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Immunological and Molecular Detection of Human Brucellosis Patients in Muthanna province

A thesis

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﴿ بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ ﴾

الرَّحْمَنُ ﴿١﴾ عَلَّمَ الْقُرْآنَ ﴿٢﴾ خَلَقَ الْإِنْسَانَ ﴿٣﴾

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Dedication

To

Who give me the power to gain this knowledge ...

"Allah"

To

Iraq's martyrs who sacrificed their lives for us

To

The one who dedicates his life for my sake
anteaches me patience, my teacher ... "dear father"

To

The source of sympathy and love ... "my mother"

To

Those who are my best support ... ", my brothers,
my sisters and my wife "

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Summary

One hundred positive rose Bengal test samples and 50 samples from healthy individuals as the control group was gathered from human blood depended on age, gender, region, taking medication, and smoking, during the extended period from December/ 2015 to July/ 2016 in Al Hussein Teaching Hospital of Muthanna Province. *Brucella melitensis* Rev1 was isolated by Lysis-centrifugation method, and diagnosis of that strain was done by the Api-20E system, Vitek 2 System, conventional Polymerase Chain Reaction (PCR), Serotyping, and Biotyping. Antibiotic sensitivity test done on growing colonies of that strain by disk diffusion method. The best treatments for *Brucella melitensis* Rev1 are Gentamicin and Tobramycin. Real Time Polymerase chain reaction technique is done on DNA samples extracted from 100 positive rose Bengal fresh blood samples, that is done by using bcp31 and omp2 as diagnostic genes, and SYBR Green I dye. The results showed 38 (38%) samples were positive and 62 (62%) were negative for both genes omp2 and bcp31. So the Real Time Polymerase chain reaction considers as a golden test in specificity and sensitivity, comparing with other parameters in the current study. 2-mercaptoethanol test done to confirm the rose Bengal test addition, differentiate between chronic and acute infections. Positive 2-Mercaptoethanol test samples were 64 (64%) and negative samples were 36 (36%) from positive Rose Bengal test samples, the number of acute infections were 17 (17%) and chronic infections were 47 (47%), at the highest sensitivity (92%), lowest specificity (53%). Indirect enzyme-linked immunosorbent assay IgG did to diagnosis human brucellosis, The results of an indirect ELISA IgG assay excluded the suspected cases (ELISA_{ex}) were 26 (26%) positive cases and 74 (74%) negative cases, at highest specificity (96%), lowest sensitivity (63%). The results of an indirect ELISA IgG included the suspected cases (ELISA_{in}) were 41 (41%) positive cases and 59 (59%) negative cases, at the intermediate estimations for sensitivity (73%) and specificity (79%). The nitroblue tetrazolium (NBT) test done to estimate the phagocytic activity in positive rose Bengal test patients, The results of (NBT) test

showed a significant increase ($P<0.05$) in phagocytic activity (266%) in the blood of patients who positive rose Bengal test, compared with (112%) in the blood of healthy individuals as a positive control. The MTT assay used for evaluating the lymphocyte transformation index % of peripheral blood leukocytes in positive rose Bengal test patients. The results of MTT assay showed a significant increase ($P<0.01$) in the lymphocyte transformation index (209%) in the lymphocytes of positive rose Bengal test patients, compared with (59%) in the lymphocytes of healthy individuals as a positive control. The total and differential count of the WBC estimate by the system (Sysmex/Japan). The results showed decreases in WBC, neutrophils, monocyte, and increase in lymphocyte at significant ($P<0.05$) in most patients with positive rose Bengal test.

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List of Abbreviations

Abbreviation	Terms
2-MET	2-Mercaptoethanol Test
ADCC	Antibody-Dependent Cell-intervened Cytotoxicity
APCs	Antigen Presenting Cells
bcs _{p31}	<i>Brucella</i> cell surface protein 31
BCV	<i>Brucella</i> Containing Vacuoles
CBC	Complete Blood Cell count
CD4 ⁺ T cell	Cluster of differentiation 4 (T-helper cell)
CFT	Complement Fixation Test
Cgs	Cyclical β -1,2-Glucan synthase
CITA	Centro de Investigación y Tecnología Agroalimentaria
CTLs	Cytotoxic T lymphocytes
C β G	Cyclic β -1,2 glucan
PrP _c	cellular prion protein
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
ELISA _{ex}	ELISA excluded the suspected cases
ELISA _{in}	ELISA included the suspected cases
ER	Endoplasmic Reticulum
ERES	Endoplasmic Reticulum Exit Site
GTPases	Guanosine 5'-Triphosphatases
HSP60	Heat Shok protein 60
IELISA	Indirect Enzyme Linked Immunosorbent Assay
IFN γ	Interferon gamma
IL-10	Interleukin-10
LAVs	Live Attenuated Vaccines
LAMP-1	lysosomal associated membrane protein 1
LPS	Lipopolysaccharide
LSD	Least significant differences

MAT	Microagglutination Test
MHC-II	Major Histocompatibility Complex Class II
MR-VP	Methyl Red – Voges-Proskauer
NAD	Nicotinamide-Adenine Dinucleotide
NBT	Nitroblue Tetrazolium
NK	Natural killer
NKT	Natural killer T cells
NPV	Negative Predictive Value
OD	Optical Density
omp2	Outer Membrane Proteins 2
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral blood Mononuclear cells
PBS	Phosphate Buffered Saline
PPV	Positive Predictive Value
qPCR	Quantitative Real Time- Polymerase Chain Reaction
RBPT	Rose Bengal plate test
RBT	Rose Bengal test
ROS	Receptive Oxygen Species
RPMI -1640 medium	Roswell Park Memorial Institute
RT-PCR	Real Time- Polymerase Chain Reaction
SAT	Serum agglutination Test
SDA	Serum–Dextrose Agar
SRs	Scavenger Receptors
STAT	Standard Tube Agglutination test
T4SS	Type IV Secretion System
TcpB	Area Containing Protein B
TGF	Transforming Growth Factor
TIR	Toll-Interleukin-1 Receptor
TLR2	Toll-like Receptor 2
TLR4	Toll-like Receptor 4
TNFα	Tumor Necrosis Factor- Alpha
TSA	Tryptose (or Trypticase) –Soy Agar
WBC	White Blood Cell

WHO	World Health Organization
$\alpha\beta$TCR	Alpha beta T Cell Receptor
$\gamma\delta$ T cells	Gamma delta T cells

1.1 Introduction

Brucellosis or Malta fever is a zoonotic disease with a worldwide spread, infecting all countries around the world, particularly in the nations of the Mediterranean and the Central East. Brucellosis is one of the most common bacterial zoonotic diseases affecting humans and animals alike, leading to a significant economic loss in livestock in addition to its effect on the public health of man because it is a multi-systemic disease (Dawson, 2008).

Brucellosis transmits from animals to humans by eating contaminated animal products or contact with embryos aborted, which are common among veterinarians, butchers and animal breeders. *Brucella* living beings can be transmitted to people through direct contact with tainted tissue by means of breaks in the skin, ingestion of sullied tissues or drink unpasteurized milk, and inward breath or mucosal introduction to aerosolized microorganisms (Poulou *et al.*, 2006).

Epidemiological studies on brucellosis were explained that disease occurs at high rates in the world annually estimated five hundred thousand cases (Dean, 2012). It is one of the common diseases in the Middle East, especially Iraq's neighboring countries (Turkey, Iran, Syria and Saudi Arabia) at a rate of 579.6 per million yearly. In Iraq, it is considered one from endemic diseases between animals and humans (Ebrahimipour *et al.*, 2012).

Control requires two steps, the first is the sero-surveillance to detect the presence of the disease in the region. Then in the second step, use of vaccines against it to limit the spread of the disease (Salih *et al.*, 2010). Historically, killed vaccines against brucellosis were weak stimulate effective T-helper 1

compared to live vaccines which recorded high degrees of success against this disease (Avila-Calderon *et al.*, 2013).

Closely diagnosis of brucellosis infections in humans is only through isolating and diagnose brucellosis, to get an accurate diagnosis of these bacteria use a combination of bacteriological, immunological and/or molecular methods (Pal, 2007; OIE, 2009).

Bacteriological methods, including growth characteristics after isolating of *Brucella* from peripheral blood of humans by enrichment culture in tryptone soya broth. Then on solid media such as blood agar base and *Brucella* medium base. Organism morphology after Gram or stamps staining, Urease, Oxidase and Catalase tests (OIE, 2009).

Immunological methods, including evaluation of innate immune response components, e.g., phagocyte cells, which measurement via Nitroblue Tetrazolium test (NBT) (Yang *et al.*, 2011), Total and differential count of white blood cell (Robert, 2007).

The measurement of humoral immune response is via serological tests, e.g., Rose Bengal test, 2-mercaptoethanol test, and Enzyme Linked Immunosorbent Assay (ELISA) (Pal, 2007; OIE, 2009). Evaluation of cell-mediated immunity by Lymphocyte transformation index in treating patients measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Wang *et al.*, 2010).

