTT CAT3`. The third PCR assay used primer sets Eri1 and Eri2. Eri1 5` GCG CCG CGA AGA ACT TAT CAA 3` and Eri2 5` CGC CAT GTT AGC GGC GGT GA 3`.

Different PCR programs were used in this study, the first PCR assay was used for detection of genus *Brucella*. The following PCR components were added in each PCR tube: B4 (2µl, 5pmole), B5 (22µl, 5pmole), DNA template (52µl), and DDW (112µl)[22, 23].All tubes were placed in the thermal cycler and amplification program started with initial denaturation at 93°C for 4 min, 40 cycles: denaturation step at 90°C at 60 sec, Annealing step at 60°C for 60 sec and Extension step at 72°C for 60 sec. the final extension at 72°C for 7 min. The amplified product was resolved using 2% agarose gel electrophoresis that is stained with redsafe and photographed by photo documentation system [22].

The second PCR assay depends on strain locus specific multiplexing [24]. PCR cocktail was used for detection of *B. melitensis* and *B. abortus* by using primer sets Ba, Bmel and IS711. The following PCR components were added in each PCR tube (20 µl reaction volume): Ba (2 µl, 20 pmole), B mel (2 µl, 20 pmole), IS711 (6 µl, 20 pmole), DNA (4 µl) and DDW (6 µl). All tubes were placed in the thermal cycler and amplification program was initial denaturation at 95°C for 4 min, 35 cycles: denaturation step at 95°C at 1.20 min, Annealing step at 55.5°C for 2 min and Extension step at 72°C for 2 min. the final extension at 72°C for 10 min. The amplified product was resolved using 1.5% agarose gel electrophoresis that is stained with redsafe and photographed by photo documentation system [22].

The third PCR cocktail assay were used primer sets (Ba, Bmel, IS711, Eri1, and Eri 2) to differentiate vaccine strain (*B. abortus* S19). Each PCR tube (20µl) contain: Ba and Bmel (2 µl, 20 pmole), IS711 (4 µl, 20pmole), Eri 1 and Eri2 primers (2 µl, 20 pmole), DNA (4 µl) and DDW (4 µl). These tested by using previously mentioned program (PCR cocktail program) and the PCR products was detected by previous method. Regarding to serological test, blood culture and PCR, all the controls represented negative results.

Results

In the serological part of this study, out of 124 peripheral blood samples obtained from patients with highly suspicion of chronic brucellosis submitted to (RBT) and (SAT). our results revealed that 36(29%) revealed strong positive results for RBT, while 60(48.4%) and 20(16.1%) represented moderate and weak positive respectively. Also, 8(6.5%) revealed negative results see table 1 .

Table (1). The distribution of strong, moderate and weak positive results of slide agglutination test (Rose Bengal test) strong study isolates.

Rose Bengal test result	The results No. %
strong positive	36 (29 %)
Moderate positive	60 (48.4%)
weak positive	20 (16.1%)
negative results	8 (6.5%)
Total	124 (100%)

On the other hand, three diagnostic thresholds were used in this study for STA. When STA≥1/320, 61 (49%) revealed positive results, when STA ≥1/160, 104(83.87%) represented positive cases and when STA titer

≥1/80118(95.2%) showed positive results see table 2.

Table (2). The distribution of positive and negative cases of standard tube agglutination test with three diagnostic thresholds among study isolates.

Standard tube agglutination test	Positive cases No. (%)	Negative cases No. (%)	P Value
STA ≥ 1/ 320	61 (49.2%)	63 (50.8%)	0.857
STA≥1/ 160	104 (83.87%)	20 (16.13%)	0.000
STA≥ 1/80	118 (95.2%)	6 (4.8%)	0.000

The study results of 2ME showed that positive cases observed in 89(71.8%) and negative cases in 35(28.2%).

Regarding to blood culture, three techniques were used in this study, monophasic blood culture method, biphasic Castaneda blood culture technique and lysis centrifugation blood culture method. Our results showed that out of 124 patients with suspected brucellosis, brucellae were isolated in 50(40.3%) and 64(51.6%) for monophasic and biphasic blood culture techniques respectively. On the other hand, only 40 patients submitted to lysis centrifugation blood culture technique where positive results observed in 35(87.5%) cases as see in table 3.

Table (3). Distribution of culture positive and negative cases among all study patients for both of monophasic ad biphasic blood culture techniques and among 40 study patients who submitted to lysis centrifugation blood culture technique.

