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Comparative Study Between Serological and Molecular Techniques in Diagnosis *Brucella melitensis*

A Thesis

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا
إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ
الْعَلِيمُ الْحَكِيمُ(٣٢)

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I would like to express my profound thanks to our God for his grace

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Zahraa Safaa

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List of Abbreviations

Abb.	Meaning
A.D	After Century.
Api20	Analytical Profile Index 20.
B.C	Before Century.
Bp	Base pair.
BrLPS	*Brucella* lipopolysaccharides.
BSL-3	Biosafety level-3.
C°	Degree Celsius.
dNTPs	Deoxynucleoside triphosphate.
DNA	Deoxyribonucleic acid .
DCs	Dendritic cells.
ELISA	Enzyme Linked Immuno Sorbent Assay .
EDTA	Ethylene diamine tetra acetate.
GC	Guanine cytosine .
HOOF	. Hyper Variable Octomeric Oligonucleotide Finger-Prints.
IL-	Interleukin.
IgM	Immunoglobulin M .
IgG	Immunoglobulin G **.**
LPS	lipopolysaccharides.
Mb	Mega base.
MLVA	multiple locus variable number analysis.
Ml	Milliliter.
µm	Micro mil litter.
NCBI	National Center Biological Information.
OIE	Office for International des Epizootics .
Omps	outer membrane proteins**.**
OPS	O polysaccharide.
ORFs	open reading frames.

PAMPs	pathogen-associated molecular patterns.
PCR	Polymerase chain reaction .
pH	Potential of hydrogen .
qPCR	Quantitative Polymerase Chain Reaction .
RNAP	RNA polymerase β sub unit.
RNA	Ribonucleic acid.
RBT	Rose Bengal Test .
Sec	Second.
Spp	Species .
TLR	Toll-like receptors.
Th1	T helper cell type 1.
Th2	T helper cell type2.
TBE	Tris borate EDTA buffer.
UV	Ultra violet .
Vir	Virulence factor.
VNTR	variable number of tandem repeats.
%	Percent.
WHO	World Health Organization.

Summary

Brucellosis is widespread zoonotic disease that afflicts both human and animals and the clinical signs and symptoms of human brucellosis are different and non –specific diagnosis of brucellosis depended on a positive assessment on laboratory based testing **.** The present study was designed to assess the validity of serological tests (Rose Bengal(RBT) and ELISA tests) and Polymerase Chain Reaction assay for diagnosis of brucellosis.

A clinical manifestation of fever cases included symptoms such as a temperature greater than 38C° on several occasion within three weeks. In patients suffering from Malta fever and according to recommendation of Ministry of Health to conduct this problem in Iraq. Blood samples were collected from the 50 patients during November 2017 to April 2018 after inform all patients about the aim of this study as a permission for ethics. Blood sera were prepared to conduct the serological tests (RBT and ELISA) then the *Brucella* was isolated on selective media such as *Brucella* agar media which confirmed molecularly by using Real-time PCR to amplify the primer of 16SrRNA *gene* and finally the method of *gene* sequencing was used to detect the species and biovars of *Brucella* by using the *rpoB gene*.

The results of screening (serological) test by using RBT showed that the occurrence percent of brucellosis in human was 45/50 (90%), while the results of ELISA was 42(84%). On the other hand ,the result of bacteria isolation on *Brucella* agar was 10(20%) , and the results of Real-time PCR was 38(76%) as a confirmation test for *Brucella melitensis* isolates after extraction of DNA and amplification of primer belong to 16SrRNA gene .

The results of *rpoB gene* amplification for *Brucella melitensis* revealed the appearance of one distinct band after electrophoresis on agarose gel that had a molecular size 1091bp .

PCR product of *rpoB gene* was used for partial sequencing and blotting the phylogenetic tree of local isolates in comparison with some pathogenic standard world strains .Five local isolates were submitted to Gene Bank- NCBI for registration of sequences of *rpoB gene* .

The Gene Bank- NCBI gave a code accession numbers which were (Banklt 2126555 Seq1 MH523634), (Banklt 2126555 Seq2 MH523635), (Banklt 2126555 Seq3 MH523636), (Banklt 2126555 Seq4 MH523637), (Banklt 2126555 Seq5 MH523638) .The phylogenetic analysis of five local isolates of *B. melitensis* showed a close related (100%) to NCBI -BLAST *B. melitensis* biovar 3(AY562180.1).

The results of specificity ,sensitivity and accuracy values of the used methods in diagnosis revealed that the technique of PCR had a sensitivity 100%,specificity 30% and accuracy 44% in comparison with bacteria culture ,sensitivity 97.3%,specifity 33.3% and accuracy 82%when compared with RBT, at the last sensitivity 100%,specifity 66.6% and accuracy 92% in compared with ELISA test.

الخلاصه

داء الحمى المالطية هو مرض حيواني واسع الانتشار يصيب الإنسان والحيوان على حد سواء، العلامات والأعراض السريرية لداء الحما المالطية البشري مختلفة ويعتمد التشخيص الغير متخصص لداء الحمى المالطية على التقييم الإيجابي للاختبارات المعتمدة في المختبر ، وقد صُممت الدراسة الحالية لتقييم دقة الاختبارات المصلية (الروز بنغال والاليزا) وسلسلة تفاعل البلمره لتشخيص داء الحمى المالطية. تضمنت المظاهر السريرية لحالات الحمى أعراضًا مثل درجة حرارة تزيد عن 38 درجة مئوية في عدة مناسبات في غضون ثلاثة أسابيع . تم جمع عينات الدم من 50 مريض خلال الفترة من نوفمبر 2017 إلى أبريل 2018 بعد إبلاغ جميع المرضى عن الهدف من هذه الدراسة كإذن اخلاقي. تم تحضير مصل الدم لإجراء الاختبارات المصلية لاجراء الاختبارات المصلية (الروز بنغال والاليزا) ، ثم تم عزل البروسيلا على وسائط انتقائية مثل وسائط أجار بروسيلا التي أكدت جزيئياً باستخدام تفاعل البلمرة التسلسلي لتضخيم الجين البادئ 16SRNA واخيرا تم استخدام طريقة تسلسل الجينات للكشف عن الاصناف والانواع الحيوية للبروسيلا بعد استخدام الجين RpoB اظهرت نتائج الكشف (المصلي) باستخدام الروز بنغال ان نسبة حدوث داء الحمى المالطية كانت 50/45 (90%) في حين كانت نتائج تقنية الاليزا 42 (84%) ومن ناحية اخرى كانت نتيجة عزل البكتيريا على اكار البروسيلا 10 (20%) وكانت نتيجة سلسلة تفاعل البلمرة 38 (76%) كاختبار توكيدي لعزل البروسيلا المالطية بعد استخراج الحمض النووي وتضخيم البادئ 16SRNA ،

أظهرت نتائج تضخيم الجين *RpoB* لـ *Brucella melitensis* ظهور فروقات مميزة واحدة بعد الترحيل الكهربائي على هلام agarose الذي يحتوي على حجم جزيئي bp1091. تم استخدام الناتج من PCR لجين *rpoB* للتسلسل الجزئي واظهرت الشجرة التحليليه المحلية بالمقارنة مع بعض السلالات المرضيه القياسيه في العالم . الخمس عزلات المحلية سجلت في بنك الجينات NCBI لتسجيل تسلسل جين *rpoB*. أعطى البنك الجيني - NCBI أرقام انضمام الكود التي كانت (Banklt 2126555 Seq1 MH523634)، (Banklt 2126555 Seq2 MH523635) (Banklt 2126555 Seq3 MH523636)، (Banklt 2126555 Seq4 MH523637) (Banklt 2126555 Seq5 MH523638). أظهر التحليل لخمسة عزلات محلية لـ B. melitensis ارتباطًا وثيقًا (100٪) بـ NCBI -BLAST B. melitensis biovar 3 (AY562180 0.1) .

أظهرت نتائج قيم الدقة والحساسية و الخصوصية للطرق المستخدمة في التشخيص أن تقنية PCR لها حساسية 100٪ ، خصوصية 30٪ ودقة 44٪ مقارنة بعزل البكتيريا ، حساسية 97.3٪ ، خصوصية 33.3٪ ودقة 82 ٪ وعند مقارنتها مع RBT ، في الاخير كانت الحساسية 100 ٪ ، والخصوصية 66.6 ٪ والدقة 92 ٪ مقارنة مع اختبار ELISA

Chapter one

Introduction
&
Review of Literature

1.Introduction and Review of literature

1.1 Introduction

Brucellosis is zoonotic disease which causes a significant health problem in both human and animals , it is prevalent in many regions of the world including Latin America ,the Mediterranean basin ,Middle east Asia and Africa(Young *et al*., 2014). More than half a million new states of infection are reported in the different countries of the world (Wyatt, 2005).

Brucella can be transmitted to human in several ways including the consumption of un pasteurized dairy ,inhalation of the microorganism and transmission through the skin (Godfroid *et al*., 2005; Nene & Kole, 2008).the genes *Brucella* descends from the α-proteo bacteria group and consists of eight species ,*B.abortus*,*B.melitensis* ,*B.ovis*,*B.canis*,*B.suis*,*B.neotomae* and the strains recently discovered in the marine mammals and in common vole (*Microtus arvalis)* and published under the respective species names of *B.pinnipedialis*,*B.ceti* and *B.microti*(Hadush *et al*., 2013; Xavier *et al*., 2009).

Most species of *Brucella* can infect animals other than their preferred hosts ,when they get in close contact .*B.abortus* ,*B.melitensis* ,*B.suis*, and *B.canis* are human pathogens, *B.melitensis* is the most virulent and causes the acute and chronic cases of brucellosis (Cutler *et al*., 2005).Since the clinical signs and symptoms of human brucellosis are different and non-specific diagnosis of brucellosis depended on appositive assessment in laboratory based testing(De Jong & Tsolis, 2012).

There are a variety of assays present for diagnosing *Brucella* in human and currently ,molecular ,serological and microbiological tests are popularly used to the goal (Hadush & Pal, 2013).Blood culture is a gold standard method for *Brucella* investigation but this method is time consuming ,elevate the risk

of disease transmission to human and suffers from an acute case sensitivity of only 15 to70(Bricker, 2002; Ocampo-Sosa *et al*., 2005; Whatmore, 2009).Also it orders a high level of skill and safety parameters. Serological screening methods detection such as the Rose Bengal commonly conducted in diagnostic laboratories (Padilla *et al*., 2010; Rock *et al*., 2016).

It is well known that lipopolysaccharides display cross reaction with the Gram-negative bacteria (Fayaz et al., 2010; Goldman, 2016).ELISA was reported as the rapid and dependable diagnostic test for brucellosis(Memish *et al*., 2002).The ELISA test has the many advantage ,it can also detect the incomplete antibodies commonly observe in brucellosis chronic phase(Araj, 2010).Molecular methods are also used for the determine of bacteria in culture, serum and blood samples. Real time- PCR has the ability to detect a very low level of bacteria in the sample and hence widely used as a tool for the diagnosis of infectious diseases(Goldman, 2016; Nadkarni *et al*., 2002; Whatmore, 2009).

On other hand , some studies have shown that serum samples are favorable to blood sample , as it can increase the sensitivity of real time – PCR (Capasso, 2002) .Several studies have been done on the comparison and evaluation different laboratory methods used for *Brucella* diagnosis but the results are very variable .In order to investigate genetic relationships with in this species and identify potential diagnostic markers sequenced multiple genetic loci from a large sample of *Brucella* isolates representing the know diversity of the genus (Moreno *et al*., 2002).

1.2 Aim of study :-

The aim present of study was undertaken to evaluate the molecular tests classical PCR, Real -time PCR and DNA sequences in comparative with the serological tests (Rose Bengal and enzyme linked immuno sorbent assay (ELISA) for diagnosis human brucellosis .

To achieve this aim ,the following objectives were conducted:-

1-Isolation and identification of *Brucella melitensis* from human blood samples using cultural and biochemical tests.

2-Detection Ag-Ab agglutination by using Rose Bengal test as a screen test for brucellosis .

3-Estimation of *Brucella* IgM Antibody by using ELISA in serum of patients with brucellosis.

4-Extraction and amplification of Genomic DNA with specific primers belong to *16SRNA gene* by using Real-Time PCR.

5-DNA sequence by using *rpoB gene* amplification.

1.3. Review of literature

1.3.1.Historical view on genus *Brucella*

In spite of the first scientific evidence that goats were the reservoir host of *B.*melitensis for centuries (Capasso, 2002).Phylogenetic studies recommend that brucellosis in goats appearance in the past 86,000 to269000 years through contact with infected sheep(Foster *et al*., 2009).Interestingly, to support this observation a late found lesions in vertebral bodies of an *Australo pithecus* Africans (who lived 2.5million years ago) in concordance with brucellosis ,where the origin of the infection could be the consumption of infected tissues from wild animals(D'Anastasio *et al*.,2009).

The closer association of human with goat and also sheep due to domestication around 10,000 years ago favored an increase in the prevalence of human brucellosis .As necessary resources for human survival ,goat and sheep herds transferred along with human communities from the fertile crescent in South western Asia to lands around the Mediterranean Sea(Zeder & Hesse, 2000).

Where Phoenician traders might have provided to the spread of *B.melitensis* infection throughout the Mediterranean littoral and islands during the first millennium B.C. It was then presented to the Americans around the 16th century by Spanish and Portuguese conquerors (Rossetti *et al*., 2017).The first written statement of goat brucellosis could be in furred from the first description of two human cases of brucellosis .In the 4th century B.C, in his Epidemics book, Hippocrats II described two cases of 120 day fever in people living in the Mediterranean island, more likely associated with the consumption of raw milk or derivatives of *B.melitensis* infected sheep and goats(Spinage, 2012) .Another testimony of the ancient evidence of brucellosis comes from preserved present from the volcanic eruption of Mount Vesuvius

in Italy on August 25th in the year 79 A.D. Scanning electron microscopy examination of remnants of carbonized cheeses founded cocci-like forms consistent with *B.melitensis* (Rossetti et al., 2017).

While an anthropological examination of human skeletal remains from that prevalence displayed an arthritic condition consistent with brucellosis References to and vivid characterization of clinical cases compatible with human brucellosis were reported in histories of military campaigns and hospital reports(Pathak, 2015).

However the investigation of the etiological agent the reservoir and the epidemiology of the infection was not unraveled until the second half of the 19th century , when the British government decided to discover a solution for these states in the island of Malta that annually suffered substantial losses caused by the so called « Malta fever».In1859 British Army Surgeon Jeffery Marston arrangement what he called «Mediterranean remittent fever» , after recovering ,he characterized his own case in great detail ,being the first author to clinically and pathologically distinguish human brucellosis from typhoid and other incidence fever(Rossetti *et al*., 2017).

In1884,theAustralian- born British physician David Bruce was deployed to Malta identification the cause of« Malta fever» .Later called brucellosis in his honor ,late in1886 using a microscope ,Bruce observed a great number of micrococci in a fresh preparation in the splenic pulp of the soldiers who had died from the infection (Rossetti *et al*., 2017; Vassallo, 1992).After one year ,Sir Bruce isolated the causative agent of « Malta fever» ,which he called *Micrococcus melitensis* and then altered name to *Brucella melitensis* from samples of four patients made culture into Koch's postulates(Weeks, 2008).

Little years later, Professor Almorth Edward Wright expanded a serum agglutination test and established the presence of specific agglutinins in the blood of infection patients, which supported differentiate those who suffered

«brucellosis» from those with typhoid ,cholera and malarial fever (Madkour, 2001). The use of this serological method in goats supplied the first insights in to the epidemiology of the disease .