Molecular methods summarized using real-time polymerase chain reaction (RT-PCR) to rapid detection and identification of *Brucella* species as well as the vaccine strains in a single step (OIE, 2009).

1.2 The aim of the Study

The aim of the study for epidemiological, bacteriological, immunological and molecular detection of human brucellosis in Muthanna province.

1.3 The objective of the study

The objective of study is based on the comparison among various tests in order to reach on accurate diagnosis and measurement of immunological response in human brucellosis.

2. Literatures review

2.1 History of *Brucella* infection in human

Brucellosis has been considered one of the most endemic diseases in Iraq since 1937, when it was first isolated by an Iraqi physician called AlZahawi (Salih, 2010).

The buried carbonised cheese, made from sheep's milk and found with the bones, revealed the presence of cocco-bacillary forms that were morphologically similar to *Brucella* spp. (Capasso, 2002).

Eighteen centuries later, Sir David Bruce isolated *Micrococcus melitensis* (now *B. melitensis*) from the spleen of a British soldier who died from a febrile illness (Malta fever) common among military personnel stationed in Malta, an island not far away from Herculaneum (Godfroid *et al.*, 2005). For almost 20 years, brucellosis was thought to be a vector-borne disease. The zoonotic nature of the brucellosis was accidentally demonstrated in 1905 by isolating *B. melitensis* from goat's milk used for the production of soft cheese in Malta (Nicoletti, 2002a; Godfroid *et al.*, 2005; Rust, 2006). It was believed that goats were not the source of infection since they did not become ill when inoculated with *Brucella* cultures. Although raw goat's milk had been used as an essential nutritional meal for hospitalized patients suffering from Malta fever, it was decided to ban it from hospitals. The public did not follow the same recommendation and consumed infected dairy products and remained exposed to the disease (Wyatt, 2005).

2.2 Taxonomy

Brucellae are facultative intracellular coccobacilli fitting in with the order Rhizobiales of the α -2 subgroup of Proteobacteria. The class Alpha- proteobacteria joins living creatures that are either mammalian or plant pathogens or symbionts (Ficht, 2011).

The family *Brucellaceae*, *Ochrobactrum* is the closest phylogenetic neighbor of *Brucella*. For the most part, *Brucellae* are isolated by host tropism, pathogenicity and phenotypic genes. Starting now, the sort involves six "exemplary" species, i.e. the *Brucella melitensis* biovars (bvs) 1–3 (generally isolated from sheep and goats), *B. abortus* bvs 1–6 and 9 (from dairy cows and other Bovidae), *B. suis* bvs 1–3 (from pigs), bv. 4 (from reindeer) and bv. 5 (from little rodents), *B. canis* (from mutts), *B. ovis* (from sheep) and *B. neotomae* (from desert wood rats). In the latest decade, a couple of "new" species have been depicted, i.e. *B. pinnipedialis* (separated from seals) and *B. ceti* (from whales and dolphins) (Foster *et al.*, 2007), *B. microti* (withdrew from the general vole and red foxes), (Hofer *et al.*, 2012; Scholz *et al.*, 2008), and *B. inopinata* (isolated from a human chest embed wound and a so far dark creature supply) (Scholz *et al.*, 2010).

An expanding number of other "atypical" strains have been separated, conceivably speaking to novel species or ancestries. *B. inopinata*-like strain (BO2), for example, have been separated from a lung biopsy test of an Australian quiet with interminable ruinous pneumonia (Tiller *et al.*, 2010a). Different strains have been refined from wild local rodents beginning from North Queensland, Australia (Tiller *et al.*, 2010b), from non-human primates (Schlabritz-Loutsevitch *et al.*, 2009), and most starting late from African bullfrogs (Eisenberg *et al.*, 2012) Table (2-1).

Despite the fact that the larger part of reported cases are brought on by *B. melitensis*, *B. abortus* and *B. suis*, in plummeting request of an event, and seldom by *B. canis*, the pathogenicity of these recently depicted species and a typical strains

from people is presently under scrutiny. A few late reports portray infections with marine well-evolved animal strains (McDonald *et al.*, 2006; Sohn *et al.*, 2003) and disengagement of *B. inopinata* (BO1) and BO2 from individuals (De *et al.*, 2008; Tiller *et al.*, 2010a).

Brucella microti is likewise known not pathogenic to mammalian host (Occhialini *et al.*, 2012). Two Gram-negative, non-motile, non-spore-forming coccoid bacteria (strains F8/08-60 (T) and F8/08-61) isolated from clinical specimens obtained from baboons (*Papio* spp.) that had delivered stillborn offspring were subjected to a polyphasic taxonomic study which assigned to the genus *Brucella* (Whatmore *et al.*, 2014).

Table (2-1): *Brucella* species, including ‘new’ atypical strains, and their pathogenicity for humans. "Sprague *et al.*, 2012"

<i>Brucella</i> species	strains	Reservoir animals	pathogenicity for humans
<i>B. melitensis</i>	1–3 3	Sheep, goats, cows, camels Nile catfish, dogs	Yes
<i>B. abortus</i>	1–6 9	Cattle, bison, buffalo, elk, yak, camels	Yes
<i>B. suis</i>	1 1–3 2 4 5	Horses Pigs, wild boar European hare Caribou, reindeer Rodent	Yes (bvs 1, 3, 4)
<i>B. canis</i>		Canines	Yes (rarely)
<i>B. ovis</i>		Rats	Not reported
<i>B. neotomae</i>		Rodents	Not reported
<i>B. ceti</i>		Whales, dolphins, porpoises	Yes
<i>B. pinnipedialis</i>		Seals	Not reported
<i>B. microti</i>		Common voles, red foxes, (soil)	Not reported
<i>B. inopinata</i>		Unknown	Yes
Baboon isolates		Baboons	Not reported
BO2		Unknown	Yes

Rodent isolates		Rodents	Not reported
Frog isolates		African bullfrogs	Not reported

2.3 Significant virulence factors

2.3.1 Immune evasion factors

2.3.1.1 BtpA (Btp1/TcpB)

Toll-interleukin-1 receptor region containing protein translocated by *Brucella* into host cells (Salcedo *et al.*, 2008). It might be substrates of the VirB type IV secretion system (T4SS) (Sengupta, 2010). Also, contains Toll-interleukin-1 receptor (TIR) space (Chaudhary, 2012). The function of these factors is a limits improvement of dendritic cells and the time of proinflammatory cytokines, also required on microtubule progress (Snyder, 2014). Btp1/TcpB is thought to enter the host cytosol by a dark system, where it intercedes ubiquitination and degradation of Mal (MyD88 connector like). A connector is required for both Toll-like Receptor 2 (TLR2) and Toll-like Receptor 4 (TLR4). This prompts proteasomal debasement and restriction of flagging through TLR2 and TLR4. (Smith *et al.*, 2013; Snyder, 2014).

2.3.1.2 BtpB

Also translocated into host cells, and it is maybe the substrates for the VirB T4SS, TIR domain-containing protein BtpB also interferes with Toll-like receptor signalling to temper the host inflammatory response, Also modulates microtubule dynamics (Smith *et al.*, 2013; Felix, 2014).

2.3.2 Intracellular survival factors

2.3.2.1 Cyclic β -1,2 glucan (C β G)

Cyclical β -1,2-glucan synthase (Cgs), the compound is responsible for the mix of cyclic β -1,2-glucans, is a 320-kDa polytopic indispensable internal layer protein with six transmembrane-traversing portions (Arellano-Reynoso *et al.*, 2005). *Brucella* cyclic β -1,2-glucan could meddle with the development of the bacterial vacuole by upsetting cholesterol-rich lipid pontoons present on phagosomal films and thus prevent the *Brucella*-containing vacuole (BCV) to combination with lysosomes, and permitting *Brucella* to come to an endoplasmic reticulum-inferred vacuole lenient for bacterial replication (Guidolin *et al.*, 2010).

2.3.2.2 Lipopolysaccharide (LPS)

Brucella possesses a non-classical LPS as compared with the so-called classical LPS from enterobacteria such as *Escherichia coli*. *B. abortus* lipid A possesses a diaminoglucose backbone (rather than glucosamine), and acyl groups are longer (C28 rather than C12 and C16) and are only linked to the core by amide bonds (rather than ester and amide bonds).

In contrast to enterobacterial LPSs, *Brucella* LPS is several hundred times less active and toxic than *E.coli* LPS. This is an evolutionary adaptation to an intracellular lifestyle, low endotoxic activity is shared by other intracellular pathogens such as *Bartonella* and *Legionella*. Plays a role in entry and early survival inside macrophages, Resistance to innate immunity, anti-bacterial responses and A modulator of the immune response (Lapaque *et al.*, 2005).