Type of blood culture technique	Positive cases No. (%)	Negative cases No. (%)	total
1- Monophasic blood culture technique	50 (40.3%)	74 (59.7%)	124
2-Biphasic blood culture technique	64 (51.6%)	60 (48.4%)	124
3- lysis centrifugation blood culture technique	35 (87.5%)	5 (12.5%)	40

In the molecular part of this study, PCR was positive in 103(83%) and only 21 (17%) represented negative cases when PCR assay was performed by primer set (B4 and B5) to indicate the presence or absence of *Brucella* organism as in the following figure.

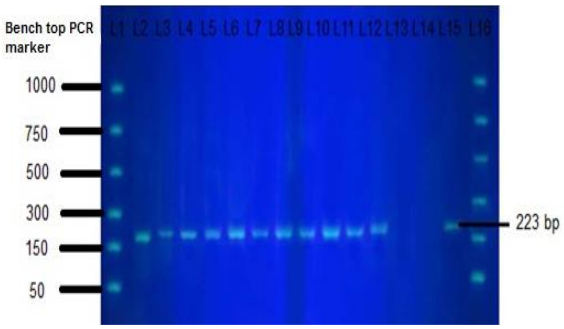

Figure (1). Detection of *Brucella* spp. DNA by polymerase chain reaction using B4, B5 primer set by agarose gel electrophoresis (2%) with Redsafe stain. Lane 1 and 16 represented DNA ladder. Lane 2,3,4,5,6,7,8,9,10,11,12 and 15 represented positive PCR band. Line 13 and 14 represented negative PCR results

All positive PCR cases submitted to PCR cocktail (1) to indicate presence of *B. melitensis* or *B. abortus*. The study results showed that out of 103 positive PCR cases, 77(74.8%) represented positive cases, while 26(25.2%) showed negative bands at specific molecular weight by used primer sets (Bmel, Ba, and IS711). The positive results revealed presence of *B. melitensis* as in following figure:

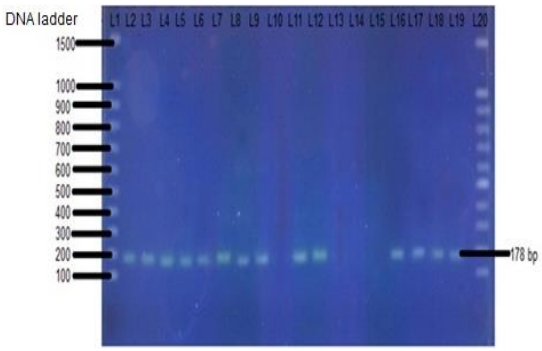

Figure (2). PCR amplification of *B. melitensis* DNA and *B. abortus* DNA by using primer set (Ba, Bmel, and IS711). Lane 1 and 20 represented DNA ladder, Lane 2 ,3 ,4 ,5 ,6 ,7 ,8 ,9 ,11 ,12 ,16 ,17 ,18 and 19: *B. melitensis*, Lane 10,13,14 and 15: neither *B. melitensis* nor *B. abortus.*

In the current study new primer set was used, Eri1 and Eri2 which used to detect and differentiate *B. abortus* S19 vaccine strain. All positive PCR cases submitted to PCR cocktail (2) by using primer sets (Bmel, Ba, IS711, Eri1, and Eri2). Out of 103 positive PCR cases, 77 (74.8%) represented positive results for *B. melitensis* while 26 (25.2%) showed negative results. These results revealed absence of *B. abortus* S19 vaccine strain as in following figure.

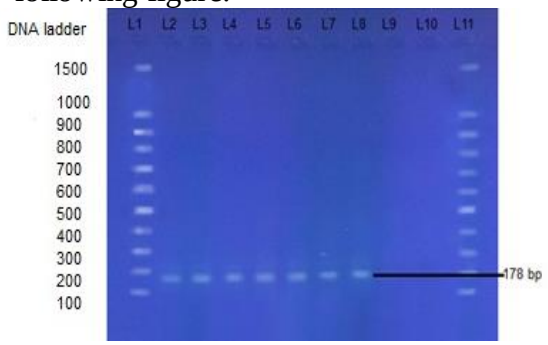

Figure (3). Polymerase chain reaction assay results by using primer sets (Bmel, Ba, IS711, Eri1 and Eri2), Lane 1 and 11 represented DNA ladder. Lane 2, 3, 4,5,6,7 and 8 revealed positive result for *B. melitensis.* Lane 9 and 10 revealed negative results.