In1904,Public Health Officer of Malta found that the blood of goats provided milk to people that has contracted« Malta fever» had agglutinins against *M.melitensis* and a posterior survey pointed that around 50% of Malta´s goats blood reacted to this microorganism (Wyatt, 2005).This observation proposed that goats were susceptible to natural infection with *M.melitensis* . Depended on all knowledge available on brucellosis the Greek physician the mistokles Zammit hypothesized that goats were often to Malta fever and that the infection spread from goat to human .Zammit fed sero negative healthy goat on the agar cultures of *M.melitensis* mixed in to their food .Goats became seropositive to *M.melitensis* after20 days or more and *Brucella* was isolated from the blood ,milk and urine of infected animals without any clinical demonstration of the infection . This observation was confirmed after its ban from the diet of the Malta garrison significantly decreased the incidence of brucellosis in naval forces and the army compared to the general population of Malta that continued to consume contaminated dairy products(DelVecchio *et al*., 2002).

in1918,Alice Evans demonstrated the same descriptions between the *M.melitensis* and the etiological agent of *Brucella abortus* ,isolated by Danish Veterinarian Bernhard Bang in 1896,and depended on that ,both agent were included belong the same bacterial genes (*Brucella)* in honor of David Bruce ,in 1920(Seleem *et al*., 2008).

1.3.2.The genus *Brucella*

Bacteriological properties *Brucella* spp. are the etiological agents of human and animals brucellosis. *Brucella* are Gram-negative bacteria, that can stain red using the modified Ziehl Neelsen technique (Fretin *et al.*, 2005)and appearing as cocci or short rod shaped cells from 0.5- 0.7x 0.6-1.5 microns in size. *Brucella* spp. live as, facultative intracellular non motile pathogens of the reticuloendothelial cells of terrestrial and marine mammal hosts(Chain *et al*., 2005). The mechanisms of their virulence and survival inside professional phagocytes partially stay an enigma as *Brucella* has not been presented to produce the virulence agents, such as cytolysins, capsules, exotoxins, secreted proteases, pili and /or fimbriae, flagella, implicated in such systems in other bacteria(Halling *et al*., 2005; Moreno *et al.*, 2002) that *Brucella* is non-motile by inactivation of some of the flagellar genes and absence of the chemotactic systems.

Despite lack of motility the presence of flagellar genes in the chromosomes has been shown to be vital in *Brucella* persistence in a murine model but not in cell culture infection(Ferguson et al., 2004). It has been suggested that the differences in the potential expression of the flagellum may explain *Brucella* adaptation to different hosts. Genome sequencing of three classical *Brucella* species has supplied information on their respiration and metabolic functions(Atluri *et al*., 2011).

The chromosomal information has shown that *Brucella* may have made to the intracellular habitat by selecting for a high-affinity respiratory mechanism and simultaneously losing useful nucleotide synthesis, sugar modification, polysaccharides compound as well as the synthesis of biotin and choline and the energy and carbon storage compounds glycogen an polyhydroxybutyrate (Chain *et al*., 2005).

Brucella respiration is made using oxygen as terminal electron acceptor with hydrogenases. A possible role for nitrate respiration under lowered redox potential has been suggested. Interestingly, D-erythritol 1-phosphate, an intermediate of the erythritol pathway, has been present to act as an electron donor to the respiratory chain (Fretin *et al.*, 2005). Given that erythritol is a main product of trophoblasts that line the placenta in the late levels of pregnancy, the attraction of *Brucella* to the gravid uterus and replication in the trophoblasts has been rationalized on this rules. An intriguing finding was the truth that *B. melitensis* strains could use glutamic acid as a sole source of nitrogen and energy demand provided that they were stimulated by CO2, or alternatively, that glucose was used for additional energy. that CO2 was needed for activating the flow of carbon to the tricarboxylic cycle.

Interestingly, the latter may link to observations concerning some *Brucella* strains that require an atmosphere containing between 5 to 10% CO2 for initial culture whereas on further sub-culture they may change CO2 independent. It is not clear, however, if this results from enzyme stimulation or sub-population selection(Seleem *et al*., 2008).

1.3.3.Genome structure of *Brucella Melitensis* :-

The genome of *Brucella melitensis* was sequenced by using a shotgun approach. The genome size is 3.29Mb was found in the two circular chromosomes in which 3,294,931 base pairs were distributed with a57%GC content . Of those two chromosomes of 2,117,144 bp and 1,177,787 bp encoding 3,197 ORFs (open reading frames) were predicted(Halling *et al*., 2005). It was also discovered that on both chromosomes ,plasmids were not

found. the origin of replication of the both chromosomes are similar to these of other alpha-proteobacteria.

there resided genes that encoded for DNA replication ,transcription, translation, protein synthesis, core metabolism and cell-wall biosynthesis (all of which were considered "housekeeping genes") (Foster *et al*., 2009). Type I, II, and III secretion systems are absent but genes encoding sec-dependent, sec-independent, adhesions, invasions, hemolysins, flagella- specific type III, type IV as well as type V secretion system were identified (Gándara *et al*., 2001; Wattam *et al*., 2009). A number of insertion and deletion events were also identified in the genomes, which led to the discovery of several fragments of unique sequences that were present in this bacteria. Several features of the *B. melitensis* genome are similar to those symbiotic *Sinorhizobium meliloti*.(Gee *et al*., 2004; Halling *et al*., 2005; Osterman & Moriyon, 2006) .

1.3.4.Virulence factors of *Brucella* species :-

As key part for stealthy to intracellular survive of *Brucella, Brucella* species is frequently called as ‘‘nasty bugs’’ depended on their unusual virulence characters (Letesson *et al.,* 2002). a long time, it was thought that *Brucella* bacterium does not contain any virulence factors that exist in other bacteria (Fugier *et al.,* 2007; Moreno *et al.*, 2002) . In recent several studies, it has been reported that *Brucella* is having chiefly five virulence factors that are essential for intracellular survival and infection, including virB T4SS (Comerci *et al*., 2001) ; cyclic β-glucan (Conde-Alvarez *et al*., 2012) . two- component sensory and regulatory system BvrS/BvrR (Gopalakrishnan *et al*., 2016), *Brucella* LPS (BrLPS) (Arellano-Reynoso *et al*., 2005) . And pathogen-associated molecular patterns (PAMPs).

Additionally, some other virulence factors have been recognized in *Brucella* spp., that are responsible for infection, counting BacA (Martín-Martín *et al*., 2012), BmaC (Posadas *et al*., 2012), outer membrane proteins (Omps)(Lim *et al.*, 2012), SagA (Del Giudice *et al*., 2013), MucR (Mirabella *et al*., 2012), BtaE (Ruiz-Ranwez *et al.*, 2013), and BetB (Lee *et al.*, 2014) ,the five virulence factors consist of :-

(I) virB type IV secretion system (virB T4SS): In *Brucella* , T4SS is one of the major virulent factors and is encoded by the virB operon which contains totally 12 *genes* (VirB1–12) located on chromosome II (De Jong & Tsolis, 2012). 15 effector proteins have been categorized in *Brucella* that regulates the intracellular and stealthy lifestyle of the pathogen (Döhmer *et al*., 2014; S. Salcedo *et al.*, 2013). This process is important for bacteria to subvert lysosome fusion and to produce *Brucella*-containing vacuole, an organelle that allow binding with the endoplasmic reticulum and replication (Celli *et al.*, 2003; Celli & Gorvel, 2004; Marchesini *et al*., 2011).

The virB operon is necessary for non-opsonized *Brucella* that continues to live within the phagolysosome and to produce a successful intracellular replicative compartment (López-Goñi & Moriyón, 2004) , and modulates *Brucella* intracellular trafficking(Comerci *et al.*, 2001; Delrue *et al*., 2001).Therefore, the T4SS plays important role for preventing host innate immune response and in stealthy intracellular persistence during infection.

(II) Two-component sensory and regulatory system BvrS/BvrR: The two-component sensory and regulatory system BvrS/BvrR are important for *Brucella* virulence, co-ordinate the outer membrane (OM) architecture, which are probability attitude to pathogen metabolism (Guzmán-Verri *et al.*, 2002; Salcedo *et al*., 2008) .

Out of these two components, BvrS is a sensor protein member of the histidine-kinase superfamily and BvrR is considered a regulator protein. This system modulates outer membrane proteins (Omp) expression which is included in invasion of host cells(Lopez-Goni *et al.*, 2002). Any dysfunction of the BvrR/BvrS sensory-regulatory system results in easy capability of *Brucella* to bactericidal cationic peptides and complement, and raised permeability to surfactants (Sola-Landa *et al.*, 1998). It alters in the bacterial outer membrane which changes cellular uptake of the organism (Manterola *et al*., 2007) . Moreover, this virulence factor plays an important role in intracellular survival of *Brucella* spp.

(III) *Brucella* lipopolysaccharides (LPS) play necessary role to evade host immune response : *Brucella* LPS is having two shapes, which are smooth and rough strains (Ronneau *et al*., 2016). Basically, rough strains obliging less or no O polysaccharide (OPS) are less pathogenic than smooth strains and can be easily controlled by complement system (J. Ko & Splitter, 2003). Gram negative bacteria *Brucella* LPS is built of lipid A and core oligosaccharide, which have less number of negatively charged sugars. All these features of BrLPS influence ability of not attach to complement, bactenecins, cathelicidins, microbicidal defenses, or any alternative cationic bactericidal molecules (Lapaque *et al*., 2005) .

The BrLPS have been characterized as virulence factor and plays action role in *Brucella* replication and survival (Barquero-Calvo *et al*., 2007). The in-built descriptions of the *Brucella* membrane envelope organization are having properties for resisting to humoral and cellular bactericidal activities of the host immune system (De Tejada *et al*., 1995).

Furthermore, this BrLPS is having high antagonism to macrophage degradation and maintenance against immune responses(Forestier *et al*.,

2000).The BrLPS alters the LPS pathogen-associated molecular pattern (PAMP) and decrease the endotoxin-related properties which are typical of LPS. In contrast to enterobacterial LPS, *Brucella* LPS is many times less action and toxic compared to E. coli LPS (Monreal *et al*., 2003) .

The BrLPS acts as a virulence factor in two positions. Firstly, *Brucella* preserves less immunogenic LPS than enterobacterial LPS (Gopalakrishnan *et al*., 2016). So, it does not stimulate host immunity to prevent *Brucella* replication. Non-pyrogenic type of BrLPS does not influence the alternative complement pathway to any notable level and is a very fragile mitogen to murine B cells (Sangari & Agüero, 1996). Secondly, BrLPS stimulates less biological activity which might be one of the cases for maintaining durability of these pathogens within phagocytic cells. The BrLPS carries a non- canonical lipid A and produce weak response to TLR-4 (Lapaque *et al*., 2006) , which are contributing to *Brucella* to provide a stealthy nature at the initial stage of infection (Sengupta *et al*., 2009). The BrLPS induces less classic TLR-4 dependent activation or require no role of TLR-2 (Barquero-Calvo *et al*., 2007; Hernández-Mora *et al*., 2009). This mechanism causes very restricted potential to induce pro-inflammatory responses in DCs (Gopalakrishnan *et al*., 2016). The LPS O chain avoids cellular apoptosis and control immune response activation(Pei, Turse *et al*., 2006). Also, the BrLPS are presenting secondary anti-inflammatory feature, which can lead to decrease deposition of complement component C3(Barquero-Calvo *et al*., 2007).

Majority of Gram-negative bacteria are enveloped by outer membrane molecules which have the PAMP, that are determined by innate immunity. However, that PAMP are not found on OM lipopolysaccharide, lipoproteins and flagellin of *Brucella* spp. (Palacios-Chaves *et al*., 2011) . So, these bacteria escape early recognition through innate immunity. Heat-killed smooth LPS *Brucella* strains can quite limit attachment with lysosomes higher than rough

mutants (Porte *et al*., 2003) . that indicates an necessary role of the O-chain in this mechanism. Despite, the smooth LPS-dependent interruption in lysosome integration is temporary and not enough to defend *Brucella* long-term durability (Martín-Martín *et al*., 2012); it means some other bacterial factors are also important for finalizing the *Brucella* intracellular cycle.

(IV) Pathogen-associated molecular patterns (PAMPs): The PAMPs have been determined as virulence factors and that are feeble inducers of toll-like receptors (TLRs) (Salcedo *et al.*, 2008; Sengupta *et al.*, 2009). which are contributing to *Brucella* in a stealthy nature at the major stage of infection (Sengupta *et al.*, 2009). *Brucella* spp. are hidden to early recognition by innate immunity, the non-presence of PAMP expression in the cell envelop *Brucella* OM lipopolysaccharide, ornithine-containing lipids, lipoproteins and flagellin (Salcedo *et a*l., 2008), which minimally activate the innate immunity (Martirosyan *et al*., 2011). *Brucella* toll-interleukin receptor (TIR) domain is detection in both the cytoplasmic regions of TLRs and adaptor proteins. TIR domain consist of BtpA and BtpB proteins, which are considered as virulence factors and are responsible for mediating the signaling cascades of innate immune recognition (Salcedo *et al.*, 2013).

(V) Cyclic β (1–2) glucan: The cyclic β (1–2) glucan is containing an osmoregulated periplasmic polysaccharide property and is made through cyclic β (1–2) glucan synthetase enzymes, which are encoded by *cgs* in *Brucella* (Briones *et al.*, 2001). But, it is not osmotically modified (Razzaq *et al*., 2014). The *cgs gene* mutant *Brucella* spp. is not present cyclic β (1–2) glucans synthetase enzyme; that can lead to deficient in production of cyclic β (1–2) glucans substance. This substance is important for inhibiting the maturation of the bacterial vacuole via inhibiting cholesterol-rich lipid rafts which are

characterized by their enrichment in flotillin-1 and consequently prevent lysosome fusion (Watarai *et al*., 2002).

The Cyclic β (1–2) glucan factor is responsible for the pathogen to accurate its final replicating slot within endoplasmic reticulum (Arellano-Reynoso *et al.*, 2005). These all the five virulence factors together, may contribute key virulence mechanisms for intracellular survival and multiplication of *Brucella*(Wang *et al.*, 2009). Additionally, documented that some molecules of *Brucella* such as transporter-like protein BacA, flagellum-like structure and phosphatidylcholine are essential for survival of *Brucella* inside the host cells.

Furthermore,(Spera *et al*.,2014) reported that chronic feature of *Brucella* is due to some virulence factors, among which are the immunomodulatory proteins such as PrpA (proline racemase protein A), included in the establishment of the chronic nature of the infectious conditions.

1.3.5.Host Interactions Pathology of Brucellosis:-

Brucella exhibit strong tissue tropism and replicate within vacuoles of macrophages, dendritic cells (DCs), and placental trophoblasts(Rossetti *et al*., 2013). However, the pathogen has the capacity to replicate in a wide variety of mammalian cell types, involving microglia, fibroblasts, epithelial cells, and endothelial cells. The intracellular life kind of *Brucella* limits exposure to the host innate and adaptive immune responses(Martirosyan & Gorvel, 2013). Sequesters the organism from the actions of some antibiotics, and drives the unique characteristics of pathology in infected hosts, which is typically divided into three vivid phases: the incubation phase before clinical symptoms are apparent, the acute phase during which time the pathogen invades and

spreads in host tissue, and the chronic phase that can result in severe organ damage and death of the host organism(Adams, 2002).