The entry and early survival stages of smooth *Brucella* are lipid raft dependent (Jimenez *et al.*, 2004). The entry-gateway seems to include *Brucella* surface-exposed Heat Shok protein 60 (HSP60)- cellular prion protein (PrPc) interaction, but also a

SR-A (class A scavenger receptor)-lipid-A interaction and is also dependent on the LPS O-chain.

And the LPS O-side ensures the *Brucella* containing vacuole (BCV) evidence of fusion with lysosomes transiently (Watarai *et al.*, 2003).

The chemical structure of *Brucella* LPS permits the bacteria to become highly resistant to anti-bacterial effectors of the innate immune system (Cardoso *et al.*, 2006). The region of Long O-side on the bacterial surface should provoke a steric hindrance leading to the formation of a protecting barrier. The presence of long O-side on the surface of *Brucella* prevents the deposition of complement at the bacterial surface. The O-side also could prevent a specific recognition of the Pathogen-associated molecular patterns (PAMPs) and as a consequence impair expression of any cytokines, both of which are known to be involved in the clearance of intracellular *Brucella* (Barquero-Calvo, 2015). The low number of anionic groups in the core lipid A, especially charged phosphate groups could both remove anionic targets and facilitate a tighter aggregation of LPS molecules via their hydrophobic fatty acids, leading to less binding and penetration of antibacterial cationic peptides (Haag *et al.*, 2010). The *Brucella* LPS forms stable large clusters with Major Histocompatibility Complex Class II (MHC-II) named macrodomians in the cell surface, interfering with MHC-II presentation of peptides to specific CD4⁺ T cells (Kim *et al.*, 2004).

2.3.2.3 Rab2 interacting conserved protein A (RicA)

The secretion system functional for RicA secretion remains to be discovered, specifically interacts with a GDP bound form of Rab2. The RicA-Rab2 interaction may affect the maturation of the *Brucella*-containing vacuole in a way that slows intracellular replication. Thereby contributing to the evasion of the innate immune system (Herrou and Crosson, 2013).

2.3.3 Regulation factors

2.3.3.1 BvrR-BvrS

These factors are homologous to the ChvI/ExoS and ChvI/ChvG two-component regulatory systems of *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* respectively (Manterola, 2005). Like their *Brucella* counterpart, the *S. meliloti* ChvI/ExoS and *A. tumefaciens* ChvI/ChvG two component regulatory systems also regulate genes involved in modulating cell envelope properties (outer membrane protein composition and lipid A modifications), but more importantly, the genes that comprise the ChvI/ExoS and ChvI/ChvG regulons are essential for the establishment and maintenance of the symbiotic state of *S. meliloti* with its plant host and disease production by *A. tumefaciens* in its plant host (Guzmán-Verri *et al.*, 2002; Rambow-Larsen *et al.*, 2009).

The function of these factors are controlled the expression of genes involved with the maintenance of cell envelope integrity. These genes encode multiple members of the outer membrane proteins 3 (Omp3) family of proteins (Omp25 (also named Omp3a), Omp22 or Omp3b) as well as gene products that modify the fatty acid composition of the lipid A moiety of the LPS. Also, they are controlling the expression of the T4SS VirB (Martinez-Nunez, 2010).

2.3.4 Secretion system factors

2.3.4.1 VirB type IV secretion system (VirB T4SS)

The characteristics of these factors are phagosome acidification which required for intracellular expression of the VirB type IV secretion system. The LuxR family regulator VjbR regulated the *virB* operon directly by bounding a fragment of the *virB* promoter containing an 18 bp palindromic motif (Uzureau *et al.*, 2007). The function of VirB T4SS is for late *Brucella* containing vacuole (BCV) maturation

events corresponding to the sustained interaction and fusion with the endoplasmic reticulum (ER) (Boschiroli *et al.*, 2001).

Brucella initiates interactions with Sar1/COPII-dependent ERES (ER exit sites), the early secretory pathway. Unlike *Legionella*, that intercepts COPI-dependent vesicular trafficking from the ER for the biogenesis of its ER-derived replicative organelle (Celli *et al.*, 2005). The acidified *Brucella* containing vacuole (BCV) interacts with late endosome/lysosomes, acquiring markers such as lysosomal associated membrane protein 1 (LAMP-1), CD63 and Rab7. Subsequently, wild-type *Brucella* is able to exclude these late endosomal/lysosomal markers from its vacuole and acquire markers of the endoplasmic reticulum (Uzureau *et al.*, 2007).

2.4 Pathogenesis and Pathogenicity of Brucellosis

Brucellae display strong tissue tropism and replicate within vacuoles of macrophages, dendritic cells (DCs), and placental trophoblasts. However, the pathogen has the ability to replicate in a wide variety of mammalian cell types, including microglia, fibroblasts, epithelial cells, and endothelial cells. The intracellular lifestyle of *Brucella* limits exposure to the host innate and adaptive immune responses (Martirosyan & Gorvel, 2013).

The organism sequesters from the effects of some antibiotics, and drives the unique features of pathology in infected hosts, which is typically divided into three distinct phases: the incubation phase before clinical symptoms are evident, the acute phase during which time the pathogen invades and disseminates in host tissue, and the chronic phase that can eventually result in severe organ damage and death of the host organism. Nonspecific influenza-like symptoms observed in humans include pyrexia, diaphoresis, fatigue, anorexia, myalgia, and arthralgia. Furthermore, increasing

evidence from endemic regions suggests that an elevated risk of human abortion is associated with exposure (Baud & Greub, 2011).

Chronic infection results from the ability of the organism to persist in the cells of the host in which *Brucellae* are distributed by way of the lymphoreticular system to eventually cause cardiovascular, hepatic, lymphoreticular, neurologic, and osteoarticular disease. Measurable splenomegaly is associated with increased lymphohistiocytic cells in the spleen, slightly reduced percentage of splenic CD4 and CD8 T cells, and major increases in the percentage of splenic macrophages (Figueiredo *et al.*, 2015).

2.5 Immunobiology of *Brucella*

Brucellae quickly translocate across the mucosal epithelium layer in vivo and are endocytosed by mucosal macrophages and DCs (Rossetti *et al.*, 2013). *Brucella* survive and replicate inside professional phagocytic cells, evade and modulate the host immune response, and disseminate to preferred tissues through cellular tropism, for example, placental trophoblasts in pregnant females, fetal lung, reticuloendothelial system, and reproductive tract (Adams, 2002).

In vitro studies were used as models to understand adhesion, internalization, intracellular trafficking, survival, and replication of *Brucella* in susceptible hosts. Thus, after attachment to the surface of mucosal epithelial cells, *Brucella* induces a zipper-like mechanism for internalization (Rossetti *et al.*, 2012). As yet incompletely defined binding molecule(s) are activated before and/or on contact with epithelial cells. *Brucella* bind to epithelial cell surface receptors that contain sialic acid and sulfated residues (Castañeda-Roldán *et al.*, 2004). These binding promotes activation of small Guanosine 5'-Triphosphatases (GTPases) that trigger a signaling cascade that reorganizes the actin cytoskeleton to induce a host cell membrane rearrangement along the surface of the pathogen that enhances invasion. Entry occurs within a few

minutes after interaction which requires full activation of a mitogen-activated protein kinase signaling pathway (Rossetti *et al.*, 2012).

Brucella survive and replicate inside nonprofessional phagocytic cells up to 72 hours in vitro and move across the epithelium in vivo by subverting the mucosal epithelial barrier function to facilitate *Brucella* transepithelial migration (Rossetti *et al.*, 2013). Simultaneously, this interaction initiates a minimal innate immune response with weak proinflammatory activity (Berg *et al.*, 2012; Rossetti *et al.*, 2012). Once translocated through the epithelium, *Brucella* are engulfed by mucosal phagocytic cells in which <10% of phagocytized bacteria survive an adaptation period. To delay being recognized by the immune system and initiating an immune response, *Brucella* reduce, modify, or cloak their pathogen-associated molecular patterns (Berg *et al.*, 2012).

However, some Toll-like receptors (TLRs; mainly TLR2, TLR4, and TLR9) initiate limited intracellular signaling that activates the transcription factor NF- κ B to control expression of inflammatory cytokine genes, although at a level that is 10-fold less than enterobacteria (Oliveira *et al.*, 2008). Inside mononuclear phagocytic cells, *Brucella* reside in a special vacuole called *Brucella*-containing vacuole (BCV), modify intracellular trafficking, and transform the vacuole into a replicative compartment or brucellosome. Experimental evidence indicates that the microenvironment inside the BCV is one of limited nutrient availability (Köhler *et al.*, 2002).

To which *Brucella* adapts soon after invasion. Initially, the pathogen undergoes quantitatively reduced gene expression and protein synthesis involved in anabolic metabolism while increasing amino acid catabolism, switching to alternative energy sources, and altering respiration to adapt to low oxygen tension (Lamontagne *et al.*, 2009).