Discussion

Brucellosis represents a prevalent disease in humans and animals. The clinical features and presentation of human brucellosis overlap with different infectious and autoimmune or neoplastic processes[25]. Since the symptoms of brucellosis are non-specific, the clinical diagnosis of the disease is difficult[8, 9].

The most popular serological tests are Rose Bengal test (RBT) and serum agglutination test (STA)[12].Regarding to RBT, the study patients grouped in to three group, strong, moderate and weak positive. The sensitivity of this test is often low. RBT test is valuable, easy to use and cost- effective screening test[26]. On the other hand, false negative and false positive represent serious limitation for this test [27].

SAT test, which is cost-effective and easy to use is the most appropriate test especially in regions endemic for brucellosis[28]. In this study three diagnostic thresholds were used, the sensitivity and specificity of STA depends on the cut- off value used, the background level of reactive antibodies in the population and endemicity [11]. The titer of SAT of ≤1/160 is problematic in areas of enedmicity, since low SAT titers may be present in healthy people who previously suffered the disease[29], during the first stage of the infection[15], and in patients suffering chronic brucellosis or a relapse[30]. The SAT titer of <1/160 cannot always be disregarded without follow up. Conversely SAT titer of ≥ 1/160 do not always signify active infection, especially in *Brucella* endemic areas[31].SAT is frequently used as a reference to which other serological tests are compared. Nevertheless, cross- reaction with various bacteria have been reported results in false positive results[32].On the

other hand, the false negative SAT results may occur in patients with a recent infection or whose serum contains blocking antibodies (Prozone) a commonly reported phenomenon in brucellosis[31].

The 2-mercaptoethanol test is taken as evidence for the presence of specific IgG antibodies at which2-ME destroys disulfide bonds of IgM antibodies so only IgG remains as agglutinable antibodies (if present)[11, 17]. This test helps to confirm the stage of the disease and response to the antibiotic treatment. Comparing to SAT and 2ME test results, it was possible to prove which antibodies were present in the sera (IgM, IgG or both)**.** Patients with positive 2ME and SAT positive had only IgG in their sera, while patients with negative 2ME and positive SAT had IgM antibodies (acute stage)[33].

Frania, (1985)[34]concluded that serological tests proved to be either too sensitive giving false positive results, or too specific giving false negative results**,** beside misdiagnosis due to cross reactivity of other gram negative bacteria such as *Yersinia enterocolitica* with smooth *Brucella* species [23].

The blood culture is method of choice, but specimens should be taken in the early stage of the disease. the study results indicated that biphasic Castaneda blood culture techniques more useful than monophasic blood culture method where the high number of positive cases showed by biphasic blood culture method. Also, forty patients submitted to lysis centrifugation blood culture technique, out of them, 35 (87.5%) revealed positive results. This technique is very useful especially in chronic stage of the disease. On the other hand, The incubation of the blood culture takes a long time, between five to 35 days. The isolation of the bacterium is proceeding of the biological hazard for the laboratory staff. Brucellosis is one of the most common laboratory acquired infections [25].

As other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis, since detection of specific DNA is a true indication of the presence of a pathogen[35].PCR, characterized by high sensitivity and specificity, short turnaround time and less hazardous, this method can overcome the limitations of conventional methodology[36]. PCR could be a more sensitive technique than blood cultures and it is more specific than conventional serological test.This technique has to be very sensitive and specific to detect small amounts of microbial DNA in eukaryotic gene material [23].

Numerous PCR based assays and different sets of primers have been developed and evaluated for the identification of *Brucella* species to improve the diagnostic capabilities ranging from general identification of the genus *Brucella* (Genus-Specific PCR assay), that is designed to explicate a single unique genetic locus that was highly conserved in *Brucella* (e.g. BCSP31) to specific identification of the *Brucella* species (differential PCR- based assay), that is depends on strain locus specific multiplexing (e.g. PCR- based on IS711)[37].

The first PCR assay was used for detection of genus *Brucella*, the second PCR assay was used for detection of *Brucella abortus* and *Brucella melitensis*, and the third PCR assay was used for differentiation *Brucella abortus* S19 from other *Brucella* species. Each of these PCR systems produces a discrete DNA

product, whose length is identical for and specific to all *Brucella* species[11].