Nonspecific influenza-like symptoms observed in humans involved pyrexia, diaphoresis, fatigue, anorexia, myalgia, and arthralgia. Furthermore, increasing evidence from endemic regions proposes that an elevated risk of human abortion is associated with exposure. Chronic infection results from the ability of the organism to persevere in the cells of the host in which *Brucella* are disseminated by way of the lymphoreticular system to eventually cause cardiovascular, hepatic, lymphoreticular, neurologic, and osteoarticular disease (Rossetti *et al*., 2012). Measurable splenomegaly is associated with increased lymphohistiocytic cells in the spleen, slightly decrease percentage of splenic CD4 and CD8 T cells, and essential increases in the percentage of splenic macrophages(Castañeda-Roldán *et al.*, 2004).

Immune Response against Stealthy *Brucella* Knowledge of protection against infection is obtained from a humans. The significance of a T helper cell type 1 (Th1) response against *Brucella* is supported by a lot studies (DelVecchio *et al*., 2002; Yongqun 2012; J. Ko & Splitter, 2003).

The roles of CD4 and CD8 T cells are important , although these results were contradictory at times. Natural killer cells play an essential role in some hosts(Yin *et al*., 2010). Passive transfer experiments recommend that antibody to LPS (O-polysaccharide) may contribute to protection, the effectiveness of the T helper cell type 2 (Th2) humral immune response remains confused, and the efficacy of rough *Brucella* vaccines contradicts the role of anti-LPS antibodies in protective immunity(Rossetti *et al*., 2010). Cytokines are played essential role in protection against brucellosis, mediating both innate and adaptive immune responses. IL-12 produced by B cells and macrophages leads to a Th1 response and stimulate of interferon-γ, which

activates macrophages. The activity of is interferon-g is maximized by tumor necrosis factor-a made by macrophages and natural killer cells. Reports also indicate that IL-1dependent stimulation of colony-stimulating factor increases neutrophil and macrophage infiltration into the spleen(Kim *et al*., 2013).

This phenomenon may also define a role for IL-6 produced by T cells. Splenocytes of infected hosts articulate higher levels of mRNA for IL-2, interferon-g, and IL-10 and decreased levels of mRNA for IL-4, consistent with a Th1 response(Lin *et al*.,2011; Liu *et al*., 2012). Increased IL-10 observed later in infection may support the capable of *Brucella* to escape immune surveillance, resulting from repression of a protective Th1 response. Interestingly, cellular and humoral immune responses against identical *Brucella* strains vary significantly among susceptible hosts. This confounding aspect of *Brucella* immunobiology has presented major challenges in the identification of reliable correlates of immune protection in tractable model animal systems(Wang *et al*., 2011). Resistance to other innate immune system components (eg, complement, phagocytic cells, opsonins, cytokines, innate lymphocytes, and other barriers) was in most cases suspected of being inherent to *Brucella* and provides passive resistance to intracellular killing mechanisms(Baldwin & Goenka, 2006).

However, on the basis of the importance of the T4SS to the long-term success of disease(Grilló *et al*., 2000; Salcedo *et al*., 2008; Mariana *et al*., 2013). it is becoming more clearly that resistance mechanisms alone are not sufficient for the success of infection. *Brucella*, and other intracellular pathogens, change the innate immune response with the immediate aim of establishing a replicative niche and long-term persistence(Adams *et al*., 2011; Rajashekara *et al*., 2006).

To restrict long-term protective immunity, the organism first escapes the innate immune response by stealthy entry into host cells. From there, the

organism controls aspects of protein secretion, intracellular trafficking, and bacterial replication ultimately changing the course of the innate and adaptive immune responses(Yin *et al*., 2010). Failure of long-term protection against *Brucella* disease is the result of a weakened adaptive immune response controlled in portion by the attenuated innate immune response. As a stealth invader, *Brucella* enters the host cell without clear activation of the innate immune response through TLR ligand interaction(Gomez *et al*., 2013).

This finding may be best explained by an abundance in host functions. However, it may also reflect that the primary goal is prevention of long-term adaptive immune response rather than save at early stages of infection. Evasion of the host induced innate immune response may allow the microorganism to gain a foothold, whereas stimulation at later times aids the spread out of infection(Fernandes *et al*., 1995; Hanna *et al*., 2013). Manipulation of the innate immune response was present for at least three factors TcpB/BtpA, BtpB, and VceC. Although many other effectors were identified(Adams *et al*., 2011; Gomez *et al*., 2013; Oliveira *et al*., 2002). Their contribution to pathogen survival remains to be demonstrated. However, it seems apparent that the wild-type *Brucella* has at its disposal a complete battery of effectors and that any delay in the innate immune response stimulated by these proteins could potentially be manipulated so as to improve the potential for more protective and safer vaccines (Arenas-Gamboa *et al*., 2012; Weiss *et al*., 2005).

1.3.6.Global distribution of brucellosis :-

Prevalence of brucellosis around the world has been reported and referenced by others (Gwida *et al*., 2010; McDermott & Arimi, 2002; Musallam *et al*., 2016; Racloz *et al*., 2013). The disease is found in 5 out of the

7 continents (South and North America, Europe, Asia, and Africa) (Lucero *et al*., 2008; Sun *et al*., 2016) . Despite being under control in major industrialized countries, it remains a most problem in the Mediterranean region, the Middle East, Central and Southeast Asia, sub-Saharan Africa, and parts of LatinAmerica (Barua *et al*., 2016; Russo *et al*., 2016; Singh *et al*., 2013).

As expected, prevalence of human brucellosis is also high in those regions where human brucellosis arises (Gaido *et al*., 2011; Poulsen *et al*., 2014). The disease has been historically underreported, potential because low-income countries prioritize other diseases or lack facilities, human capabilities, or specific tests that would otherwise underpin diagnosis and research(Montie *et al*.,l2013).

Over the last 15 years, the infection has re-emerged, in particular in Eastern Europe, the Balkans, and Eurasia (Herrera, *et al*., 2011). (i.e., found of anti-*Brucella* antibodies, *B. melitensis* isolation, or *Brucella* DNA detection from brucellosis in humans, due to *B. melitensis* infection have been reported in recent years (Obradović & Velić, 2010). Historically, *B. melitensis* biovar 1 is predominant in Latin America (Likov *et al*., 2010; Nenova *et al*., 2015), while biovar 2 is predominant in the Middle East together with biovar 3, which is also widespread in European and African Mediterranean countries, Eurasia, and China (De Massis *et al*., 2015; Karagiannis *et al*., 2012; Mick et al., 2014; Naletoski *et al*., 2010) , biovars 1 and 3 seem to be equally present in India (Coelho *et al*.,2013; Kirandziski *et al*., 2010).

Unfortunately, there are few studies addressing the characterization of isolates from sub-Saharan countries. 1In the Americas, *Brucella melitensis* was most likely occurred around the 16th century via the infected goats and sheep of Spanish and Portuguese conquerors (Markovic-Denic *et al*.,2010). Today, *B. melitensis* is endemic in some areas of Mexico, Peru, and Argentina (Álvarez *et al*., 2011) and has also been reported in Ecuador and Venezuela (Porphyre *et*

al., 2010). brucellosis is apparently absent in Central America, , Paraguay, Bolivia, and Brazil, although this epidemiological situation is not confirmed (Rossetti *et al*., 2017). Goat herds from the USA, Colombia, Canada, Chile, and Uruguay are free from *B. melitensis* infection, and human cases in these countries are clearly associated with international travelers or infected food necessary from endemic regions (Islam *et al*., 2013) .

Despite intense joint efforts to eliminate *B. melitensis* from goat flocks in Europe, the disease still happens in Portugal, Spain, France, Italy, the Balkans, Bulgaria, and Greece. Northern and Central European countries like the United Kingdom, Belgium, the Netherlands Germany, Austria, , Denmark, Switzerland, the Czech Republic, Hungary, Poland, Romania, Sweden, Norway, and Finland, among others, are officially free of the disease (Pérez-Sancho *et al*., 2014).

In Asia, brucellosis is broadly distributed. Except for Japan and the Republic of Korea (South Korea), where the disease has never been reported, brucellosis is officially recognized in several countries on the continent, such as Turkey, Israel, , Iraq, , Jordan, Iran, Armenia, Georgia, Afghanistan, Russia, and Mongolia, among others Table(1-1) ,and is also known to be endemic in countries like Syria, Lebanon, India, China, Indonesia, Myanmar, etc., where no public information is present or the distribution of the information is restricted (Akbarmehr & Ghiyamirad, 2011; Mamisashvili *et al*., 2013; Pishva *et al*., 2015; Sadhu *et al*., 2015).

In Africa, brucellosis is endemic in Mediterranean countries like Morocco, Algeria, Tunisia, Libya, and Egypt, and also in those countries placed in the eastern part of the continent, such as Sudan, Eritrea, Ethiopia, Somalia, Kenya, Uganda, and Tanzania. Unfortunately, there is no information present from Central and West African countries like Chad, Congo, Angola, Zambia, Cameroon, Mali, , Guinea, and Senegal, among others, where goats are

abundant (Ebrahimi, *et al*., 2014). Altogether, the information above indicates that the knowledge regarding distribution of brucellosis as well as the existence of *B. melitensis* around the world is sparse, especially in some regions of the Americas, Asia and Africa. The deficiency of useful epidemiological data must induce official veterinary services and public health officers to collect and share data for designing control and eradication plans(Al-Tae & Al-Samarrae, 2013; Bechtol *et al*., 2011). Table (1-1) shows those countries where brucellosis.

Table (1-1) Brucellosis incidence by country (cases per 100000 person –years) (Dean *et al*., 2012)

Country	Incidence per 1000000 per years
Central Asia	
Kyrgyzstan	88.00
North Africa and Middle east	
Saudi Arabia	137.61
Iraq	52.29-268.81
Jordan	25.70-130.00
Palestine	8.00
Turkey	6.00-149.54
Iran	0.73-141.60
Sub-Saharan Africa	
Chad	34.86
Central and Southern Latin America	
Mexico	25..69
Argentina	12.84
Western Europe	
Greece	4.00-32.49
Italy	1.40
Germany	0.03
North America	
USA	0.02-0.09

1.3.7.Distribution of brucellosis in Iraq:-

Data from Office for International des Epizootics were built present for the incidence of human brucellosis in Iraq ,underlining the huge endemicity of the disease in this area ,the endemicity of the infection in this region may raise concerns ,since a purely endemic brucellosis case in an international soldier stationed in Iraq might cause alarm of a potential bio warfare incident (Pappas *et al*., 2006). the first bacteriological isolation of *Brucella* in Iraq was made by Salem (1977).The isolate *B.melitensis* biotype 1 and 2from milk samples and aborted goat fetuses from a herd of goats in Baghdad . From that time until recently there were reports recorded available of the disease in human (Al-Ouqaili, 2006) . While (Al-Thwani, *et al*., 2001) in a study conducted 100 samples of patients (having the clinical signs of brucellosis and RBPT+ve) obtained 23 *Brucella* isolates and found that *B.melitensis* was the commonest.

1.3.8. Diagnostic techniques:-

Brucellosis is difficult to diagnose depended on clinical symptoms of the disease, which are nonspecific and often atypical signs(Bricker, 2002; Hadush & Pal, 2013).Therefore, the diagnosis mostly trusts on the results of laboratory testing. Culture of the organism is the diagnostic method of choice; however, cultures include risk of infection and require special precautions in the laboratory(Ocampo-Sosa *et al*., 2005; Whatmore, 2009).An infectious dose for *Brucella* in humans is 10 to 100 organisms; therefore, diagnostic laboratory personnel who cultivate these organisms are at significant hazard of accidental exposure. Brucellosis is one of the most commonly described laboratory-acquired infections. Present methods of testing cultures for *Brucella* are time-consuming and lack sensitivity, particularly in chronic infections(Nielsen, 2002; Poester *et al*., 2010).

Most laboratories apply serological tests that do not make available suitable sensitivity and specificity for this organism. Enzyme-linked immunosorbent assay (ELISA) methods that identify immunoglobulin G (IgG) are sensitive(Rich *et al*., 2000) but have low specificity(Gwida *et al*., 2010). Measurement of specific immunoglobulin M (IgM) levels has lower sensitivity than IgG but is more specific (Seleem *et al*.2010).

Molecular diagnostic assays minimize the risks related with handling potentially infectious specimens and increase the sensitivity, specificity, and rapidity of testing, while some studies have reported only moderate sensitivity (50%) using these methods(Boschiroli *et al* 2001; Gwida *et al*., 2010). However, rare laboratories diagnose brucellosis using culture methods because cultures have imperfect sensitivity, are time-consuming, and require specially biosafety equipment(Boschiroli *et al*., 2001). Therefore, data on the frequency on *Brucella* infections are often undependable. Recently, multiplex polymerase chain reaction (PCR) protocols that overcome these problems have been defined(Geresu & Kassa, 2016).

1.3.9 .Bacteriological diagnosis:-

Isolation of the organism is thought the gold standard diagnostic method for brucellosis since it is specific and permits biotyping of the isolate, which is related under an epidemiological point of view (Celli & Gorvel, 2004; De Miguel *et al.*, 2011). Though, in spite of its high specificity, culture of *Brucella spp*. is challenging. *Brucella* spp. is a fastidious bacterium and needs rich media for primary cultures. Furthermore, its isolation wants a large number of viable bacteria in clinical samples, good storage and quick delivery to the diagnostic laboratory(Sam *et al*., 2012; Singh *et al* 2015).

Contamination of clinical samples is a complicating element for *Brucella* spp. isolation., the use of brain heart infusion broth is considered a rich media supplemented that can isolation of *Brucella* spp. (Her *et al*., 2010; Mariana *et al*., 2009).Another limiting element for culturing *Brucella* spp. is the requirement for suitable laboratory conditions and personnel exercise so the procedure can be executed safely *Brucella* spp (Her *et al*., 2010; Scholz *et al*., 2008). is categorized as a Biosafety level 3 organism, whose management should be performed in biosafety level-3 laboratories (Scholz *et al*., 2009). Importantly, brucellosis is one of the most communal accidental laboratory infections, mainly in research laboratories (Scholz *et al*., 2009; Scholz *et al*., 2008). Samples for *Brucella* spp. isolation from human blood, also from slaughterhouses include mammary, iliac, pharyngeal, parotids and cervical lymph nodes, and spleen.

Samples should be immediately sent to the laboratory, preferentially frozen at -20°C, and they must be recognized as suspect of *Brucella* spp. infection (Christopher *et al* 2010).The isolation is more difficult, often resulting in false negative results(Araj, 2010).

Brucella spp. colonies are elevated, transparent, curved, with intact borders, smooth, and a brilliant surface. The colonies have a honey color under diffused light. Optimal temperature for culture is 37°C, but the organism can grow under temperatures extending from 20°C to 40°C, while optimal pH ranges from 6.6 to 7.4. Some *Brucella* spp. needs CO2 for growth. Typical colonies seems after 2 to 30 days of incubation, but a culture can only be reflected negative when there are no colonies after 2 to 3 weeks of incubation (Sathyanarayan *et al*., 2011). False negative results should be considered in the deficiency of bacterial growth since the sensitivity of culture is little(Agasthya *et al* 2012).

Ordinarily, solid media such as chocolate agar ,dextrose agar, tryptose agar, and trypticase soy agar, are suggested for primary isolation of *Brucella* (Godfroid, *et al* 2010),also we make biochemical test such as oxidase ,catalase and API 20 test.