2.6 Intracellular trafficking

Brucellae evades intracellular destruction by restricting fusion of the BCV with the lysosomal compartment. Some BCVs that harbor internalized *Brucella* traffic from endocytic compartments to a replicative niche within BCVs that contain markers of the endoplasmic reticulum (ER). BCV seizure of ER membranes and components is accompanied by structural characteristics and functional restructuring of the ER (Smith *et al.*, 2013). BCVs later accumulate autophagic features and exhibit LAMP1 positivity, constituting a distinctive aspect of the intracellular *Brucella* lifestyle. The VirB T4SS regulates *Brucella* intracellular trafficking and organisms that lack this system fail to establish an intracellular replicative niche in vitro (Comerci *et al.*, 2001; Delrue *et al.*, 2001). The T4SS of *Brucella* is thought to secrete effector molecules that control the intracellular and stealthy lifestyle of the pathogen (Salcedo *et al.*, 2013).

2.7 The innate immune system of *Brucella*

2.7.1 Macrophages and dendritic cells (DCs)

Macrophages and dendritic cells (DCs) play a fundamental role in innate immunity, in recognition and in the induction of robust adaptive immunity against intracellular bacteria such as *Brucella* spp. many lines of evidence support the bidirectional relationship between these two cell types and *Brucella* spp. Both cell types have various inducible mechanisms to eliminate bacteria while bacteria can invade these phagocytic cells to avoid the cellular immune response and establish infections for prolonged periods. In order to establish chronic infection *Brucella* spp. express virulence factors or PAMPs that interfere with, or do not alert, the antimicrobial arsenal and antigen-presenting potential of the host macrophages/DCs (Maria-Pilar *et al.*, 2005; Comstock & Kasper, 2006; Martirosyan *et al.*, 2011; Skendro *et al.*, 2011; Velásquez *et al.*, 2012).

This stealthy mechanism, portrayed by low stimulatory action and toxicity for antigen presenting cells (APCs), gives a "crevice" that allows the foundation of an intracellular replicative specialty before the actuation of Th1 immune response, *Brucella* is uptake by means of routine zipper such as phagocytosis through lipid pontoon communications utilizing different phagocytosis advancing receptors (FcR, C3bR, scavenger receptors [SRs]). Taking after passage into macrophages and DCs, a large portion of the microscopic organisms (roughly 90%) is killed inside of an initial couple of hours. Be that as it may, some of them survive and achieve their replicative specialty (Watarai *et al.*, 2002; Rafiei *et al.*, 2006; Martirosyan *et al.*, 2011).

Brucella guarantees its survival and harmfulness by redirecting endocytic pathway trafficking and framing exceptional phagosomes of endoplasmic reticulum (ER) source called BCVs these speak to the intracellular replication compartments. The BCVs escape combination with late endosomes and lysosomes, before they move to the ER and wire with the ER layer (Zwerdling *et al.*, 2008).

Autophagy is a dynamic cellular homeostatic process by which cytoplasmic targets are sequestered within double-membraned vesicles called autophagosomes and subsequently delivered to lysosomes for degradation (Kuballa *et al.*, 2012). Recently, substantial evidence has demonstrated autophagy to be an important innate immune mechanism resulting in direct elimination of various intracellular pathogens through hydrolytic degradation in autophagolysosomes (Kuballa *et al.*, 2012; Skendros & Mitroulis, 2012).

Pathogen engulfment by APCs, TLR signalling and IFN γ secretion by Th1 cells are potent inducers of the autophagic machinery against intracellular bacteria (Skendros & Mitroulis, 2012). Notably, recent experimental data on murine macrophages suggest that *B. abortus* and *B. melitensis* subvert autophagy or exploit autophagic machinery to their advantage. In particular, BCVs that display structural features of autophagy are required for *B. abortus* to complete their intracellular

lifecycle and spread from cell to cell (Starr *et al.*, 2012). Moreover, the pharmacological inhibition of autophagy *in vitro* has been shown to significantly reduce the intracellular replication of *B. melitensis* (Guo *et al.*, 2012).

2.7.2 Neutrophils

Neutrophils have a key role in innate immunity and they are the first cells recruited in vast numbers to the site of inflammation. Neutrophils encounter and kill microbes intracellularly upon phagocytosis, when their antimicrobial granules fuse with the phagosome. Furthermore, they release lytic enzymes and reactive oxygen species (ROS) that destroy pathogens (Nauseef, 2007).

It has been shown that *B. abortus* promptly activates human neutrophils and prolongs neutrophil survival *in vitro*. This effect is associated with lipoprotein (L-Omp) (Elfaki & Al-Hokail, 2009), indicating the proinflammatory role of lipoproteins in the innate immunity against brucellosis (Zwerdling *et al.*, 2008). *Brucella* does not replicate within neutrophils, although it seems to resist killing (Conde-Álvarez *et al.* 2012).

2.7.3 Innate lymphocytes

Innate lymphocytes are at the interface between innate and adaptive immunity, and include mainly natural killer (NK) cells, natural killer T (NKT) cells and $\gamma\delta$ T cells. In comparison with conventional antigen-specific T cells ($\alpha\beta$ TCR CD4⁺ and CD8⁺ T lymphocytes), they are a smaller proportion of the peripheral blood cell populations and recognize non-peptidic antigens (glycolipids and phospholipids) without MHC restriction. Many studies support the important role of innate lymphocytes as rapid and early producers of IFN γ in immunity to intracellular pathogens, before the expansion of specific Th1 responses (Dieli *et al.*, 2003; Sada-Ovalle *et al.*, 2008; Kubota, 2010; Nyirenda *et al.*, 2010).

2.8 Adaptive immunity in brucellosis

2.8.1 Specific T-cell immunity

Adaptive immunity expands after the activation of innate immunity in order to mount and sustain an antigen-specific response aimed at eradicating bacteria and protecting the host (Barquero-Calvo *et al.*, 2007). The Th1 immune response against *Brucella* leads to IFN γ secretion by antigen-specific T lymphocytes. Most studies indicate that CD4⁺ T lymphocytes are the major producers of IFN γ , although other cell subsets such as CD8⁺ T lymphocytes, $\gamma\delta$ T lymphocytes and NK cells also participate in IFN γ production (Yingst & Hoover, 2003; Dean *et al.*, 2012). The IFN γ activates the bactericidal machinery of macrophages, promotes the expression of antigen presenting and costimulatory molecules on APCs, stimulates CTL-mediated cytotoxicity and potentiates the apoptotic death of infected macrophages (Yingst & Hoover, 2003; Skendros *et al.*, 2011).

The bactericidal efficiency of IFN γ - activated murine macrophages against *Brucella* is hampered by the Th2 cytokine IL-10. It has also been reported that Br-LPS induces the expression of IL-10 by human PBMCs (Amina *et al.*, 2002). Data suggest that *Brucella* disturbs IFN γ signal transduction in infected human macrophages, although the aetiological mechanism has not yet been clarified (Castaneda-Roldán *et al.*, 2004).

Substantial evidence demonstrates the protective role of CD8⁺ CTLs in several models of experimental brucellosis. Activated CTLs contribute to protective immunity against *Brucella* through Fas- or perforin-mediated cytotoxicity and IFN γ secretion (Durward *et al.*, 2010; Durward *et al.*, 2012b).

Recent data obtained in IFN γ and IL-12/ β 2-microglobulin knockout mice demonstrate that lack of endogenous IFN γ is more important to control of brucellosis

than CTLs (Brandão *et al.*, 2012). However, in the murine brucellosis model, CTLs were found to increase during the chronic stage of infection (Adone *et al.*, 2012).

In a similar manner, human clinical studies have also demonstrated increased numbers of CTLs in the peripheral blood of patients with chronic/relapsing brucellosis (Moreno-Lafont *et al.*, 2002; Skendros *et al.*, 2007). It could be presumed that this phenomenon is a compensatory effect of the defective CD4⁺ T-cell responses that are characteristic of chronic disease. Recent data from a murine model of chronic brucellosis indicate that CTLs also display a low quality responding capacity, characterized by suppression of Th1 cytokines (IFN γ , TNF α and IL-2). This deficit is associated with the *B. melitensis* Toll/IL-1 receptor (TIR)-domain-containing protein (TcpB), which inhibits CD8⁺ cytotoxic killing of target cells expressing antigenic peptides and constitutes a novel strategy for *Brucella* to evade adaptive immunity (Durward *et al.*, 2012b).

2.8.2 B lymphocytes and *Brucella* infection

The B lymphocytes govern the humoral arm of adaptive immunity, characterized by production of antigen-specific antibodies. In addition to their neutralizing effect, antibodies act as opsonins that facilitate the phagocytosis of bacteria by APCs, activate complement and promote antibody-dependent cell-mediated cytotoxicity (ADCC) by macrophages, neutrophils and NK cells. Under certain circumstances, B cells perform antigen presentation which can activate cellular immunity (Baldwin & Goenka, 2006).