Regarding the first PCR assay which was used primer sets (B4 and B5), to indicate the presence of *Brucella* organism. This primer sets used for amplification was specific for the genus encoding a 31 KDa *Brucella* outer membrane protein (BCSP31) with molecular weight 233 bp. It is conserved in all species and biovars of *Brucella*[33]. In this study, 103(83.06%) revealed positive results. this result reflect the superiority of PCR compared with traditional techniques. Also, Queipo- Ortuno and co- workers (1997)[38]founded that 100% sensitivity and 98.3% specificity using B4 and B5 primer pair.

PCR could be particularly useful in patients with chronic brucellosis and specific complications such as neurobrucellosis, or other localized infections, since cultural serological testing often fail in such patients [11, 39.]

For species-specific surveillance programs, differential PCR programs and different primer sets are needed [32].In this study all positive PCR cases using B4 and B5 primers were submitted to PCR cocktail for identification of *Brucella* species. In the second PCR assay, the primer sets (Bmel, Ba and IS711) were used for differentiation *B. melitensis* from *B. abortus* at which Bmel primer indicated presence of *B. melitensis* whileBa primer indicated presence of *B. abortus*. PCR cocktail was based on the existence of specific DNA sequence for each species and IS711 copies which found in unique chromosomal location for each of *Brucella* species [33].

In this study, out of 103(83%) positive PCR cases submitted to PCR cocktail, 77(74.8%) revealed positive results for *B. melitensis*, while 26(25.2%) represented negative cases. The negative cases could be explained by many factors such as presence of PCR inhibitors, number of *Brucella* organism below the detection threshold, degradation of target DNA in the samples and inefficient DNA extraction[40].

It is well known that primer cocktail used to identify more number of *Brucella* biovars and also to discriminate between *B. abortus* vaccine strains and other isolates of *Brucella* [41]**.** So, the third PCR assay was carried out using primer sets (Bmel, Ba, IS711, Eri1, and Eri2).Eri1 and Eri2 were used to differentiate *B. abortus* S19 vaccine strain[37]. Regarding to our study there was no *B. abortus* S19 (vaccine strain) detected in patients who submitted to PCR cocktail. These results indicating that all positive samples identified as *Brucella* species were *B. melitensis.* Similar results were previously reported by (Gupta *et al*., 2006; Ica, *et al*, 2012)[40, 42].Regarding to the relationship between STA, blood culture and PCR, the study results reflected the superiority of PCR technique. These results prove the role of PCR as good tool for diagnosis of brucellosis when other tests fail in the diagnosis of brucellosis.

Conclusions

The study suggested that combination of Rose Bengal test and serum agglutination test ensured the diagnosis of brucellosis. Also Castaneda biphasic blood culture method had improved the rate of isolation and reduce the period of incubation. On the other hand, lysis centrifugation blood culture technique showed increase in the rate of isolation especially inchronic stage. Further, the

current study suggested that PCR has several advantages over the conventional methods for the diagnosis of human brucellosis such as speed, safety, high sensitivity and specificity. Further, PCR is very specific and highly sensitive technique that can be used not only for detection of *Brucella* antigen in any stage of the disease but also in differentiating *Brucella* species by using PCR cocktail which used different sets of primers.