1.3.10.Rose Bengal plate test:-

The Rose Bengal test (RBT) is a rapid, slide-type agglutination assay performed with a stained *B. abortus* suspension at pH of 3.6-3.7 and plain serum(Díaz *et al* 2011). Its simplicity prepared it an best screening test for small laboratories with limited resources. The disadvantages of RBT include: low sensitivity particularly in chronic cases, relatively low specificity in endemic regions and prozones make strongly positive sera perform negative in RBT (Ruiz-Mesa *et al*., 2005).The overall sensitivity is 92.9%, so the use of RBT should be reflected carefully in endemic areas, mainly in individuals exposed to brucellosis and those having past of *Brucella* infection (Nielsen *et al*., 2008). Rose Bengal plate test [RBT] is an agglutination test that is depended on reactivity of antibodies against smooth lipopolysaccharide (LPS). As sensitivity is high, false negative results are seldom encountered. To increase specificity, the test may be applied to a serial dilution (1:2 through 1:64) of the serum samples (Asaad & Alqahtani, 2012). The current World Health Organization (WHO) guidelines recommend the confirmation of the RBT by other assays such as serum agglutination tests (Di Febo *et al*., 2012; Díaz *et al*., 2011).

1.2.11.Enzyme linked immunosorbent assay:-

Enzyme linked immunosorbent assay (ELISA) has become prevalent as a standard assay for the diagnosis of brucellosis, serologically. It measures IgG, IgA and IgM antibodies and this allows a recovered interpretation of the

clinical situation(Gall *et al*., 2003). The diagnosis of brucellosis is based on the recognition of antibodies against the smooth LPS. Detection of IgG antibodies is more sensitive than detection of IgM antibodies for diagnosing cases of brucellosis but specificity is comparable(Agasthya *et al*., 2012; Mantur *et al*., 2010). Compared to the conventional agglutination techniques, ELISA is more sensitive in acute and chronic cases of brucellosis and it suggestions a significant diagnostic advantage in the diagnosis of brucellosis in endemic areas. For case finding and an accurate diagnosis of suspected cases, the combination of ELISA IgM and IgG tests should be used as this combination of laboratory tests has been exposed to be the most efficient technique in the detection and diagnosis of brucellosis. For follow-up and checking of prognosis, ELISA Ig M is more promising (Gall *et al*., 2001; K. Y. Ko *et al*., 2012; Perrett *et al* 2010).

Enzyme linked immunosorbent assay (ELISA) is an excellent technique for screening large populations for *Brucella* antibodies and for differentiation between acute and chronic phases of the disease (Mantur *et al*., 2010). It is the test of select for complicated, local or chronic cases chiefly when other tests are negative while the case is under high clinical suspicion. It can expose total and individual specific immunoglobulins (IgG, IgA and IgM) within 4-6 hours with high sensitivity and specificity. In addition to the recognition of immunoglobulin classes, ELISA can also detect *Brucella*-specific IgG subclasses and other *Brucella* immunoglobulins such as IgE(Ko *et al*., 2012).

Polymerase chain reaction (PCR) and/its variants, based on amplification of specific genomic sequences of the genus, species or even biotypes of *Brucella* spp., are the most approximately used molecular technique for brucellosis diagnosis (Scott *et al*., 2007). The technique is chosen based on the type of biological sample and the aim, i.e., diagnosis or molecular characterization or epidemiological survey. Most of the molecular diagnostic

methods for brucellosis have sensitivity reaching from 50% to 100% and specificity between 60% and 98%. The DNA extraction protocol, type of clinical sample, and detection restrictions of each protocol, are factors that can influence the efficiency of the technique ((Huber *et al* .,2009).

Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic description, requires Biosafety level-3 (BSL-3) protocols for the high risk of laboratory-acquired infections (Mitka *et al*.,2007), molecular methods have been investigated in order to overcome these difficulties. Furthermore, the PCR-based assays have detectable a higher sensitivity with respect to the standard microbiological assay for the diagnosis of brucellosis (Lopez-Goñi *et al*., 2008).

1.3.12.Real-time PCR:-

Real-time PCR is more rapid and more sensitive than conventional PCR. It does not require post amplification handling of PCR products, thereby decreasing the risk of laboratory contamination and false positive results. Real-time PCR assays have been recently defined in order to test *Brucella* cells (Redkar *et al* ., 2001), serum(Winchell *et al* .,2010), blood, and paraffinembedded tissues (Foster *et al*., 2009).

The primer and probe used in these real-time PCRs derived from the, 16SrRNA whereas the *B. melitensis* primer and Taq man probe are selected from unique species or biovar-specific chromosomal loci(Whatmore *et al* .,2007). A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR

assays, including 16S rRNA, the 16S-23S intergenic spacer region, omp2, and bcsp31 (Wareth *et al* .,, 2014; Wojno *et al*., 2016). The most frequently described PCR target for the diagnosis of human brucellosis is the16S rRNA gene conserved *Brucella melitensis*. (Redkar *et al*., 2001). described real-time PCR assays for the detection of, *B. melitensis*,.

These PCR assays target the specific 16SrRNA within the genome of the respective *Brucella* species or biovar. The assays, however, were designed to be tested in separate PCRs., we have developed a real-time triplex assay that permits rapid confirmation of *Brucella* spp. .The primer and TaqMan probe were design in this study using NCBI-Genbank database. For *Brucella melitensis* identification, the primers and probe target L26166.1 *Brucella melitensis* 16S ribosomal RNA (16S rRNA) *gene*. The nucleic acid targets for *B. melitensis* identification are similar to those described by (Wojno *et al*., 2016).

1.3.13.Gene variation:-

In the last years the availability of microbial genome sequences has facilitated the development of multilocus sequence-based typing approaches such as multiple locus variable number of tandem repeats (VNTR) analysis (MLVA)(Huynh *et al*., 2008). The VNTR, allelic hyper variability related to variation in the number of tandemly repeated sequences observed at several genomic loci in the *Brucella* genomes, were used for the discrimination of bacterial species that display very little genomic diversity(Sayan *et al*., 2011).

The first application of VNTR based typing to *Brucella* was the HOOF-Prints scheme (Hyper Variable Octomeric Oligonucleotide Finger-Prints) published by (Bricker, 2002; Sayan *et al*., 2011). The approach was based on a

comparison of the newly completed genome sequences of *B. melitensis* along with a draft *B. abortus* sequence which identified an eight base pair tandem repeat sequence at nine distinct genomic loci (Awwad *et al* .,2011).In a recent study in which reference *B. melitensis* isolates were investigated ,the *rpoB gene* coding the DNA dependent RNA polymerase β sub unit (RNAP) was found to be useful for genotyping *Brucella* strains and biovars (Din *et al*., 2013).In this study, we investigated the efficacy of single nucleotide poly-morphism (SNP) analysis of the *rpoB* gene by sequencing in the genotyping *Brucella melitensis* strains(Bamaiyi *et al*., 2012; Shevtsov *et al*., 2015).

2. Materials and Methods

2.1. Materials

2.1.1. Instruments and Equipment:

Table(2.1.) shows the instruments and equipment that used in this study.

Table (2-1):Instruments and equipment and their remarks that used in the present study:

Instrument / equipment	Company / Country
Autoclave	Gallen Kaamp (England)
Conical flasks	BBL\USA
Digital camera	Samsung/ china
Disposable syringe 10 ml, 5ml and 3ml	Sterile EO. / China
Disposable Petri dishes	Al-Hani(China)
Disposable Syringes	Superestar(India)
Elisys Uno Human	Kottermann (Germany)
Eppendorf tubes	Sigma(England)
Eppendorf tubes	Bioneer/ Korea
Exispin centrifuge	Bioneer/ Korea
Exispin vortex centrifuge	Bioneer/ Korea
Gel electrophoresis	Shandod Scientific/ UK
Glass slides	Superestar(India)
High speed Cold Centrifuge	Eppendorf/ Germany
Incubator	Memmert (Germany)
Laminar flow cabinet	Labtech(South Korea)
Micropipettes (different volumes)	Eppendorf / Germany
Miniopticon Real Time PCR	Bio-Rad/ USA
Millipore filter paper 0.22µm	Nalgene(USA)µm
Mixing stick	Superestar(India)
Nanodrop	Thermo Scientific/ USA

Refrigerator	Concord/ lebanon
Sensitive balance	Gallen Kaamp (England)
Standard wire loop	John Bolten\England
Sterile test tube	Superestar/ India
Sterilized Swabs cotton	Sterellin L td.(England)
Test tubes	Superestar(India)
Thermocycler PCR	MJ-Mini BioRad/ USA
Tips	Sterile EO .\China
UV Transilluminator	ATTA/ Korea
Vortex	CYAN/ Belgium
Water bath	Kottermann (Germany)

2.1.2.Culture Media and Reagents

Culture Media and Reagents that used in this study are listed in table (2-2):

Table (2-2): Culture Media and Reagents with their remarks.

Culture Media /Reagents	Company / Country
Blood base agar	Himedia (India)
Brain heart infusion broth	APCO/ USA
Brucella agar	Himedia
Catalase reagent 3%	Samara/Iraq
Chocolate agar	Himedia
Oxidase reagent	Himedia

2.1.3. Kits

Table (2-3):Shows the kits used in this study with their companies and countries of origin.

Table (2-3): The diagnostic kit that used in the study with their remarks.

No.	Kit	Company	Country
1	Api20 E Kit	Biomerieux	France
	VP1 reagent		
	VP2 reagent		
	JAMES reagent		
	TDA reagent		
	Potassium iodide		
	Iodine		
	Brilliant green		
2	Rose Bengal Kit	Girona	Spain
	Rose Bengal *Brucell* Antigen Suspension		
	Positive control		
	Negative control		
3	ELISA Kit for *Brucella* IgM	Kiel-Wellsee	Germany
	Microtiter Strips:12 strips with 8 breakable wells coated with a Brucella antigen		
	. Calibrator A (Negative Control)		
	Calibrator B (Cut-Off Standard)		
	Calibrator C (Weak Positive Control)		
	Calibrator D (Positive Control)		
	. Enzyme Conjugate, anti-human-IgM-HRP (rabbit)		
	Substrate		
	Stop Solution		

	Sample Diluent		
	Washing Buffe, 10x concentrate		
	Plastic Foils		
	Plastic Bag		
2	gSYAN DNA Extraction Kit	Geneaid Biotech Ltd.	Taiwan
	GST buffer		
	GSB buffer		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	GD column		
	Collection tube 2ml		
2	NEXpro™ qPCR Master Mix (Probe)	Genes Laboratories	USA
	Taq DNA polymerase		
	DNTPs		
	10X qPCR buffer		
3	NEXpro™ 2X PCR Master Mix	Genes Laboratories	USA
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0		
	KCl		
	$MgCl_2$		
	Stabilizer and Tracking dye		

2.1.4. Primers and Probe:-

The primers and probe specific for detection *B. melitensis* based on 16SrRNA gene and phylogenetic variants rpoB primers were design in this study using NCBI-Genbank database and primer3 plus. These primers and probe were provided by (Bioneer company, Korea) as table (4):

Table (2- 4) The primers and probe used in the study:

Primer or Probe	Sequence (5'-3')		Product Size
16SrRNA *B. melitensis* primers	F	ACACACGTGCTACAATGGTG	108bp
	R	TTCATGCACTCGAGTTGCAG	
16SrRNA *B. melitensis* probe	FAM-GTGACAGTGGGCAGCGAGCA-TAMRA		
rpoB- Sequence *B. melitensis* primers	F	TCGACATCTACCGCGTCATG	1091bp
	R	AACCTGATCGACGATACCGC	

Standard strain of *B. melitensis* for NCBI

1- L26166.1 *Brucella melitensis* 16S ribosomal RNA (16S rRNA) gene
2- *Brucella melitensis* strain RevI DNA-dependent RNA polymerase beta chain (*rpoB*) *gene*, partial cds GenBank: DQ086119.1

2.1.5. Chemicals

The chemical and biological materials used in this work are listed in table (2-5).

Table (2-5):Chemical and biological materials with their remarks

Chemical	**Company and Origin**
Absolute Ethanol	BDH (England)
Agarose	BioBasic (Canada)
Ehidium Bromide	BioBasic (Canada)
Free nuclease water	Biolab/ USA
Gram stain	BDH/UK
Proteinase k	BioBasic (Canada)
PCR water	Bioneer (Korea)
TBE buffer	BioBasic (Canada)
100 bp DNA Ladder	Biolab/ England

2.2: Methods

2.2.1. Samples collection

To conduct the current work on antibody detection and molecular tests of *Brucella melitensis* detection in human a total of 50 blood sample was collected from human . The samples collection based on clinical symptoms for suspected cases were noted by body temperature ,headache ,sweating , arthritis and abdominal or back pain …etc. were recorded

(Appendix 1) .A total of 50 of patient blood samples were collected during November \2017 to April 2018 from different regions through Laboratory/ Samawah Gynecological and pediatrics Teaching hospital and private laboratories. Five to ten ml of blood samples were withdrawn by disposable syringe under aseptic condition from each patient ,5ml from blood was placed in a sterile brain heart infusion broth bottle for incubation ,then the residues of blood sample was separated by centrifugation at 3000 rpm for 5 minutes to make serological tests from apart of serum . Then stored by freezing (-20℃) until used. The figure (2-1) shows the study design of the present study.

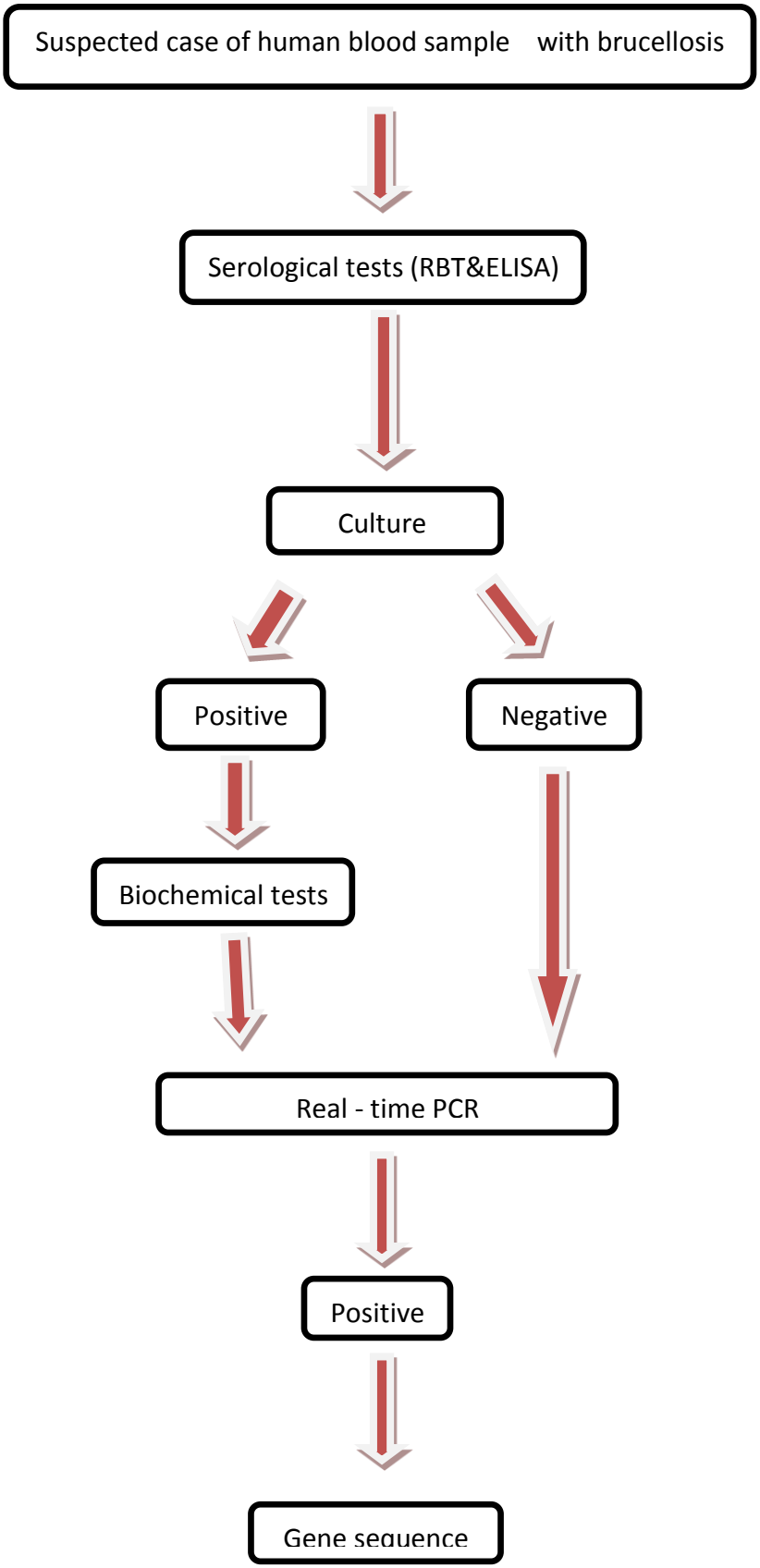


Figure (2-1):The study design of present work

2.2.2.Preparation of culture media:-

2.2.2.1.Ready –manufacture media :-

Ready prepared media used in recent study were prepared according to manufacturer's instructions ; all media were autoclaved at 121°C and pressure 15 Ib/inch² for 15 min. After sterilizing and cooling to 50 °C, the blood base agar was supplemented with 10% human blood .These media include the following :

- Blood base agar
- Brain-heart infusion

2.2.2.2.Preparation of Chocolate Agar:-

A volume of horse or sheep blood was heated that is 5% of the total volume of media was prepared very slowly to 56°C in a water bath, then dispensed into sterile Petri dishes, then allowed the media to solidify and condensation to dry, in the last placed the plates in sterile plastic bags and store at 4ºC until use.(Ferreira *et al*., 2010).