The role of humoral immunity against intracellular bacterial infections is limited and not protective. Antibody-mediated opsonisation by immunoglobulin (IgM, IgG1, IgG2a and IgG3) enhances phagocytic uptake of bacteria, limiting the level of initial infection with *Brucella*, but has little effect on the intracellular course of *Brucella* infection (Bellaire *et al.*, 2005; Baldwin & Goenka, 2006). From a clinical

perspective, detection of antibodies against Br-LPS is commonly used for the diagnosis of brucellosis (using seroagglutination tests) in livestock and humans (Al Dahouk *et al.*, 2003). Recently, a study of B-cell-deficient mice provided new insight into the regulatory role of B cells in brucellosis, illustrating the critical role of Th1 responses in host resistance. During the early phase of disease, B cells produce IL-10 and transforming growth factor β (TGF β), which attenuate IFN γ -mediated Th1 responses and promote persistence of infection. Absence of B cells is associated with marked, antibody-independent resistance to *Brucella* (Goenka *et al.*, 2011).

2.9 Epidemiology of brucellosis

2.9.1 Worldwide scenario

Brucellosis is found worldwide, but it is well controlled in most developed countries. The clinical disease is still common in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and the Caribbean. *Brucella species* vary in their geographic distribution. *B. abortus* is found worldwide in cattle-raising regions except in Japan, Canada, some European countries, Australia, New Zealand and Israel, where it has been eradicated. Eradication from domesticated herds is nearly complete in the USA. *B. abortus* persists in wildlife hosts in some regions, including the Greater Yellowstone Area of North America. *B. melitensis* is particularly common in the Mediterranean. It also occurs in the Middle East and Central Asia, around the Arabian Gulf and in some countries of Central America. This organism has been reported from Africa and India, but it does not seem to be endemic in northern Europe, North America (except Mexico), Southeast Asia, Australia or New Zealand (CFSPH, 2007).

2.9.2 Brucellosis in center East

The center east has generally been considered as an endemic region. In reality, five of the ten nations with the most astounding occurrence of human brucellosis are around there.

Syria has the most astounding yearly frequency around the world, coming to a disturbing 1603 cases for every million every year, as per information from the world organization for Animal Health (OIE) (OIE, 2009).

An extension in the yearly number of cases reported has been furthermore found in Turkey, surpassing 15000 cases in 2004 (OIE., 2009). Brucellosis is particularly endemic in the poor eastern ranges. Different reports have tended to the investigation of infection, transmission of the disorder in isolated scopes of this region (Yildiz *et al.*, 2005). Despite the fact that an organized national methodology is as yet missing. The connection of poor financial status and brucellosis rate, as sketched out in the EU, most likely applies here as well: various studies concentrating on seroprevalence, abridged by Cetinkaya and colleagues demonstrate that the nearness of a region either to Europe or to Ankara is joined by lower seroprevalence rates, perhaps because of upgrading utilization of the Turkish Brucellosis Challenge Project that has been running for as far back as 20 years (Cetinkaya *et al.*, 2005).

According to data from the National Commission on Communicable Diseases Control of an Iranian condition. In 1989, the yearly recurrence surpassed 1000 cases for every million in 2003 according to the Regional Animal Disease Surveillance and Control Network and Nations in 2005, the yearly rate had tumbled to 238.6 cases for every million. (Pappas *et al.*, 2006a) Still, human brucellosis remains an enormous weight for this country.

Brucellosis is endemic in the southern territory of Oman, with a yearly recurrence surpassing 1000 cases for each million (Pappas *et al.*, 2006a).

Furthermore, Saudi Arabia is an incomprehensible storehouse of human brucellosis. Regional endemicity contracts in Saudi Arabia, to some degree according to air figures: a stormy season in a locale would achieve the extending grass advancement and along these lines downpours of shepherds and flocks (Elbeltagy, 2001).

In the United Arab Emirates, most cases are represented from Dubai a celebrated overall travel destination, underlining the centrality of the disease in the field of travel medicine (Pappas *et al.*, 2006a).

Kuwait is a prime representation of the change of the investigation of illness, the transmission of human brucellosis by political components. 20 years earlier, the yearly recurrence surpassed 500 cases for every million, on a very basic level as a result of vast animal brucellosis according to the Regional Animal Disease Surveillance and Control Network and Nations in 2005. Massive loss of animals during the 1991 Iraqi attack brought about a significant decline in human cases in the next years; in any case, this abatement was not managed over time, and the region is still endemic (Salih, 2010).

In Jordan, the assessed frequency of human brucellosis surpassed 300 yearly cases for each million in the late past (Abo-Shehada and Abu-Halaweh, 2013).

Brucellosis is endemic in the southern district of Oman, with a yearly recurrence surpassing 1000 cases for each million (Pappas *et al.*, 2006a).

Endemicity of the ailment in Lebanon has been relentless throughout later years, especially in the Beqaa area, as shown by data from the Ministry of Public Health (Pappas *et al.*, 2006a).

2.9.3 Iraq

Iraq is in both the northern and eastern sides of the equator, it is arranged in the Middle East, an apparent geological area of southwestern Asia. The country is boarded by the Arabian Gulf, and the countries of Kuwait, Saudi Arabia, Jordan, Syria, Turkey, and Iran. Its property zone is 432,162 sq km (166,859 sq miles).

Data from OIE were recently made available for the incidence of human brucellosis in Iraq, underlining the huge endemicity of the disease in this region, despite ongoing attempts to control both animal and human disease. Whether control can be achieved in a state still plagued by war and famine is questionable. Moreover, the endemicity of the disease in this area may raise concerns, since a purely endemic brucellosis case of an international soldier stationed in Iraq might cause alarm of a possible biowarfare incident (Pappas *et al.*, 2006a).

In Muthanna city the study was in the AL-Samawa city in the time from January to December (2005), to focus on the rate of Brucellosis. In this study, 368 cases with Brucellosis were disengaged investigated from the patient, the otherworldly related of reasons the disorder was *Brucella melitensis* and *Brucella abortus* the age – social affair of (<1–5 years) was the most normal get-together. More than 66% of the cases were females. The events of the infection among patients were from natural reach, case records at hot months more than during the cold months (Zayadi, 2002).

2.10 Transmission

Brucella organisms are present in the reproductive tissues and products of parturition at extremely high concentrations. The organisms also concentrate in the udders of animals that produce the milk used for human consumption. *Brucella* organisms can be transmitted to humans through direct contact with infected tissue via breaks in the skin, ingestion of contaminated tissues or milk products, and inhalation or mucosal exposure to aerosolized bacteria. Other routes, including in

utero transmission, person-to-person transmission, and tissue transplantation-associated transmission (Poulou *et al.*, 2006).

Kotton have been identified or suggested, but are much less common. Among nonhuman animals, the predominant route of exposure to smooth strains of *Brucella* is through ingestion or inhalation of organisms that are present in fetal fluids or other birth products. Herds are typically exposed following the introduction of an infected animal that subsequently gives birth or aborts a fetus, whereupon pasture or water becomes contaminated by these excretions(Kotton, 2007).

2.11 Pathogenesis-informed approaches to therapeutics

Human *Brucella* infection is typically treated with combination antibiotic therapy. But therapeutic relief from the infection is neither certain nor rapid, because the intracellular location reduces antibiotic efficacy. Treatment can be prolonged and associated with undesirable side effects. Moreover, relapse after antibiotic therapy constitutes a substantial risk to the treated patient. As such, the recommended treatment usually entails a combination of drugs for at least 30 days, each of which falls into Food and Drug Administration pregnancy categories C (rifampin) or D (tetracycline/doxycycline) that are poor to unsuitable for use in gravid women (Mile *et al.*, 2012).

To address these issues, development of novel therapeutic strategies to address brucellosis has been pursued. Recent advances in our understanding of the molecular mechanisms that mediate interactions between *Brucella* and host cells have facilitated this pursuit. For example, Villamil Giraldo *et al* has spearheaded the development of novel antivirulence compounds that target virulence functions while leaving essential cell functions unharmed. One advantage of the antivirulence approach is that molecules that do not target essential functions will fail to induce selective pressure for antibiotic resistance (Villamil Giraldo *et al.*, 2012).

To identify novel compounds that inhibit the virulence of *Brucella*, a high throughput small molecule screen was performed for compounds that inactivated the function of VirB8 (Paschos *et al.*, 2011). A central component of the bacterial T4SS, which is essential for virulence and pathogenicity of *Brucella*, and a wide variety of other animal and plant pathogens. This endeavor identified several potent and specific inhibitors of T4SS function (Paschos *et al.*, 2006).

X-ray crystallography and docking studies were used to demonstrate that VirB8 inhibitors bind to a surface groove opposite to the dimerization interface, thereby providing insight into the mechanism of action of this potential therapeutic (Paschos *et al.*, 2011; Smith *et al.*, 2012).