References

1- Bret K. Purcell, PhD, MD; David L. .Hoover, MD; and Arthur M. Friedlander, MD. 2009. Brucellosis. In: Medical Aspects of biological warfare.
2- BG Mantur, SK Amarnath and RS Shinde. 2007. Medical journal. 25: 188- 202.
3- MenachemBanai and Michael Corbel. 2010. Taxonomy of *Brucella*. The open veterinary Science journal. 4: 85- 101.
4- Supriya Christopher. Umapathy BL, Ravikumar KL. 2010. Brucellosis: Review on the recent trends in pathogenicity and laboratory diagnosis. Journal of laboratory physicians. 2: 55-60.
5- M. J. Corbel. 2006. Brucellosis in humans and animals. Food and agriculture organization of the united nations. WHO library cataloguing in publication data.
6- Robert A. Salata. 2007. Brucellosis. In: Cecil medicine. By: Lee Goldman, Dennis Ausiello and Saunders Elsevier. Chapter 331. 23 edition. Elsevier Inc.
7- FrenandoPasillaPoester, Klaus Nielsen, Luis Ernesto Samartion and Wei Ling Yu. 2010. Diagnosis of brucellosis. The open veterinary science journal. 4:46- 60.
8- M. Kechagia, S. Mitka, E. Papadogiannakis, V. Kontos and C. Koutis. 2011. Molecular detection of *Brucella* spp. DNA in patients with manifestations compatible with emotional disorders. The open infectious disease journal. 5: 8- 12.
9- Sascha Al Dahouk and KarstenNockler. 2011. Implications of laboratory diagnosis on brucellosis therapy. Expert Rev. Anti-Infect. Ther. 9: 11: 833- 845.
10- Clavijo E, Diaz R, Angita A, Garcia A, Pinedo A, Smits HL. 2003. Comparison of a dipstick assay for detection of *Brucella*- specific immunoglobulin M antibodies with other tests for serodiagnosis of human brucellosis. ClinDiag Lab Immunol. 612- 5.
11- Baddour M.M. 2012. Diagnosis of brucellosis in humans. A review. Journal of verterinary advances. 2: 4: 149- 156.
12- Al Dahouk S., Tomaso, H., Nockler, K., Neubauer, H., Frangoulidis, D. 2003. Laboratory-based diagnosis of brucellosis- a review of the literature. Part II: Serological tests for brucellosis. Clinical laboratory journal. 49: 557.
13- Kattar MM, Zallou PA, Araj GF, Samaha- Kfoury J, Shbaklo H, Kanj SK. 2007. Development and evaluation of real- time polymerase chain reaction assays on whole blood and paraffin- embedded tissues for rapid diagnosis of human brucellosis. Diagn. Microbiol. Infect. Dis. 59: 23-32.

14- Quipo- Ortuno MI, Colmenero JD, Bravo MJ, Garcia- Ordonez MA, Morata P. 2008. Usefulness of a quantitative real- time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis. Clinical microbiology infections. 14: 1128- 34.

15- Young EJ. 1991. Serological diagnosis of human brucellosis: analysis of 214 cases by agglutination tests and review of literature. Rev Infect Dis. 13: 359- 72.

16- Alton GG, Jones LM, Angus RD, Verger JM. 1988. Techniques for the brucellosis laboratory. Institute National de la Research Agronomic. 147. Paris.

17- Edward J. Young. 2006. *Brucella* spp. In: principles and practice of clinical bacteriology. By: Gillespie and Hawky.2nd edition. John Wiely and Sons, Ltd. England.

18- Brooks G, Butel JS, Morse SA, Jawetz, Melnick and Adelberg's medical microbiology.2004. Lange medical books/ McGraw- Hill. 23 edition. 284- 6.

19- Eltemadi H, Raissadat A, Picket MJ, Zafari Y, Vahedifar P. 1984. Isolation of *Brucella* spp. From clinical specimens. Journal of clinical microbiology. 20.

20- Mantur BG, and Mangalgi SS. 2004. Evaluation of conventional Castaneda and lysis centrifugation blood culture techniques for diagnosis of human brucellosis. Journal of clinical microbiology. 42: 9: 4327- 4328.

21- Baron EJ, Peterson LR, and Finegold SM. 1994. In: Baily and Scott's diagnostic microbiology. 9th edition. P: 408- 410. Toronto. C. V. Mosby company.

22- Sambrook, J., Fritsch, E.F. andManiatis, T. (1989). Molecular cloning: a laboratory manual. 2nd Ed., cold spring harbor laboratory press, cold spring harbor, New York.

23- Moussa I. M., Omnia M. E., Amin A. S., Ashgan M. H. and Selim S. A. 2011. Evaluation of the currently used polymerase chain reaction assays for molecular detection of *Brucella* species. African journal of microbiology research. 5: 12: 1511- 1520.

24- Redkar R, Rose S, Bricker B, Del Vecchio V. 2001. Real- time detection of *Brucella abortus*, *Brucella melitensis* and *Brucella* suis. Mol. Cell. Probes. 5: 1: 43- 52.

25- Maida Sisirak and MirsadaHuki, 2009. Evaluation and importance of selected microbiological methods in the diagnosis of human brucellosis. Bosnian journal of basic medical sciences. 9: 3: 199- 203.