2.2.2.3.Preparation of *Brucella* agar:-

A total of43 g of the medium was suspended in one liter of purified water, then heated with frequent agitation and boil for one minute to completely dissolve the medium, then autoclaved at 121°C for 15 minutes, dispensed into sterile petri dishes while liquid(Vandepitte *et al*., 2003).

2.2.3.Bacterial Isolation Methods:-

2.2.3.1. Blood culture of specimens :-

Five ml of the patient blood sample was taken and planted it in the brain heart infusion broth media and placed in the incubator at a temperature of 37% for 4 weeks with sub culturing every few days(Frangoulidis et al., 2003).

Sub culture of bacterial growth were grown on brain heart infusion broth on blood base agar, chocolate agar and *Brucella* agar, the rate of two dishes for each sample from each these media then incubated the dishes at a temperature of 37 and left dishes for 2 to 3 days(MacFaddin, 1985).

2.2.3.2.Identification of bacterial isolates

A .Morphology of colonies :

Its appeared smooth ,mucoid, colonies ,may be colourless or grey–white. For the purpose of identifying the isolated bacteria, some of the colonies were transferred to two glass slides, then stained with gram stain and examined under the oil lens to investigate the shape and arrangement of the bacteria. showing the rod bacilli of the negative to gram stain(Sam *et al*., 2012).

B. Biochemical tests:-

1. API 20E system:-

The following steps were done according to the manufacturer's instructions (Biomerieux).

- Incubation box was prepared by using of 5ml of D.W. into the small wells of the tray to make a humid atmosphere.
- The strip was placed in the incubation tray.
- A single young isolated colony was removed from an isolation plate by a pipette and carefully emulsified in 5ml ampule of API Nacl 0.85% medium to obtain a homogeneous bacterial suspension.
- By a sterile pipette, the bacterial suspension was distributed into the microtubes of the strip according to certain instructions.
- The incubation tray was closed and incubated at $36^0C \pm 2^0C$ for 18-24 hours.

- After the incubation period, the strip was read according to the reading table. The spontaneous reactions were recorded on the result sheet and then detected the tests which require the addition of reagents.
- The results was obtained with numerical profile and matched with analytical profile index (Elsaghir & James, 2003).

2. Oxidase test:-

- A filter paper was soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride
- The paper was moisten with a sterile distilled water
- The colony to be tested was picked up with wooden or platinum loop and smear in the filter paper
- Inoculated area of paper was observed for a color change to deep blue or purple within 10-30 seconds that due to that bacteria positive to the test(Tarrand & Gröschel, 1982).

3. Catalase test (Slide Test):-

- A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using a loop or sterile wooden stick
- A drop of 3% H_2O_2 was placed on to the slide and mix.
- A positive result was the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.
- A negative result was no bubbles or only a few scattered bubbles.

- A slide was disposed in the biohazard glass disposal container (Taylor & Achanzar, 1972).

2.2.4. Serological tests:-

2.2.4.1.Rose Bengal Test

It is frequently used as a screening test in human brucellosis and would be optimal for small laboratories with limited means. False-negative reactions occur mainly stages of acute infection.

Procedure of Rose Bengal Plate Test:

- Test Serum (0.03 ml) was mixed with an equal volume of antigen on a white tile or enamel plate to create a zone approximately 2 cm in diameter.
- The mixture was agitated mildly for four minutes at ambient temperature, and then observed for agglutination.
- Any visible reaction was reflected to be positive (Naureen, Saqib, Muhammad, Hussain, & Asi, 2007).

2.2.4.2.ELISA Technique :-

The Diagnostic Automation Inc. *Brucella* IgM Antibody ELISA Test Kit has been designed for the the detection and the quantitative determination of specific IgM antibodies against *Brucella* in serum and plasma. Further applications in other body fluids are possible and can be requested from the Technical Service of Diagnostic Automation by using Elisys Uno Human(Bricker, 2002) .

The syringe pump is used to make precise dilutions. The syringe measures volumes of 2.5 ml or less. The single probe moves left and right as well as vertically. It is equipped with a liquid surface detection mechanism

that stops the probe automatically when the tip is sufficiently submerged. Probe washing uses de-ionised H2O from the prime bottle and drains to the bottle below. Each of the two racks and the plate move independently toward the front and back of the instrument. Commonly referred to as a reagent rack, a Sample rack, and a Reaction plate. However, reagents can be placed in the Sample rack, or two racks can be used to perform pre-dilutions. Each rack has an arrangement of holes or grooves configured to hold different types of tubes, bottles, micro tubes, microwells, and other containers. Racks are identified in the software in order to tell the instrument which configuration is to be used. They are also displayed graphically.

The incubator plate/well can be set to heat to 25°C, 37°C, or remain at ambient room temperature. The plate/well will heat to 25°C providing the ambient room temperature is below 25°C. (It should be noted that the option of heating the plate/well to 25°C should only be used when the ambient room temperature is consistently below 20°C.) When the probe carries a reagent to an incubated reaction plate, the temperature-controlled coil can be set to pre warm the liquid before dispensing. Reagent racks can be loaded and unloaded with bottles from run to run. The location of each reagent is indicated using a colour-coded computer screen.

Alternately, preferred reagent rack setups can be stored in panels. For convenience, multiple pre-loaded racks can be stored in the refrigerator ready to load and use. When taking an optical reading, the reaction plate automatically positions itself under the 4-channel optical system. Four lamps are aligned to simultaneously shine down through four wells. A filter wheel with eight filters rotates constantly below the plate. The filter wheel is designed so that four filters align with the four lit wells for absorbance readings. Depending on the setup, reports may be displayed or printed to create permanent lab records and we report.

2.2.5. Molecular test:-

2.2.5.1.Genomic DNA Extraction

Genomic DNA was extracted from blood ,serum, broth culture samples by using Genomic DNA Mini Kit (Geneaid. USA) and done according to company instruction as following steps:

Step1: Cell lysis:-

- A 1000µl bacteria broth samples were placed in 1.5 ml microcentrifuge tube, and the supernatant discarded
- A 200µl serum samples were placed in 1.5 ml microcentrifuge tube, 200µl GSB Buffer was added to each tubes and then mix by shaking vigorously.
- A 200µl blood, samples were placed in 1.5 ml microcentrifuge tube, 200µl GSB Buffer was added to each tubes and then mix by shaking vigorously.
- A 200µl GSB Buffer was added to each tubes and then mix by shaking vigorously.
- The samples incubated at 60ºC for 10 minutes and inverted the tube every 2 minutes.

Step2: DNA Binding

A 200µl absolute ethanol was added to mixture and mixed well by pipetting, and then briefly spin down to get the drops clinging under the lid.

1. The lysate was carefully transferred into spin column that fitted in a 2 ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for 1 minute.

Step3: Wash

- Throughout lysate was discarded in disposal bottle, and then 500µl Washing buffer 1 (W1) was added to each spin column, and centrifuged at 8000 rpm for 1 minute.

- Throughout Washing buffer 1 was discarded in disposal bottle, and then 500µl Washing buffer 2 (W2) was added to each spin column, and centrifuged at 8000 rpm for 1 minute.
- Throughout Washing buffer 2 was discarded in disposal bottle, and then the tubes were centrifuged once more at 12000 rpm for 1 minute to completely remove ethanol.

Step4: Elution

After that, spin column that containing genomic DNA was transferred to sterile 1.5ml microcentrifuge tube, and then added 100µl of elution buffer and left stand the tubes for 5 minutes at room temperature until the buffer is completely absorbed into the glass filter of spin Binding column tube.

Finally, all tubes were centrifuged at 8000 rpm for 1 minute to elute DNA, and storage at -20℃freezer.

2.2.5.2. Estimation of DNA concentration and purity

Nanodrop DNA examination.

The extracted DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) and concentration ng/µl as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, dsDNA).
2. A dry wipe was taken and cleaned the measurement unit several times. Then carefully pipet 2µl of free nuclease water and place on the measurement unit for blank the system.
3. After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

2.2.5.3. Amplification of*16S RNA gene* in *Brucella melitensis* using Real-Time PCR:-

Real Time PCR was performed for detection of *Brucella melitensis* from blood, serum and bacteria broth samples by using the specific primers and TaqMan probe specific for 16S ribosomal RNA *gene* specific for *Brucella melitensis* this technique was carried out according to method described by (Sun et al., 2016) as following:

qPCR master mix was prepared by using **NEXpro™ qPCR Master Mix (Probe)**and this master mix done according to company instructions as following table(2-6) .

Table (2-6)q PCR Master Mix:

PCR Master mix	Volume
DNA template 5-50 ng/ µL	5µL
16S ribosomal RNA gene (10pmol)F	1µL
16S ribosomal RNA gene (10pmol)R	1µL
16S ribosomal RNA gene probe (20pmol)	1 µL
qPCR master mix	10µL
PCR water	2 µL
Total volume	20µL

After that, these PCR master mix component that mentioned in table above transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in Real-time PCR Thermocycler (BioRad . USA).

Real-Time PCRthermocycler conditions was set according to primer annealing temperature and RT-PCR TaqMan kit instructions by Biorad Real-Time PCR thermocycler system as table (2-7):

Table (2-7): RT PCR thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	55 °C 30 sec	
Detection (Scan)		

qPCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification of *Brucella melitensis* in Real-Time PCR cycle number.

2.2.5.4.Amplification of *rpoB gene* using PCR for DNA sequences

PCR technique was performed for detection *rpoB gene* that use phylogenetic tree analysis study by DNA sequencer method. The method was carried out according to method described by (Sun *et al*., 2016) as following steps:

PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions Table(2-8) :-

Table(2-8) Master Mix

PCR Master mix	Volume
DNA template	5µl
Forward primer (10pmol)	1.5µl
Reveres primer (10pmol)	1.5µl
PCR water	12µl
Total volume	20µl

After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (MJ-Mini BioRad. USA).

PCR thermocycler conditions were done by using conventional PCR thermocycler system Table(2-9) :-

Table(2-9) : PCR thermocycler conditions

PCR step	Temp.	Time	Repeat
Initial Denaturation	94C	5min	1
Denaturation	94C	30sec.	30 cycle
Annealing	58C	30sec	
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

2.2.5.5.PCR product analysis :-

The PCR products was analyzed by agarose gel electrophoresis following steps:

1- 1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µl of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- PCR products were visualized by using UV Transilluminator.

2.2.5.6.DNA Sequencing method:-

DNA sequencing method was performed for genotyping biovar of *Brucella melitensis* isolates based *rpoB gene* using Phylogenetic tree analysis and homology sequence identity between local *Brucella melitensis* isolates and NCBI-BLAST *Brucella melitensis* biovar isolates then the identified isolate were submitted for NCBI-Genbank data base. The PCR product of *rpoB gene* were sent to Macrogen Company in Korea for performed the DNA sequencing by AB DNA sequencing system. Phylogenetic analysis was performed based on NCBI-Blast Alignment identification and Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

2.2.6.Statistical Analysis:-

Sensitivity: is the conditional probability that a diseased person has a positive result. Its value can be changed by changing the cut-off point for positive test results.

This was measured by dividing true-positive test results over all patients with the disease. {=a/ (a+c)}.

Specificity: is the conditional probability that a disease free person has a negative test result. This was measured by dividing true- negative test results over all patients without the disease. {=d/ (b+d)}.

Negative predictive value (NPV): is the conditional probability that a person with a negative test result is truly free of the disease. This was measured by dividing true-negative test results over all negative test results. {=d/(c+d)}.

Positive predictive value (PPV): is measured by dividing true- positive test results over all positive test results. {=a/ (a+b)}.

Overall accuracy was measured by dividing true-positive + true negative test results over all tests. {(a+d)/ (a+b+c+d)}.

Where by:

a= True positive. c= False negative.

b= False positive d= True negative.

3.Results and Discussion:-

3.1.Serological Tests:-

3.1.1.Rose Bengal Test:-

This test was carried out on 50 samples of patients suspected of brucellosis 45 (90%) of which were positive. This test was used as a screening and diagnostic test because it is an easy, fast and efficient test.(Abbas *et al*., 2012) The effectiveness of this test is specifically limited to the detection of immunoglobulin IgG1 and depend in the sensitivity to the immunoglobulin IgM and IgG also this test detects the infection in the early stages(Al-Ouqaili, 2006).

There are several studies indicated the approaching the percentage of brucellosis like Abbas (2012) who recorded cases for the brucellosis suspected samples were 58 Cases (58%) that positive for Rose Bengal test which in the acute phases from100 samples .Agasthya (2012) showed that found (15.69%) serum samples were positive out of 652 suspected case from veterinary personnel.Al-Ouqaili,(2006) who examined 84 of tested samples he found 78(92.9%)patients with acute brucellosis and 80(82.5%)out of 97 patients with chronic brucellosis gave positive results for Rose Bengal test.Al-Bayatti & Al-Thwani (2009)found that 40 tested samples (80%)were positive out of 50 suspected cases in Baghdad city .

The above studies reveal differences in their results which can be clarified considering the next trails such as site of studies ,number of samples , study duration and RBT is still the main stay of serological diagnosis of acute Brucellosis. So the Prozone phenomenon sometimes occur in this test. The immunoglobulin M(IgM) is major agglutinating antibody formed especially in first week , followed by IgG and IgA antibodies in chronic infection .All these antibodies are active in RBT test. Prozone phenomenon due to IgG and IgA can give false–negative. .