2.12 Pathogenesis-informed approaches to vaccines

Nonviable *Brucella* vaccines historically have a poor record of success that started to heat-killed *Brucella*, crude extracts, and then subunit and DNA vaccines. This poor track record is primarily explained by a failure to induce an effective Th1 response comparable with live, attenuated vaccines (LAVs) (Avila-Caldern *et al.*, 2013).

In contrast, LAVs have a long history of successful use against brucellosis and other intracellular pathogens in laboratory animal models and in target species, such as ruminants. However, given the safety concerns associated with human use, including the threat of persistent infection or reversion to virulence extreme, caution must be used in LAV development (Figueiredo *et al.*, 2015).

Prevention of animal disease was found to provide substantial protection against human disease. Historically, the focus of immune protection against *Brucella* infection in animals has been the use of spontaneously attenuated strains (Figueiredo *et al.*, 2015).

Attenuated virulence may be derived from simple point mutations or from genetic rearrangements, including gene deletion. Although the potential for reversion of a

mutant that bears a complete gene deletion is already small, the potential for reversion to virulence can be made infinitesimal by the introduction of a secondary mutation. Optimally, the additional mutation would affect the same pathway as the primary mutation, serving only as a backup to prevent reversion, but inducing no additional reduction in virulence or negative effect on protective immunity. Obviously, care must be taken so that a balance is struck that supports the survival sufficiently to enhance immune protection without posing a risk of inducing disease. One approach to this task is encapsulation of the attenuated vaccine strain to release the organism over time and to provide the added advantage of providing a natural booster response (Arenas-Gamboa *et al.*, 2011; Arenas-Gamboa *et al.*, 2012).

This approach uses a vaccine depot from which the attenuated *Brucella* is gradually released over a 30-day period and significantly enhances immune protection by using highly attenuated LAVs to improve both efficacy and safety (Wu *et al.*, 2006). In addition, recent cell biology findings have revealed the dependence of *Brucella* infection (Qin *et al.*, 2008; Smith *et al.*, 2013).

This dependence may be exploited in an effort to provide LAVs that provide enhanced immune protection. ER stress and TLR signaling provide a synergistic stimulation of the proinflammatory response (Martinon *et al.*, 2010).

2.13 Diagnosis of Brucellosis in human

2.13.1 Clinical features

Brucella spp. are endocytosed by macrophages and polymorphonuclear cells, within which they survive and multiply (Seleem *et al.*, 2008). Thereafter, they move into the lymph nodes and the blood circulation, settling down in several organs and tissues (liver, spleen and bone marrow) where they continue to multiply intracellularly and spread to other organs. Depending on the duration and progress of the disease, the following phases can be observed (von Bargen *et al.*, 2012).

- Acute disease phase (duration <3 months).
- Subacute disease phase (duration 3-12 months).
- Chronic disease phase (duration >12 months).

The disease presents with a great variety of clinical signs, depending on the phase and focal localization. Relapses are mostly related to incomplete/improper therapy (von Bargen *et al.*, 2012).

2.13.2 Identification of the agent

2.13.2.1 Staining methods

Brucella are coccobacilli or short rods measuring from 0.6 to 1.5 μm long and from 0.5 to 0.7 μm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident (Dawson *et al.*, 2008).

Brucella are nonmotile. They do not form spores, and flagella, pili, or true capsules are not produced (Percin, 2013). *Brucella* are Gram negative and usually do not show bipolar staining. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method. This is the usual procedure for the examination of smears of organs or biological fluids that have been previously fixed with heat or ethanol, and by this method, *Brucella* organisms stain red against a blue background (Kadhum, 2015).

A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used (Kumar, 2013). The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms are presumptive evidence of brucellosis. However, these methods have a low sensitivity in blood and dairy products where *Brucella* are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules (Percin, 2013).

2.13.2.2 Culture

2.13.2.2.1 Basal media

Direct isolation and culture of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants (Percin, 2013).

However, the use of liquid media may be recommended for voluminous samples or for enrichment purpose. A wide range of commercial dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)–soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* biovar 2, and many laboratories systematically add serum to basal media, such as blood agar base (Oxoid) or Columbia agar (BioMérieux), with excellent results (OIE, 2009).

Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol dextrose agar, can be used. SDA is usually preferred for observation of colonial morphology (Madkour Monir, 2014). A nonselective, biphasic medium, known as Castañeda's medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is usually advised. Castañeda's medium is used because *Brucellae* tend to dissociate in broth medium, and this interferes with biotyping by conventional bacteriological techniques (Mangalgi & Sajjan, 2014).

2.13.2.2.2 Selective media

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*. The most widely used selective medium is the Farrell's medium (Vicente *et al.*, 2014), which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymyxin B sulphate

(5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg). A freeze-dried antibiotic supplement is available commercially (Oxoid). However, nalidixic acid and bacitracin, at the concentration used in Farrell's medium, have inhibitory effects on some *B. abortus* and *B. melitensis* strains (Rodríguez *et al.*, 2015).

Therefore the sensitivity of culture increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin medium (Ferreira *et al.*, 2012).

Briefly, the modified Thayer–Martin's medium can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methanesulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]) (Yagupsky, 2013).

Contrary to several biovars of *B. abortus*, growth of *B. melitensis* is not dependent on an atmosphere of 5–10% CO₂ (Lonsway *et al.*, 2010). Enrichment can be carried out in liquid medium consisting of serum–dextrose broth, tryptose broth (or trypticase)–soy broth (TSA) (Use *et al.*, 2010).

The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO₂ for up to 6 weeks, with weekly subcultures on to solid selective medium (Godfroid *et al.*, 2013).

If preferred, a biphasic system of solid and liquid selective medium in the same bottle (Castañeda's method) may be used to minimise subculture. The results demonstrated that the (CITA) and Farrell media inhibited the growth of contaminants better than the *Brucella* agar (Vicente *et al.*, 2014).

A selective biphasic medium composed of the basal Castañeda's medium with the addition of the following antibiotics to the liquid phase, is sometimes recommended for isolation of *Brucella* in milk and blood (quantities are per litre of medium):

polymyxin B (sulphate) (6000 units = 6 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); amphotericin B (1 mg); vancomycin (20 mg); D-cycloserine (100 mg) (Mantur & Mangalgi, 2004).

2.13.3 Identification and typing

Any colonies of *Brucella* morphology should be checked using a Gram-stained (or a Stamp-stained) smear. As the serological properties, dyes and phage sensitivity are usually altered in the non-smooth phases, attention to the colonial morphology is essential in the typing tests described below (Percin, 2013). The recommended methods for observing colonial morphology are Henry's method by obliquely reflected light (Turse *et al.*, 2011). The acriflavine test described by Braun & Bonestell (Rambow-Larsen *et al.*, 2008). Or White & Wilson's crystal violet method of staining colonies (Saeedinia *et al.*, 2015).

Identification of *Brucella* organisms can be carried out by a combination of the following tests: organism morphology after Gram or Stamp's staining, colonial morphology, growth characteristics, urease, oxidase and catalase tests, and the slide agglutination test with an anti-*Brucella* polyclonal serum (OIE, 2009).

Species and biovar identification requires elaborate tests (such as phage lysis and agglutination with anti-A, -M or -R monospecific sera), the performance of which is left to reference laboratories with expertise in these methods (Dongyou Liu, 2014).

The simultaneous use of several phages e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and rough species of *Brucella* (Zhu *et al.*, 2009). However, several characteristics, for example added CO₂ requirement for growth, production of H₂S (detected by lead acetate papers), and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, are revealed by routine

tests that can be performed in moderately equipped nonspecialised laboratories (Affi *et al.*, 2011; Ankit *et al.*, 2013; Sahin *et al.*, 2008).

2.13.4 Molecular identification

Molecular studies have found that most *Brucella* strains have (2) circular chromosomes encoding approximately (3.2) kb with (2) replicons, Several features of the *B. melitensis* genome are similar to those of the symbiotic *Sinorhizobium meliloti* (DelVecchio *et al.*, 2002). Bacteriophages have been isolated from *Brucella* strains (Hammerl *et al.*, 2016).

Overall, the genome can be considered to have two main components: a 'core', which is higher in G+C, is mostly chromosomal, is shared with related organisms, and has a consistent phylogeny; and an 'accessory' component, which is sporadic in distribution, lower in G+C, and located on the plasmids and chromosomal islands. The accessory genome has a different nucleotide composition from the core despite a long history of coexistence (Young *et al.*, 2006).

Polymerase chain reaction (PCR) assays can be used to detect *Brucella* DNA in pure cultures and in clinical specimens, i.e. serum, whole-blood and urine samples, various tissues, cerebrospinal, synovial or pleural fluid, and pus (Colmenero *et al.*, 2005, 2010; Debeaumont *et al.*, 2005; Queipo-Ortuño *et al.*, 2005a, 2006, 2009).

Direct detection of *Brucella* DNA in brucellosis patients is a challenge because of the small number of bacteria present in clinical samples and inhibitory effects arising from matrix components (Queipo-Ortuño *et al.*, 2008a).