26- Mesa, J. D. R., Gonzales, J. S. and Reguera, J. M. 2005. Diagnostic yield and use for the diagnosis of human brucellosis in emergency departments in endemic areas. Journal of clinical microbiology infections. 11: 221- 225.

27- Serra, J. and Vinas M. 2004. Laboratory diagnosis of brucellosis in a rural endemic area in northeastern Spain. International microbiology. 7: 53- 58.

28- OzanPabuccuoglu, TalatEcemis, Sibel EI, Abdullah Coskun, SinemAkcali and Tamer Sanlidag. 2011. Evaluation of serological tests for diagnosis of brucellosis. Jpn journal of infectious disease. 64: 272- 276.

29- Orduna A, Almaraz A, Rado A, Gutierrez MP, Garcia- Pasual A,

Duenaz A, Cuervo M, Abad R, Hernandez B, Lorenzo B, Bratos MA and Torres AR. 2000. Evaluation of an immunocapture- agglutination test (Brucellacapt) for serodiagnosis of human brucellosis. Journal of clinical microbiology. 38: 4000- 4005.

30- Pellicer T, Ariza A, Foz R, Pallares R, Gudiol F. 1988. specific antibodies during relapse of human brucellosis. Journal of infectious diseases. 157: 918- 24.

31- Basappa G. Mantur, Mallanagouda S. Biradar, Rajendra C. Bidri, Mallanna S. Mulimani, Veerappa K., PiaraylalKariholu, Siddanagouda B. Patil and Smita S. Mangalgi. 2006. Journal of medical microbiology. 55: 897- 903.

32- Sascha Al Dahouk, Heinrich Neubauer and Herbert Tomas. 2001. *Brucella*. In: molecular detection of human bacterial pathogens. By: Dongyou Liu. CRC press. Taylor and Francis group.

33- VasoTaleski. 2010. An overview of introducing various laboratory tests for diagnosis of human brucellosis in the republic of Macedonia. Mecedonian journal of medical sciences. 3: 3: 239- 245.

34- Frania R. 1985. Current serological methods in *Brucella melitensis* diagnosis. *Brucella melitensis*. J. M. Verger and M. Plommet (ed) INRA, Nouzilly, France.

35- Falguni Mukherjee, Jainendra Jain, Vipul Patel and Mrinalini Nair. 2007. Multiple genus specific markers in PCR assays improve the specificity and sensitivity of diagnosis of brucellosis in field animals. Journal of medical microbiology. 56: 1309- 1316.

36- Zerva L, Bourantas K, Mitka S, Kansouzidou A, Legakis NJ. 2001. Serum is the preferred specimen for diagnosis of human brucellosis by PCR. 39: 1661- 4

37- Ocampo- Sosa AA, Aguero- Balbin J, Garcia- Iobo JM. 2005. Development of a new PCR assay to identifu*Brucellaabortus*biovars 5, 6 and 9 and the new sub group 3b of biovar 3. Veterinary microbiology. 110: 40- 51.

38- Queipo- Ortuno, M.I., Morata, P., Ocon, P., Manchado, P. and Colmenero, J.D. 1997. Rapid diagnosis of human brucellosis by peripheral blood PCR assay. Journal of clinical microbiology. 35: 2927- 2930.

39- Colmenero JD, Queipo- Ortunoo MI, Reguera JM, Baeza G, Salazar JA, Morata P. 2005. Real time polymerase chain reaction: a new powerful tool for the diagnosis of neurobrucellosis. Journal of Neurol. Neurosurg. Psychiatry. 76: 1025- 1027.

40- Morata P, Queipo- Ortuno MI, Reguera JM, Garcia- Ordonez MA, Cardenas A, Colmenero JD. 2003. Development and evaluation of a PCR enzyme linked immunosorbent assay for diagnosis of human brucellosis. Journal of clinical microbiology. 41 :144- 8.

41- Tuba Ica, FuatAydin, K. SemithGumussoy, DuyguPercin, AhmetBulentSumerkan, FulyaOcak, SecilAbay, H. OkanDogan, ArzuFindik, AlperCiftci. 2012. Conventional and molecular biotyping of *Brucella*strains isolated from cattle, sheep and human. Ankara Univ Vet FakDerg. 59: 259- 264.

42- Gupta VK, Verma DK, Rout PK, Singh SV, Vihan VS. 2006. polymerase chain reaction for detection of *Brucella melitensis* in goat milk. Small Ruminant Res. 65: 79- 84.