Also false–positive test due to immunological cross-reactivity have been associated with *Brucella* skin testing , *Cholera* vaccination , or infection with *Vibrio cholera, Francisella tularensis , Yersinia enterocolitica* duetomsimilar O-antigen side chain of lipopoly saccharide of *Brucella* with these microbes (Al-Bayatti & Al-Thwani, 2009; Al-Ouqaili, 2006).

3.1.2.ELISA Test :-

An enzyme-linked immunosorbent assay (ELISA) designed at detecting anti *Brucella* IgM antibody was also used to test the 50 patient samples that had previously been examined by the Rose Bengal Test. Of the 45(90%) samples that had positive the Rose Bengal results, 42(84%) were also positive in the ELISA. 42 serum samples were found to contain IgM antibodies against *Brucella* antigen. The 5 serum samples that were negative with the Rose Bengal test were also negative in ELISA test. Results of this study showed that ELISA test was the best technique in diagnosis of acute infection of *Brucella* microorganism.

These results elucidate that the test was more sensitive than Rose Bengal test. Because the ELISA test was very sensitive and could as simply be made specific for antibodies(Agasthya *et al*., 2012). Investigation from other studies of patients with acute brucellosis showed that the ELISA was the most sensitive diagnosis test such (AL-Kha,2012), showed that found 150(100%) patients of acute infection with *Brucella* microorganism were positive. Ahmed (2010)showed serum samples a high seropositivity of 95 (43%) of the 221 positive samples positive for IgM.

Alim (2015) showed in *Brucella* ELISA test results, 96 (44.2%) IgM antibodies out of 217 cases. I- ELISA. This high seropositivity exclusively to I-ELISA could only be best ascribed in its nature being a primary binding assay which can detect 1/100 of the antibodies to those detected by secondary binding assay

(Sharma, 2016).I-ELISA present study make it a highly sensitive test in brucellosis serology. Further, the above finding was supported by the work of (Sharma *et al*., 2017) who revealed that some culture Were negative while positive by I-ELISA.

3.2.Culturing and identification of bacteria :-

The results showed that of the total of 50 samples blood of the patients suspected of brucellosis, 10 (20%) samples were isolated a positive result of *Brucella* while 40(80%) samples were given negative result, all these samples were cultured on brain heart infusion broth through (48-72) hours and also perform sub culture in the week on blood agar ,chocolate agar, *Brucella* agar.

After isolation of bacteria make slides and stained with Gram -stain, where the results showed that is the colonies bacilli or cocco bacilli negative to Gram –stain in the following figure (3-2) After the work of swabs of isolates ,while it was results of Api 20 , oxidase ,catalase (Biochemical tests) matching As described (Foulongne *et al*.,2000) in the following figure (3-3).

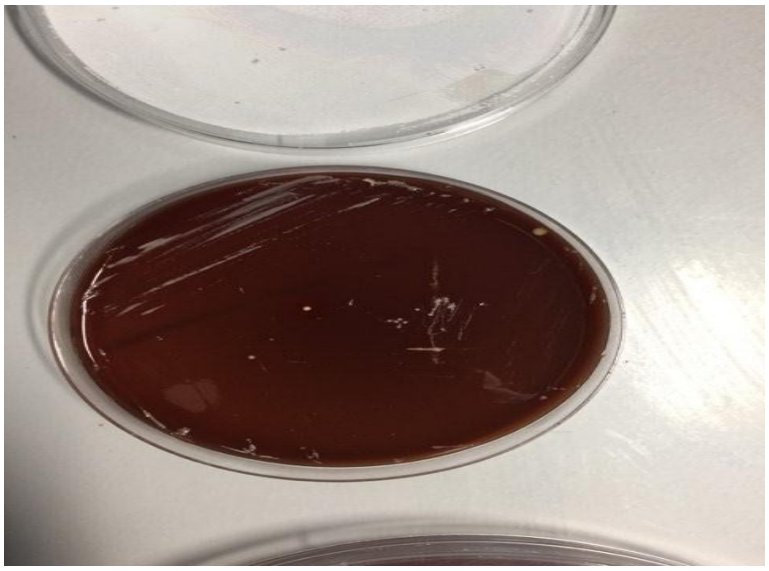
The results of isolation of *Brucella* indicated that the percentage of isolation in patients examined was different compared to other studies such as study(Yong *et al*., 2015) found a total of 41(100%) *Brucella* spp. isolates from blood culture of 41 patients were obtained from various parts of Malaysia .(Tabibnejad *et al*., 2016) showed a total of 100 patients with suspected brucellosis 39 cases (39%) had positive results when tested by the BACTEC system, and 61 cases (61%) became negative 23 culture. (Abbas *et al*., 2012) presented the rate isolation of brucellosis that (20 %).

These above differences may be related to the patients had been taken antibiotics before made the culture , to the different percentage of brucellosis habited according to the geographic region ,also to the technique used to the isolation bacteria(Bryan, 1989).

The results of culture on the blood agar, chocolate agar, *Brucella* agar showed after culturing during period (24-72) hour in 37C growth colonies of *Brucella* In the form of colonies yellow and yellow ovaries with a convex appearance and is not shiny and dry and large size about 3 mm and irregular edges as in the following forms. On the other hand, the results of the microscopic examination of the colonies after the staining of the Gram-stain showed the existence of a bacilli-rod bacteria that result in groups of parallel, negative, to, Gram-stain. They may appear in coccobacilli form, as well as single and chains, short chains or small clusters, and their axis is straight and the ends are rounded and the sides are parallel or convex in the following figure(3-1).

The biochemical tests were conducted on the bacteria that grew on the selective medium, since the isolated *Brucella* was positive for catalase and oxidase and produced a H2S gas. These results are compatible with what was indicated(Scholz *et al*., 2008).

(A) **(B)**



(C)

Figure (3-1) *Brucella* colonies on different culture media:-

A: *Brucella* colonies on Chocolate agar showing, yellow not shiny irregular colonies.

B: *Brucella* colonies on *Brucella* agar showing colonies yellow smooth a convex appearance.

C: *Brucella* colonies on Blood agar showing slowly growth.

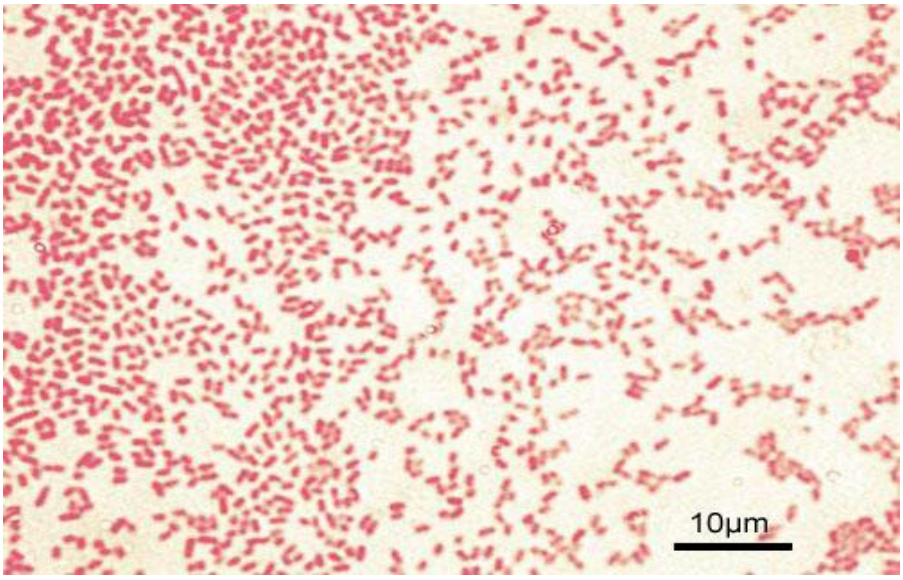


Figure (3-2):- Gram's stain smear of *Brucella* colonies. (100 X) showing Gram negative rods and coccobacilli.

Figure (3-3): A nalytic Profile Index (Api20) . The strip refer to code number of positive and negative biochemical tests of *Brucella spp.*

3.3.Molecular tests :-

3.3.1.Real-Time PCR:-

Real-time PCR assay targeting the 16S rRNA gene detected infection in 38 (76%) from 50 patients blood samples. Serological tests showed different results RBT90%, I-ELISA 84%and only 10% of blood samples were positive with all three tests, which makes interpretation of the serological results very complicated.

Mustafa (2017) found the results of real- time PCR assay with 16SrRNAgenes of all (75) isolates from patients belonged to genus *Brucella* and species *B. melitensis*. (Pelerito *et al*., 2017)examined 259 samples were tested by real –time PCR assay43 (16.6%) were positive for *Brucella* spp., being *B. melitensis* the only species detected in the analyzed cases as described, the real-time PCR assay will allow the confirmation of bacterial isolates as *Brucella* spp., or *B. melitensis* within 2 to 3 h. The inclusion of a genus specific primers-probe set assists in the recognition of infrequently isolated *Brucella* species and the identification of atypical *Brucella* strains.

Conventional methods for *Brucella* isolation and detection may take days to weeks to perform and often need the preparation of heavy suspensions of these highly infectious pathogens. Laboratory performs Gram stain, oxidase, and catalase testing as a primary screening test for suspected *Brucella* isolates. If a slow-growing, gram-negative coccobacillus is observed, the isolate is then tested by real-time PCR method described here uses heat to inactivate the microrganisms and greatly reduces the hazard of laboratory-acquired infection with *Brucella*. Finally, the multiplex format of the technique will reduce reagent cost and staff time required to perform testing for brucellosis.

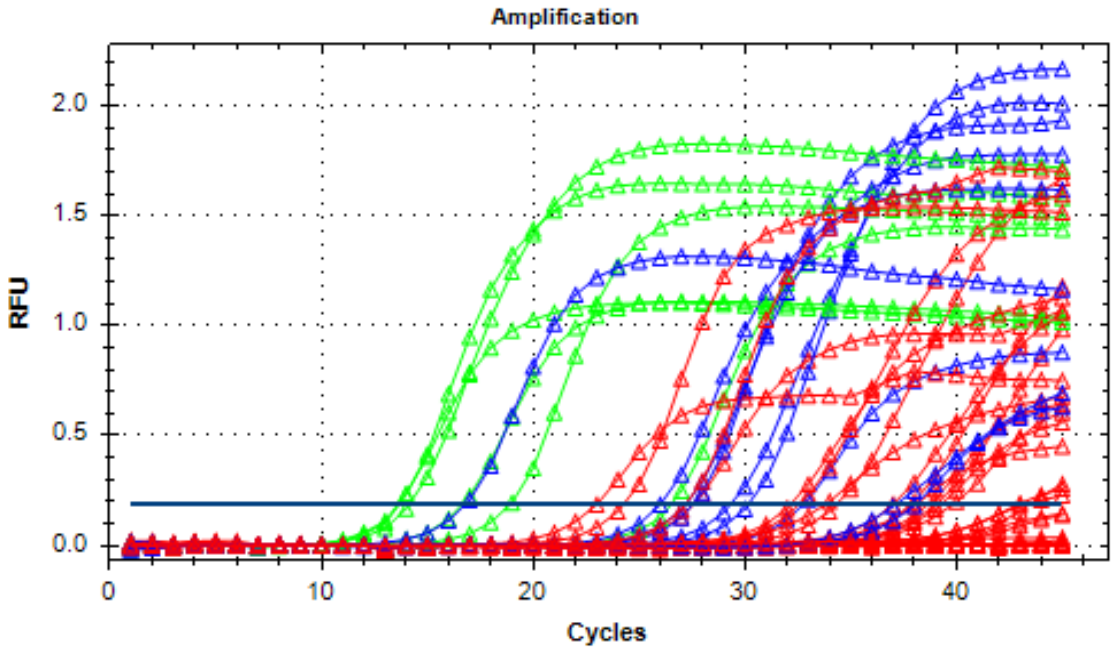


Figure (3-4) : Real-Time PCR Amplification plot of 16SrRNA gene that used in detection *Brucella melitensis*. Where, Red plot from blood samples, Blue plot from serum samples, and Green plot from culture isolates samples.

Results obtained were compared the diagnosis of brucellosis in human in four different tests bacteria isolation, serological tests RBT and ELISA ,and real-time PCR ,the seven blood samples for which the result was obtained with RBT and four samples in ELISA , inhibited in the real-time PCR ,while twenty three samples wasn't detection in culture ,as a follow table (1)

Table(3-1) Occurrence of brucellosis in human by RBT,ELISA, culture and PCR tests.

Technique	No. tested	Patients			
		Sample- positive		Sample-negative	
		No.	%	No.	%
RBT	50	45	90	5	10
ELISA	50	42	84	8	16
Culturing	50	10	20	40	80
PCR	50	38	76	12	24

A.The percent Sensitivity , specificity and accuracy of culture by comparing with RBT:-

The (3-2) table shows the sensitivity, specificity and accuracy of the methods used (culture and RBT) in the diagnosis of brucellosis in humans. Of the 50 samples examined by the test of the Rose Bengal test and culturing of bacteria were 10 positive samples of the RBT and the culture and 35 samples of the positive in the RBT and the negative in the culture, the sensitivity , specificity and accuracy (100%) (12.5)%, (0.3%) respectively

Table (3- 2): Sensitivity and specificity of techniques that used (Culture and RBT) in diagnosis of *Brucella melitensis*

Test / *RBT*	*Culture*		Total	Sensitivity %	Specificity %	Predictive value %		Accuracy rate %
	Positive	Negative				Positive	Negative	
Positive	10 T.P	35 F.P	45	100%	12.5%	22.2%	100%	0.3%
Negative	0 F.N	5 T.N	5					
Total	10	40	5o					

* T.P= True positive ** F.P = False positive

*** T.N= True negative **** F.N = False negative

B. The percent Sensitivity , specificity and accuracy of culture by comparing with ELISA.

The (3-3) table shows the sensitivity, specificity and accuracy of the methods used (culture and ELISA) in the diagnosis of brucellosis in humans. Of the 50 samples examined by the test of the Enzyme Linked Immuno Sorbent Assay and culturing of bacteria were 10 positive samples of the ELISA and the culture and 32 samples of the positive in the ELISA and the negative in the culture, the sensitivity , specificity and accuracy (100%) (20)%, (36%) respectively

Table (3 -3): Sensitivity and specificity of techniques that used (Culture and ELISA) in diagnosis of *Brucella melitensis*

Test / *ELISA*	*Culture*		Total	Sensitivity %	Specificity %	Predictive value %		Accuracy rate %
	Positive	Negative				Positive	Negative	
Positive	10 T.P	32 F.P	42	100%	20%	23.8%	100%	36%
Negative	0 F.N	8 T.N	8					
Total	10	40	5o					

* T.P= True positive ** F.P = False positive

*** T.N= True negative **** F.N = False negative

C.The percent Sensitivity , specificity and accuracy of culture by comparing with PCR:-

The (3-4) table shows the sensitivity, specificity and accuracy of the methods used (culture and PCR) in the diagnosis of brucellosis in humans. Of the 50 samples examined by the test of the Polymerase Chain Reaction and culturing of bacteria were 10 positive samples of the PCR and the culture and 28 samples of the positive in the PCR and the negative in the culture, the sensitivity , specificity and accuracy (100%) (30)%, (44%) respectively

Table(3-4): Sensitivity and specificity of techniques that used (Culture and PCR) in diagnosis of *Brucella melitensis*

Test / *PCR*	*Culture*		Total	Sensitivity %	Specificity %	Predictive value %		Accuracy rate %
	Positive	Negative				Positive	Negative	
Positive	10 T.P	28 F.P	38	100%	30%	26.31%	100%	44%
Negative	0 F.N	12 T.N	12					
Total	10	40	5o					

* T.P= True positive ** F.P = False positive

*** T.N= True negative **** F.N = False negative

D.The percent Sensitivity , specificity and accuracy of ELISA by comparing with RBT.