Basic sample preparation methods should diminish inhibitory effects and concentrate the bacterial DNA template. Residual PCR inhibition in complex matrices can be unmasked by the use of an internal amplification control (Al Dahouk *et al.*, 2010).

The QIAamp™ DNA Mini Kit (Qiagen Inc., Valencia, California, USA) and the UltraClean™ DNA BloodSpin Kit (MO BIO Laboratories Inc., Carlsbad, California, USA) are among the many commercial kits that have been successfully used to extract *Brucella* DNA from whole-blood, serum and tissue samples (Maas *et al.*, 2007; Queipo-Ortuño *et al.*, 2008b; Vrioni *et al.*, 2008).

The advantages of PCR are numerous. Independent of the disease stage, it is more sensitive than blood cultures and more specific than serological tests. Various PCR assays targeting different gene loci have been successfully used for the diagnosis of human brucellosis (Asaad & Alqahtani, 2012; Rundell *et al.*, 2014). By increasing the number of molecular markers, both sensitivity and specificity can be increased accordingly. Molecular assays targeting the IS711 insertion element, which is found in multiple copies within *Brucella* chromosomes, also improve analytical sensitivity (Hini *et al.*, 2008; Bounaadja *et al.*, 2009).

Moreover, real-time PCR enables high-throughput screening of clinical samples and delivers results within a few hours. Genus-specific PCR assays are generally adequate for the molecular diagnosis of human brucellosis (Al Dahouk & Nöckler, 2011). The *bcs*p31 gene, coding for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* spp., is the most common molecular target in clinical applications (Zhang *et al.*, 2012). Such a genus-specific PCR can help to avoid false-negative results in patients infected with unusual species and biovars. However, a primary molecular diagnosis must always be confirmed using a second gene target (Al Dahouk *et al.*, 2007). For confirmation and distinction from closely related microorganisms, 16S rRNA gene sequencing can be used (Fadrosh *et al.*, 2014). Differential PCR assays, e.g. the conventional Bruce-ladder PCR (Morales-Estrada *et al.*, 2012) or multiple real-time PCR assays (Tatti & Tondella, 2013).

The analytical sensitivity can be further increased by using real-time PCR assays, which can detect as few as five bacteria per reaction (Wareth *et al.*, 2015). Can also

be used for confirmatory identification and differentiation of *Brucella species*. Subtyping at the strain level can be useful for differentiating re-exposure from relapse (Sandberg *et al.*, 2012).

Molecular detection of *Brucella* DNA can be a sign of acute or chronic brucellosis and can also be detected in asymptomatic subjects with a history of brucellosis (Castaño & Solera, 2009). Despite successful antibiotic therapy, *Brucella* DNA remains detectable in the majority of brucellosis patients throughout treatment and treatment follow-up, as well as years after clinical cure and in the absence of any symptoms indicative of chronic disease or relapse (Navarro *et al.*, 2006; Maas *et al.*, 2007; Vrioni *et al.*, 2008).

2.13.4.1 Real-time polymerase chain reaction (RT-PCR)

Compared with the standard PCR, real-time PCR is a valuable technique in determining the quantification of nucleic acids in individual blood samples, as well as in automating the data. With the decreasing prices of real-time PCR thermocyclers and the reagents, many more people now have access to this technology to measure DNA copy number, mRNA expression levels and viral titers (Ginzinger, 2002).

Recently, real-time PCR for the rapid detection and differentiation of *Brucella species* in clinical samples has recently been developed, targeting 16S-23S internal transcribed spacer region (ITS) and the genes coding omp25 and omp31 (Kattar *et al.*, 2007), BCSP 31 (Colmenero *et al.*, 2005; Queipo-Ortuño *et al.*, 2008a), and IS711 (Cerekci *et al.*, 2011; Zhang *et al.*, 2013).

Real-time PCR seems to be highly reproducible, rapid, sensitive and specific. Additionally, this assay is easily standardized and minimizes the risk of infection in laboratory workers. It is therefore a useful method for both the initial diagnosis of

human brucellosis and the differentiation among inactive, seropositive, and active states (Queipo-Ortuño *et al.*, 2008b).

Reported that the sensitivities of a SYBR Green I Light Cycler-based real-time PCR assay with serum samples was 93.3%, which is higher than 90% and 65% obtained by PCR-ELISA with whole blood samples and blood cultures, respectively (Queipo-Ortuño *et al.*, 2005b). This group further developed a LightCycler-based real-time PCR assay to detect *Brucella* DNA in serum samples. This assay was found to be 91.9% sensitive and 95.4% specific when tested with 65 negative control samples and 62 serum samples from patients with active brucellosis (Queipo-Ortuño *et al.*, 2005b).

2.13.5 White blood cell differential and total counts

The white blood cell count (WBC) is a component of a complete blood cell count (CBC) and is the enumeration of white blood cells in a small volume of whole blood. The testing is performed on an automated hematology analyzer. The white blood cells (leukocytes) are further divided into phagocytes or myeloid (neutrophils, eosinophils, basophils, monocytes) and immunocytes or lymphoid (lymphocytes) (Robert Hutchison, 2007).

The reference range for adults (males and females) is as follows: Total leukocytes: $4.00-11.0 \times 10^9$ cells/L, Neutrophils: $2.5-7.5 \times 10^9$ cells/L, Lymphocytes: $1.5-3.5 \times 10^9$ cells/L, Monocytes: $0.2-0.8 \times 10^9$ cells/L, Eosinophils: $0.04-0.4 \times 10^9$ cells/L and Basophils: $0.01-0.1 \times 10^9$ cells/L. WBC check of under 4×10^9 cells/L shows leukopenia. A WBC count of more than 11×10^9 /L indicates leukocytosis (Kim *et al.*, 2014). Decreased WBC count, leukopenia, is seen when supply is depleted by infection or treatment such as chemotherapy or radiation therapy, or when a hematopoietic stem cell abnormality does not allow normal growth/maturation within the bone marrow, such as myelodysplastic syndrome or leukemia. Leukopenia (decrease in WBC) is most often due to a lower number of neutrophils, referred to as

neutropenia. Characteristically, the neutrophil count is less than 1.5×10^9 cells/L (Kim *et al.*, 2014; Gulack *et al.*, 2015).

An abnormal white blood cell count usually results from an infection, cancer, or other conditions that trigger systemic inflammation responses (Zhang *et al.*, 2015).

2.13.6 Nitroblue tetrazolium (NBT) assay

A semi-quantitative microscopic nitroblue tetrazolium (NBT) assay is used to determine the production of superoxide anion O_2^- in various phagocytic cells. This microscopic assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, NBT by O_2^- (Choi *et al.*, 2006).

The purpose of study was to evaluate the ability of spermatozoa and leukocytes in semen to produce reactive oxygen species (ROS) by using nitroblue tetrazolium (NBT) staining and to examine the association between NBT staining and levels of ROS as measured by chemiluminescence (Esfandiari *et al.*, 2003). The neutrophil function was assessed by performing nitroblue tetrazolium (NBT) reduction test in thalassemia major (TM) patients and compare it with the control group (Ghaffari *et al.*, 2008).

The reduction of tetrazolium salts to coloured formazans is often used as an indicator of cell metabolism during microbiology studies, although the reduction mechanisms have never clearly been established in bacteria. The objective of the study was to identify the reduction mechanisms of tetrazolium violet (TV) in *Lactococcus lactis* using a mutagenesis approach, under two experimental conditions generally applied in microbiology: a plate test with growing cells, and a liquid test with non-growing (resting) cells (Tachon *et al.*, 2009).

The investigation of disease related oxidant-antioxidant imbalance is difficult due to the limited availability of specific biomarkers of oxidative stress, and the fact that measurement of individual antioxidant may give a misleading picture because

antioxidants work in concert through chain breaking reactions. Therefore, analysis of total antioxidant capacity may be the most relevant investigation. As the total blood is continuously exposed to oxidative stress, the aim of the study was to investigate the total blood antioxidant capacity in healthy and periodontitis patients by using novel Nitroblue Tetrazolium reduction test (Konuganti *et al.*, 2012).

Study the efficacy of Nitroblue tetrazolium test (NBT), which is a simple test to evaluate neutrophilic functions in bacterial and non-bacterial infectious diseases, The NBT test proved to be useful as a simple diagnostic aid in differentiating patients with bacterial infections from individuals with non bacterial diseases. The proportion of absolute numbers of NBT positive neutrophils serves as a measure of the adequacy of the host response to infection (Mohammed Moid Afzal and Anunayi Jeshtadi, 2014).

2.13.7 Antibody detection of *Brucella*

2.13.7.1 Rose Bengal test

The Rose Bengal test (RBT) is a rapid slide-type agglutination assay performed with a stained *B. abortus* suspension at pH 3.6–3.7 and plain serum. Because of its simplicity, it is often used as a screening test in human brucellosis and would be optimal for small laboratories with limited means. However, there is confusion about the value of this test so that present WHO guidelines recommend that RBT results be confirmed by other tests (Diaz *et al.*, 2011). The total sero-prevalence of brucellosis in humans ranged between 5% and 8%, with no significant differences with respect to different seasons of the year. The high prevalence rates of human brucellosis call for strict application of hygienic measures to prevent the spread of brucellosis from cattle and other livestock to humans (Samaha *et al.*, 2009).