The (3-5) table shows the sensitivity, specificity and accuracy of the methods used (RBT and ELISA) in the diagnosis of brucellosis in humans. Of the 50 samples examined by these tests were 42 positive samples of the ELISA and the RBT and 3 samples of the positive in the RBT and the negative in the ELISA, the sensitivity , specificity and accuracy (100%) (62.5)%, (94%) respectively

Table(3-5): Sensitivity and specificity of techniques that used (RBT and ELISA) in diagnosis of *Brucella melitensis*

Test	*ELISA*		Total	Sensitivity %	Specificity %	Predictive value %		Accuracy rate %
RBT	Positive	Negative				Positive	Negative	
Positive	42 T.P	3 F.P	45	100%	62.5%	93.3%	100%	94%
Negative	0 F.N	5 T.N	5					
Total	42	8	5o					

* T.P= True positive ** F.P = False positive

*** T.N= True negative **** F.N = False negative

E.The percent Sensitivity , specificity and accuracy of PCR by comparing with RBT.

The (3-6) table shows the sensitivity, specificity and accuracy of the methods used (RBT and PCR) in the diagnosis of brucellosis in humans. Of the 50 samples examined by these tests were 37 positive samples of the PCR and the RBT and 8 samples of the positive in the RBT and the negative in the PCR, while, one sample was positive in the PCR but negative in the RBT, the sensitivity , specificity and accuracy (97.3%) (33.3)%, (82%) respectively

Table(3-6): Sensitivity and specificity of techniques that used (RBT and PCR) in diagnosis of *Brucella melitensis*

Test / RBT	*PCR*		Total	Sensitivity %	Specificity %	Predictive value %		Accuracy rate %
	Positive	Negative				Positive	Negative	
Positive	37 T.P	8 F.P	45	97.3%	33.3%	82.2%	80%	82%
Negative	1 F.N	4 T.N	5					
Total	38	12	5o					

* T.P= True positive ** F.P = False positive

*** T.N= True negative **** F.N = False negative

F.The percent Sensitivity , specificity and accuracy of PCR by comparing with ELISA.

The (3-7) table shows the sensitivity, specificity and accuracy of the methods used (ELISA and PCR) in the diagnosis of brucellosis in humans. Of the 50 samples examined by these tests were 38 positive samples of the PCR and the ELISA and 4 samples of the positive in the ELISA and the negative in the PCR, the sensitivity , specificity and accuracy (100%) (66.6)%, (92%) respectively

Table (3-7): Sensitivity and specificity of techniques that used (ELISA and PCR) in diagnosis of *Brucella melitensis*

Test	*PCR*		Total	Sensitivity %	Specificity %	Predictive value %		Accuracy rate %
ELISA	Positive	Negative				Positive	Negative	
Positive	38 T.P	4 F.P	42	100%	66.6%	90.4%	100%	92%
Negative	0 F.N	8 T.N	8					
Total	38	12	5o					

* T.P= True positive ** F.P = False positive

*** T.N= True negative **** F.N = False negative

3.3.2.Comparison of the used serological tests and PCR in the detection of brucellosis :-

The serological tests are believe good laboratory test which it is used as screening test because it is high sensitivity and accuracy . The sensitivity and specificity percent of the PCR compared with RBT and ELISA techniques in human is significant (Authority, 2009) found higher sensitivity and specificity of the PCR than the serological methods to the diagnosis of brucellosis among the 50 patients whose nested PCR assays were initially positive, 43 (86%) were negative 6 months after completing treatment. Relapse founded in five (10%) patients within 6 months after treatment and all were PCR positive. PCR and indirect-ELISA give a significant advantage over conventional serological techniques in the diagnosis of brucellosis in endemic geographical area. The PCR test results can be particularly significant in human with clinical symptoms and signs, and negative serological tests, permitting the rapid confirmation of the brucellosis(M. J. Corbel, 2006).

Brucella have slow growth time, and the culture result are not obtainable for several days or weeks. The number of bacteria in clinical samples may vary broadly, with the isolation of *Brucella* being highly dependent on the phase of disease (acute vs. chronic), antibiotic pretreatment, the existence of an suitable clinical specimen and the culturing methods used(M. Corbel & Banai, 2005). Lysis centrifugation method and automated blood culture systems enhanced the speed of finding but are still too slow to create a rapid diagnosis, (Millar *et al*., 2007) polymerase chain reaction (PCR) is fast and can be performed on any clinical sample(Dreier *et al*., 2007).

Although PCR is very hopeful, standardization of extraction methods, infrastructure, equipment and expertise are absent, and a better understanding of the clinical significance of the results is still wanted , PCR-based laboratory

tests have been projected, they cannot be considered a routine diagnostic technique yet. These limitations make serology for antibody recognition the most useful instrument for the laboratory diagnosis of brucellosis(Constance, 2010).

Serological tests are used for the first diagnosis of human brucellosis as well as throughout treatment follow-up. In our study, RBPT had a sensitivity and specificity of compared to ELISA. The RBPT can be used as a screening test in endemic region especially in rural population. But one should remember its presentation is poor in patients formerly and/or repeatedly showing to the agent(Authority, 2009). In high-risk populations, testing of diluted sera using the RBT might be a sensible alternative, as this would reduce the need for a significant number of confirmatory tests(Al Dahouk *et al*.,2013).

Laboratories can use ELISA as it is the most sensitive test. Though RBT is cheaper and easier test turnaround time is longer. In chronic and acute cases, ELISA is more domino effect in acute cases. The detection of the IgG antibody class by ELISA is more sensitive than IgM detection(Al Dahouk & Nöckler, 2011).

ELISA techniques are low cost, need less time to complete and less preparation for interpretation compared with RBT(Nielsen, 1998). These advantages give explanation their widespread use in recent years. However the sensitivity and specificity of ELISA for recognition of antibodies against *Brucella* spp. differ among studies. (Gomez *et al*., 2013) give a sensitivity of 60% for IgM and 84% for IgG, while the combined specificity for IgG and IgM was 100% .However (Mantur *et al*., 2010) found a combined IgG and IgM ELISA sensitivity of 100% but joint specificity of 71.3% . (Welch & Litwin,

2010)reported a 92.3% combined sensitivity and a combined specificity of 55%.

The results of specific finding of IgG and IgM antibodies should be interpreted with caution (Gomez *et al*., 2013; Welch & Litwin, 2010) since the antibody positivity is not always indicative of acute brucellosis, and its negativity does not eliminate the disease.

The presence of specific IgM is considered investigative of acute or recent infection. However, IgM antibody recognition in the absence of IgG may guide to an erroneous diagnosis of acute brucellosis(Corbel, 2006) and may be a cause of controversy. IgM antibodies can be recognized because of cross-reaction in other clinical conditions, and also in the existence of rheumatoid factor. Pre-absorption of rheumatoid factor is needed before the determination of IgM antibodies(Muhammad, 2009).

This study is to assess the validity of the discovery of IgM anti *Brucella* when IgG is negative, by describing a sequence of patients in whom this serological pattern was establish. We analyzed symptoms, medical history and clinical development, in order to determine whether these patients had acute brucellosis or not, and to better interpret such a result in clinical practice. In our study we described a series of patients with suspected acute brucellosis in whom ELISA serology determined IgM but not IgG anti *Brucella* antibodies. Most patients had symptoms associated to the musculoskeletal system such as arthralgia or back pain. some of them were treated with antibiotics, but the clinical picture and the outcome were not indicative of active infection caused by *Brucella* spp(Gomez *et al*., 2013).

IgM antibodies are considered evocative of acute infection and appear about a week after the onset of the disease, reaching a peak stage one to three months later. IgG antibodies show approximately three weeks after disease

onset, reaching a maximum after six to eight weeks. Some studies give a specificity of 100% for the finding of IgM by ELISA for the diagnosis of acute brucellosis(Franco *et al*., 2007; Lucero *et al*., 1999).

However, other studies performed on the usefulness of diverse serological methods detected isolated states with positive IgM in patients without brucellosis (Gomez *et al*., 2013; Hasanjani Roushan *et al*., 2005) .

In one of these studies the presence of cross-reactions was postulated, and the significance of a possible over-diagnosis in an area where other conditions such as malaria, tuberculosis, typhoid or rheumatoid arthritis can induce clinical brucellosis was highlighted(Gomez *et al*., 2013).

False positives in the determination of anti-*Brucella* IgM may be due to the found of cross-reactions. These cross-reactions are due to antigenic resemblance of the lipopolysaccharide of the cell wall with other Gram-negative bacteria. Cross-reactions with *Escherichia coli* O157, *Francisella tularensis*, *Yersinia enterocolitica*, *Vibrio cholerae* and *Salmonella* species have been classified. Most of the antibodies responsible for these cross-reactions are IgM (Corbel & Banai, 2005). These cross-reactions are possibly not responsible for the IgM anti *Brucella*- antibodies in the patients of our series.

Furthermore, false positives in the detection of IgM antibodies may also be due to the presence of rheumatoid factor. (Díaz *et al*., 2011) described that situation in three cases of chronic hepatosplenic suppurative brucellosis. Although in two of these cases IgM anti *Brucella* reactions were determined at first, other study found that IgM became negative when the rheumatoid factor was pre-absorbed with an antiserum. Although the frequency of rheumatoid factor in patients with brucellosis presents to be low, in those with chronic and focal disease that have a powerful antigenic stimulation it may be more

frequent. Millar (2007) founded rheumatoid factor positivity in 8.8% of patients with osteoarticular brucellosis .and in only 0.2% of the patients lacking this complication. Although the above mentioned states were patients with brucellosis, routine removal by pre-absorption of rheumatoid factor before detection *Brucella* IgM antibodies is suggested, as it may interfere with the test result(Dreier *et al*., 2007). The pre-absorption of rheumatoid factor was made in the sera samples from the patients of our sequence according to the orders from the manufacturer of the commercial kit used . who described a series of patients with suspected acute brucellosis in whom ELISA serology detected IgM but not IgG anti *Brucella* antibodies(del Pozo *et al*., 2014).

Mitka (2007) described that PCR is a very useful apparatus not only for the diagnosis of acute brucellosis, but also as a predictive indicator for the course of the disease and the post treatment follow-up, which is valuable for the early determining of relapses. In the present study, there was statistically insignificant difference as regards results of PCR in relation to ELISA and this agrees with (O'Leary *et al*., 2006)who recommended that there was advantage in using PCR methods over standard serological and bacteriological methods in the detection of *B. melitensis* ,samples may take from whole blood or lymph nodes. However, molecular techniques have been shown to be more suitable, accurate, highly sensitive, rapid, and simple to need small sample volumes to reduce the risks of handling tissues and more specific for the diagnosis of brucellosis from whole blood samples or from sera (Bounaadja *et al*., 2009).

3.3.3.Gene sequence:-

In the subsequent study , make certain the genetic variation (gene sequence) of *Brucella melitensis* to distinguish *Brucella* biovars, a molecular characterization of the *rpoB* gene was also performed. In difference to the *16S* rRNA locus, which lacks sufficient sequence variability for differentiation of *Brucella* spp, the *rpoB* gene appears sufficient polymorphism to distinguish all *Brucella* species and their biovars; the exceptions are *B. abortus* biovars 1 and 4 and *B. abortus* biovars 5, 6 and 9, which appear the same *rpoB* sequence (Huber, 2010).

Brucella strains were subjected to whole-genome sequencing on NCBI-Gene bank for other purposes than the ones of the present study, All 4134 bp *rpo*B gene sequences were retrieved from every draft genome and were compared with that of the published *B. melitensis* 16M genome (Georgi *et al*., 2017). *B. melitensis* strains are classified in three *rpo*B types (biovar 1, biovar 2, and biovar 3) according to the presence or absence of mutations in *rpo*B gene targeting the specific codon residues 629, 985, 1249 and 1309. essentially, a strain was classified as phenotypically belong to biovar 1, if *rpoB* is 100% the same to that of the *B. melitensis* 16M genome. The occurrence of nucleotide substitutions GCG to GTG at codon 629, GCC to GTC at codon position 985 and CTG to CTA at codon position 1309 underlies the classification as biovar 2. The existence of the nucleotide replacement ATG to ATA at codon position 1249 leads to the classification as biovar 3(Pelerito *et al*., 2017).

In which reference *Brucella* isolates were investigated, the *rpoB* gene coding the DNA depended RNA polymerase β sub unit (RNAP) was found to be useful for genotyping *Brucella* strains (Huber, 2010).In this study determined the efficiency of single nucleotide poly-morphism (SNP) analysis of the *rpoB* gene by sequencing in the genotyping of the *Brucella melitensis* strains.

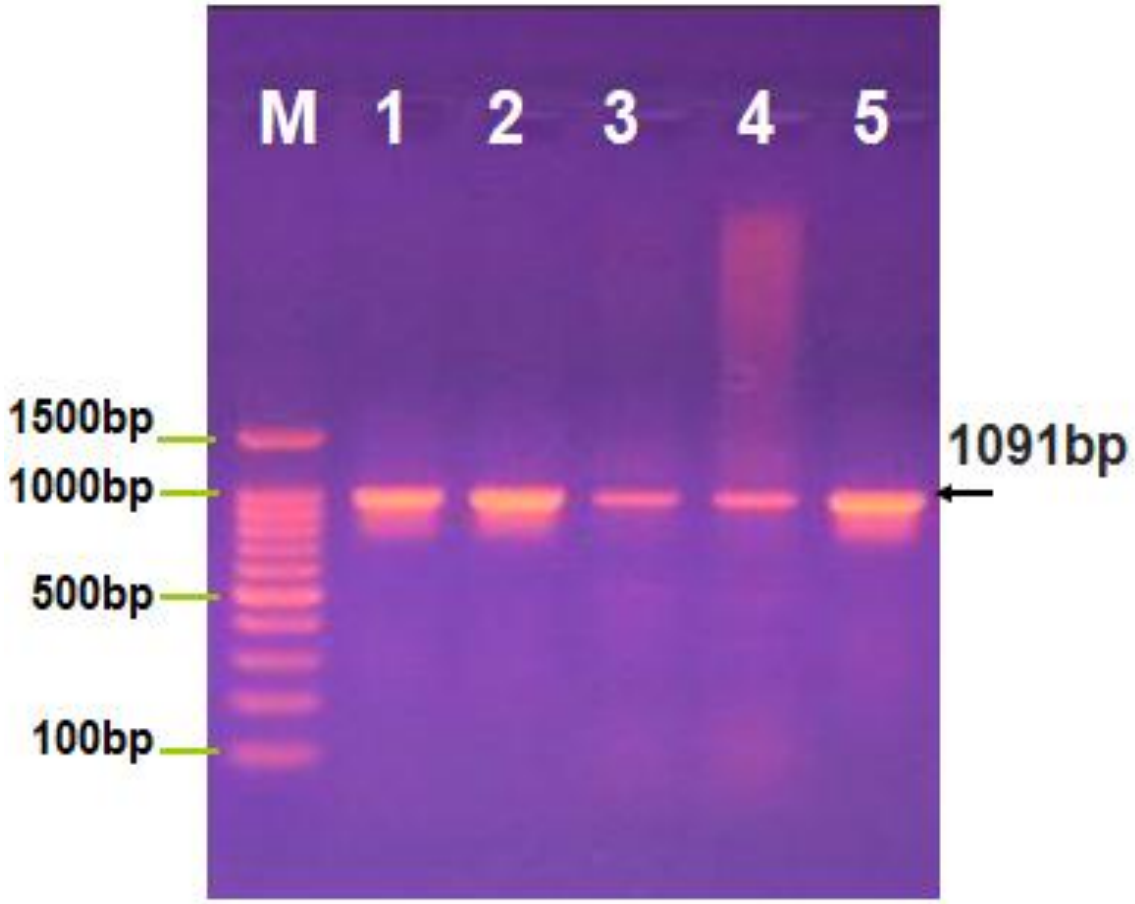
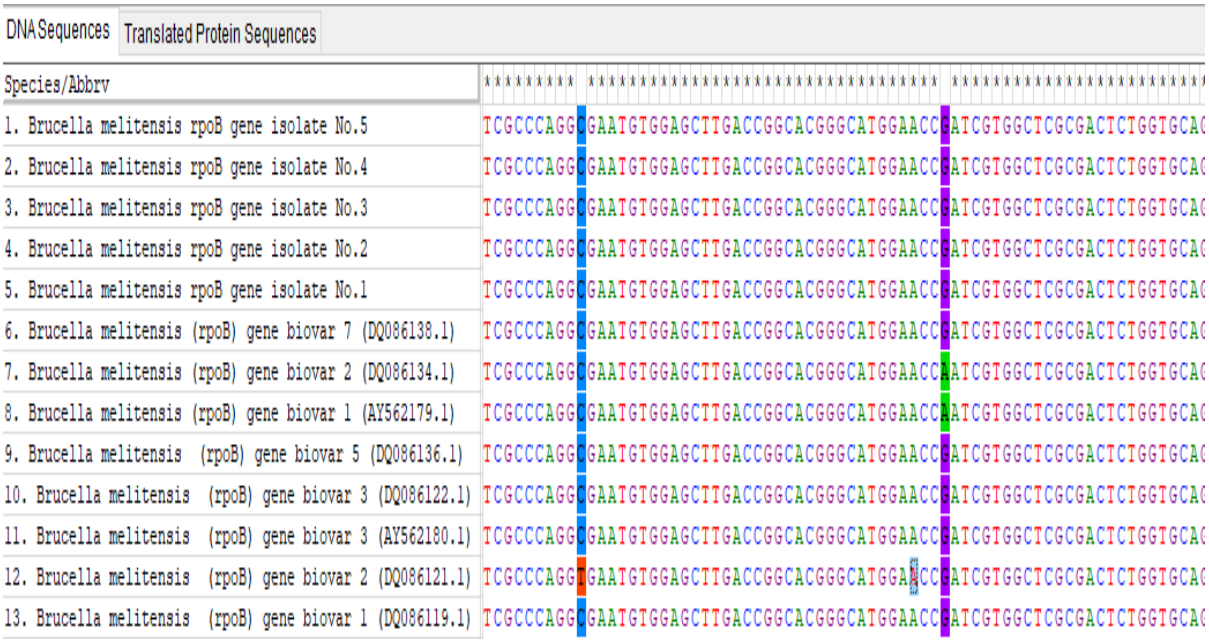


Figure (3-5): Agarose gel electrophoresis image that show the PCR product analysis of rpoB gene in *Brucella melitensis* isolates. Where M: marker (1500-100bp), lane (1-5)positive *Brucella melitensis* isolates at (1091bp) PCR product size.

It is done by selecting five isolates of the *Brucella melitensis* to made gene sequencing where it showed a close related to NCBI-Blast *Brucella melitensis* biovar 3 (AY562180.1). Whereas, the *Brucella melitensis* isolates was showed less related to NCBI-Blast *Brucella melitensis* biovar 2.The accession numbers for the nucleotides sequences :- (Banklt 2126555 Seq1 MH523634), (Banklt 2126555 Seq2 MH523635), (Banklt 2126555 Seq3 MH523636), (Banklt 2126555 Seq4 MH523637), (Banklt 2126555 Seq5 MH523638) .

Table(3-8): Homology sequence Identity of local *B.melitensis* isolates and NCBI-Blast *B. melitensis* isolates using NCBI-BLAST alignment tool.

NCBI-Blast *B. melitensis* isolate No.	NCBI-Genbank Accession number	Homology sequence Identity (%)	
		B.melitensis biovar AY562180.1	**Identity (%)**
B.melitensis isolate No.1	Banklt 2126555 Seq1 MH523634	Biovar 3	**100%**
B.melitensis isolate No.1	Banklt 2126555 Seq2 MH523635	Biovar 3	**100%**
B.melitensis isolate No.1	Banklt 2126555 Seq3 MH523636	Biovar 3	**100%**
B.melitensis isolate No.1	Banklt 2126555 Seq4 MH523637	Biovar 3	**100%**
B.melitensis isolate No.1	Banklt 2126555 Seq5 MH523638	Biovar 3	**100%**



Figure(3-6): Multiple sequence alignment analysis of the partial rpoB gene sequence in local *Brucella melitensis* (No.1-No.5) and NCBI-Genbank *Brucella melitensis* biovar isolates based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (biovar 3)in *rpoB* nucleotide sequences.

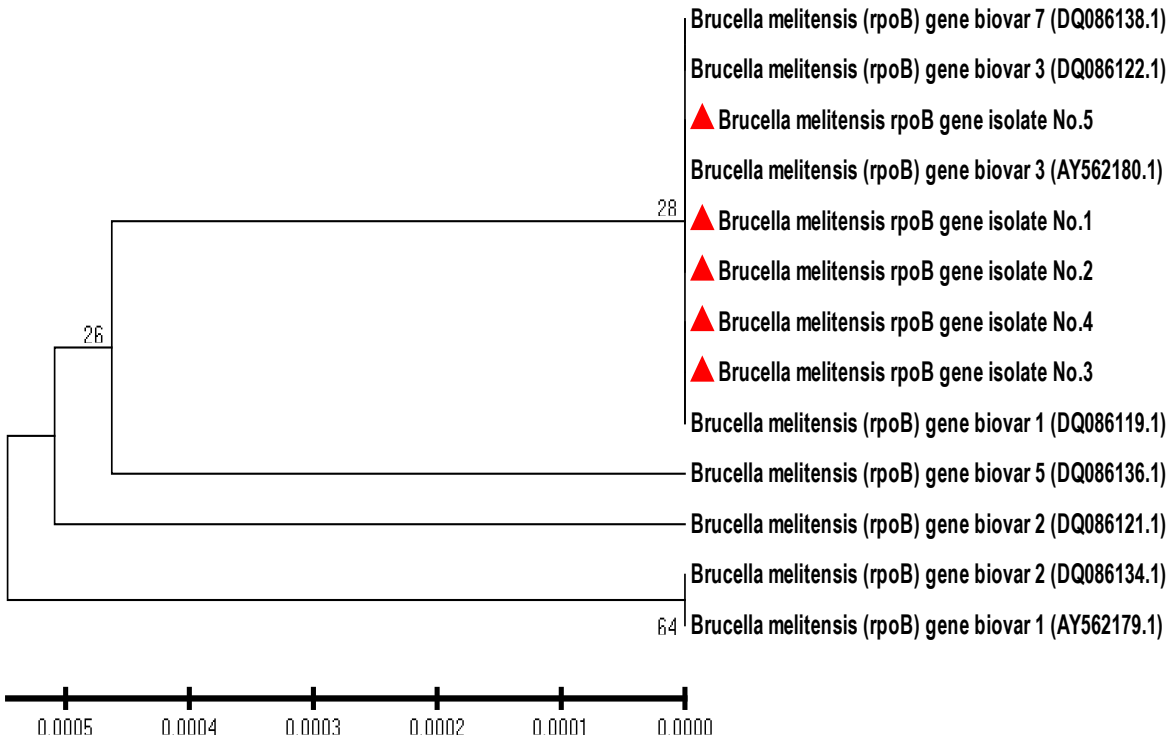


Figure (3-7): Phylogenetic tree analysis based on DNA-dependent RNA polymerase *(rpoB) gene* partial sequence that used for genotyping of *Brucella melitensis* isolates from Human samples.

The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The *Brucella melitensis* (No.1-No.5)isolate showed a close related to NCBI-Blast *Brucella melitensis* biovar 3 (AY562180.1). Whereas, the *Brucella melitensis* isolates showed less related to NCBI-Blast *Brucella melitensis* biovar 2.

The mainly of the PCR positive cases belonged to biovar 3, pointing it as clearly the most common species/biovar involved in the human disease in Portugal.(Menshawy *et al*., 2014) found most(13/17) of the isolates in Egypt were identified as a *Brucellamelitensis* biovar 3.(AL-Hamdawee, 2017) show theisolates of *Brucellamelitensis* biovar 3 from human and sheep higher percentage from than the other biovar when who used genotyping of *Brucella melitensis*.

Conclusions:-

1.The clinical symptoms alone reappear insufficient method to diagnosis of the brucellosis.

2.Serological methods gave rapid screening test for determining of brucellosis.

3.The PCR and real –time PCR considered a main technical for diagnosis and differentiating among *Brucella* spp.

4. The using gene sequence method for detection biovars of *Brucella melitensis* that was most specific and sensitive.

5.The specificity ,sensitivity and accuracy of PCR was higher when we compared with serological tests.

Recommendations:-

1.The Rose Bengal tests still effective as a screening test for the brucellosis detection .

2. The depended on using ELISA technique as an accurate examination of infected human of brucellosis and differentiation between acute and chronic brucellosis with in our health institutions adding to the availability of this technology in most hospitals.

3.The PCR and real-time PCR techniques can be used either to confirm the conventional methods also differential among *Brucella* spp.

4.The culturing method was not depended for diagnosis brucellosis but it can used to purpose epidemic and treatment .

5.The depended on gene sequence to know mutation or diversity of *Brucella melitensis* strains and biovars that imported for detection pathogenesis of *Brucella*.

6.The epitope mapping of brucellosis can be depended to diagnosis of disease.

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(Appendix 1)

Questioners about brucellosis

Differential signs

1.Undulate fever Yes…….or No…….

2.Chills Yes…….or No…….

3.Myalgia Yes……or No……..

4.Sweats with no coughing Yes……or No……..

5.Joints and back pain Yes……or No……..

6.Nervous disorders Yes……or No……..

7.Anothers symptoms ……..

(Appendix II)

Accession numbers for nucleotide sequences

GenBank MH523634-MH523638

gb-admin
to hassan_iq84, me
Jun 25 View details

Dear GenBank Submitter:

Thank you for your direct submission of sequence data to GenBank. We have provided GenBank accession numbers for your nucleotide sequences:

BankIt2126555 Seq1	MH523634
BankIt2126555 Seq2	MH523635
BankIt2126555 Seq3	MH523636
BankIt2126555 Seq4	MH523637
BankIt2126555 Seq5	MH523638

The GenBank accession numbers should appear in any publication that reports or discusses these data, as it gives the community a unique label with which they may retrieve your data from our on-line servers. You mav

(Appendix III)

qPCR-Results

1	Blood	FAM	B.melitensis	16SrRNA	29.31	2.01	(+) Positive
2	Blood	FAM	B.melitensis	16SrRNA	40.45	0.36	(+) Positive
3	Blood	FAM	B.melitensis	16SrRNA	40.89	0.38	(+) Positive
4	Blood	FAM	B.melitensis	16SrRNA		0.10	
5	Blood	FAM	B.melitensis	16SrRNA	38.64	0.57	(+) Positive
6	Blood	FAM	B.melitensis	16SrRNA	23.01	0.76	(+) Positive
7	Blood	FAM	B.melitensis	16SrRNA	36.99	0.89	(+) Positive
8	Blood	FAM	B.melitensis	16SrRNA	33.78	0.64	(+) Positive
9	Blood	FAM	B.melitensis	16SrRNA	38.07	0.42	(+) Positive
10	Blood	FAM	B.melitensis	16SrRNA	43.23	0.17	
11	Blood	FAM	B.melitensis	16SrRNA			
12	Blood	FAM	B.melitensis	16SrRNA	39.25	0.47	(+) Positive
13	Blood	FAM	B.melitensis	16SrRNA	38.37	0.40	(+) Positive
14	Blood	FAM	B.melitensis	16SrRNA			
15	Blood	FAM	B.melitensis	16SrRNA	32.15	1.08	(+) Positive
16	Blood	FAM	B.melitensis	16SrRNA			
17	Blood	FAM	B.melitensis	16SrRNA	39.82	0.47	(+) Positive
18	Blood	FAM	B.melitensis	16SrRNA			
19	Blood	FAM	B.melitensis	16SrRNA	39.03	0.70	(+) Positive
20	Blood	FAM	B.melitensis	16SrRNA	34.32	1.51	(+) Positive
21	Blood	FAM	B.melitensis	16SrRNA	37.84	0.92	(+) Positive
22	Blood	FAM	B.melitensis	16SrRNA	32.55	1.52	(+) Positive
23	Blood	FAM	B.melitensis	16SrRNA	18.91	1.50	(+) Positive
24	Blood	FAM	B.melitensis	16SrRNA	24.27	1.53	(+) Positive
25	Blood	FAM	B.melitensis	16SrRNA	27.20	1.71	(+) Positive
26	Blood	FAM	B.melitensis	16SrRNA		0.01	
27	Blood	FAM	B.melitensis	16SrRNA		0.00	
28	Blood	FAM	B.melitensis	16SrRNA		0.00	
29	Blood	FAM	B.melitensis	16SrRNA	27.53	1.92	(+) Positive
30	Blood	FAM	B.melitensis	16SrRNA	37.64	0.67	(+) Positive
31	Blood	FAM	B.melitensis	16SrRNA	24.01	0.66	(+) Positive
32	Blood	FAM	B.melitensis	16SrRNA	36.29	0.88	(+) Positive
33	Blood	FAM	B.melitensis	16SrRNA	32.78	0.74	(+) Positive
34	Blood	FAM	B.melitensis	16SrRNA	14.13	1.73	(+) Positive
35	Blood	FAM	B.melitensis	16SrRNA			
36	Serum	FAM	B.melitensis	16SrRNA	38.06	0.56	(+) Positive
37	Serum	FAM	B.melitensis	16SrRNA	37.12	0.59	(+) Positive
38	Serum	FAM	B.melitensis	16SrRNA			
39	Serum	FAM	B.melitensis	16SrRNA	30.15	2.15	(+) Positive
40	Serum	FAM	B.melitensis	16SrRNA			
41	Serum	FAM	B.melitensis	16SrRNA	13.66	1.59	(+) Positive

42	Serum	FAM	B.melitensis	16SrRNA	26.01	1.62	(+) Positive
43	Serum	FAM	B.melitensis	16SrRNA	27.21	1.78	(+) Positive
44	Serum	FAM	B.melitensis	16SrRNA	37.61	0.77	(+) Positive
45	Serum	FAM	B.melitensis	16SrRNA	16.78	1.18	(+) Positive
46	Bacterial	FAM	B.melitensis	16SrRNA	27.56	1.00	(+) Positive
47	Bacterial	FAM	B.melitensis	16SrRNA	16.63	1.05	(+) Positive
48	Bacterial	FAM	B.melitensis	16SrRNA	32.76	0.86	(+) Positive
49	Bacterial	FAM	B.melitensis	16SrRNA	26.51	1.44	(+) Positive
50	Bacterial	FAM	B.melitensis	16SrRNA	13.60	1.02	(+) Positive