The Rose Bengal test was highly sensitive in acute and long evolution brucellosis cases and those related to its ability to detect IgM, IgG and IgA, to the absence of

prozones, and to the agglutinating activity of blocking IgA at the pH of the test. RBT was also highly specific in the sera of persons with no contact with *Brucella*. No test in this study outperformed RBT, and none was fully satisfactory in distinguishing contacts from infected patients (Diaz *et al.*, 2011). 241 serum samples from individuals exposed to brucellosis were subjected to the Rose Bengal plate test (RBPT), the titre was estimated by standard tube agglutination test (STAT), with positive ≥ 80 IU/ml. The clinical history coupled with a combination of two or more tests will reduce diagnostic errors (Yohannes *et al.*, 2012).

Agglutination tests occasionally yield false results. Superagglutination test reduced false results, had higher sensitivity (95.88%) and negative predictive value (95.83%) than Rose Bengal plate test (RBPT), Standard Tube Agglutination test (STAT), ELISA, and Complement Fixation test (CFT) has specificity (89.32%) and positive prescient gene (89.42%) higher than RBPT and STAT (Chothe and Saxena, 2014). RBT is based on lipopolysaccharide antigen of *Brucella*, which may cross react with other gram-negative bacteria and produce false positive results (Thepsuriyanont *et al.*, 2014).

2.13.7.2 2-Mercaptoethanol test (2-ME)

The use of 2-ME in the STA destroys the agglutinating ability of IgM antibodies and allows measurement of only IgG (Keid *et al.*, 2009). IgG as detected by the 2-ME test is used as a marker for active infection. The routine *Brucella* agglutination test is the most frequently used type of serological test for this purpose; however, it does not differentiate between active and inactive disease because it does not differentiate between between IgG and IgM agglutinins. The IgM titer can be eliminated by treating the serum specimen with 2-mercaptoethanol (2-ME), which breaks the disulfide bonds and depolymerizes the IgM. Depolymerized IgM does not contribute to agglutination; therefore, the titer is due primarily to 2-ME-resistant

antibody (IgG). The IgG antibody titer can be determined by treating the serum with 2-mercaptoethanol to inactivate the IgM *Brucella* antibodies while leaving the IgG *Brucella* antibodies intact (Zeytinoğlu *et al.*, 2006).

Due to the neutralizing effect of mercaptoethanol on IgM, 2ME-MAT inhibits the serological response to *Yersinia enterocolitica* O:9, as well as many other false positive serologic response producers (Dashti *et al.*, 2012).

2ME-MAT was used to compare and confirm the results obtained with SAT and MAT. A major objection to the use of the 2-ME test is that, 2-ME irritates the eyes and mucous membranes. It also has a very strong and offensive odor (Sareyyupoglu *et al.*, 2010).

Patients with an SAT titer of 1/80 or greater and a 2ME titer of 1/20 or greater were considered to have brucellosis, and the remaining patients were considered to have other febrile illnesses mimicking brucellosis. Such criteria would increase the overall diagnostic specificity at the expense of sensitivity. Since these studies compared patients with brucellosis with patients with other febrile illnesses that should be discriminated from brucellosis, the results of a study are potentially more useful in practice (Dashti *et al.*, 2012).

2ME easily denatures the IgM class of antibodies, so if a decrease in the titer is seen after using this agent, it means that the contribution of IgM has been removed leaving the IgG component. This differentiation of antibody classes is important; as it allows for the distinction of a recent from an old infection (Sendil and Prakash*, 2016).

2.13.7.3 Enzyme-linked immunosorbent assay (ELISA)

The diagnostic value of enzyme-linked immunosorbent assay (ELISA) was evaluated when blood specimens of 92 patients suspected of brucellosis underwent the ELISA (IgM and IgG). ELISA has been found to be more sensitive in acute (28% higher sensitivity; $P < 0.02$) and chronic (55% higher sensitivity; $P < 0.01$) cases. For accurate diagnosis in suspected brucellosis cases detection, we recommend both ELISA IgM and IgG tests (Mantur *et al.*, 2010). Detection of IgG antibody was more sensitive than detection of IgM antibody for diagnosing brucellosis cases, but the specificity was comparable. Serology results need to be considered in tandem with patient history, clinical signs and other test results (Fadeel *et al.*, 2011).

ELISA is more reliable than conventional methods because of the sensitivity and the possibility of distinguishing between specific IgG and IgM immunoglobulins (Hassan *et al.*, 2011). ELISA can also determine specific antibody classes against *Brucella*. It is a sensitive, simple and rapid test, The cut-off point of 53 IU/ml of ELISA-IgG yielded the maximal sensitivity and specificity comparable to the other levels of ELISA-IgG, and was considered the best cut off-point of ELISA-IgG to diagnose acute brucellosis (Dashti *et al.*, 2012).

Hasibi *et al.* researched the accuracy of ELISA tests for diagnosis of human brucellosis and determined the optimal cut-off value for ELISA results, ELISA IgG test was more reliable than ELISA IgM test in diagnosis of human brucellosis in Iran (Hasibi *et al.*, 2013). Both the (IgG + IgM) ELISA and Brucellacapt tests demonstrate a high specificity in this study. According to the results of the current study, it is found that both tests are valuable tools for diagnosis of brucellosis in Iran as an endemic area of brucellosis. It is strongly suggested that a combination of both tests to be used for the diagnosis of brucellosis (Peeridogaheh *et al.*, 2013).

All methods tested in our study need to be used to ensure accurate diagnosis of brucellosis, although ELISA displayed the highest level of efficiency. Also, *B*

melitensis showed a higher frequency rate than *B. abortus* in our cohort (Hajia *et al.*, 2013). The sensitivity and specificity of ELISA to diagnose human brucellosis was higher when combined ELISA (IgM/IgG or both) was used. Mean IFN- γ levels were lower, but not significantly, in the chronic phase of the disease than in the sub acute phase and healthy persons (Abo-Shadi *et al.*, 2014).

2.13.8 MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) is a colorimetric assay for assessing cell metabolic activity. Nicotinamide-Adenine Dinucleotide (NAD) (P) H-dependent cellular oxidoreductase enzymes may under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color (Meerlo *et al.*, 2011). After some incubation, the water-insoluble formazan dye forms crystals, which can be dissolved in an organic solvent and the amount can be determined semi-automatically using a microplate reader. Absorbance readings are related to the number of cells, therefore providing the possibility to use the MTT assay as a proliferation assay to assess cell growth after irradiation (Buch *et al.*, 2012).

This colorimetric microplate assay is cost effective because of the number of tests which can be performed at one time without the problem of radioisotopes and contaminated materials disposal. Applications of the MTT assay are used to the cell growth and cell survival offers major advantages in speed, simplicity, cost, and safety over conventional cell counting assays (Chapdelaine, 2011). To reduce the interference in the MTT reduction assay, Wang *et al.* developed an improved protocol for evaluating the viability of *Escherichia coli* cells by conducting an MTT reduction in 1.5-mL centrifuge tubes and Formosan dissolution in test tubes. The study is helpful in developing protocols for measuring the viability of other gram-

negative bacteria (Wang *et al.*, 2010). Results demonstrated that MTT was useful for determining the direct killing effect of *Lysobacter enzymogenes* on fungal cells, and also for differentiating antagonistic activity between the wild type strain and an impaired mutant strain of the bacterium (Patel *et al.*, 2013).

Lymphocyte proliferation response to the antigen was evaluated by MTT test and measure up to that of nonimmunized mice lymphocyte culture, Proliferation rates of lymphocytes from rOmp19 immunized mice were significantly higher than lymphocytes from non immunized mice (Farahi *et al.*, 2012). MTT assay used to assess damage induced by *Staphylococcus aureus* in human umbilical vein endothelial cells (HUVECs). MTT reduction by intracellular *S. aureus* was negligible and therefore permits assessment of *S. aureus* induced EC damage (Seidl & Zinkernagel, 2013).

MTT assay is commonly used to evaluate the cytotoxicity potential of drugs vehicled by liposomes. However, liposome delivering drugs could produce inconsistent values of MTT absorbance (Angius & Floris, 2015).

3. Materials and methods

3.1 Materials

3.1.1 Types of equipment and Instruments

Table (3-1): Equipment's and Instruments with their origin

NO.	Equipment's OR Instruments	Company/ (origin)
7	Anaerobic Jar	Hirayama (Japan)
1	Autoclave	Hirayama (Japan)
21	Blood collection tubes with heparin	Afco (Jordan)
5	Burner	Sundown (England)
24	Camera	Sony (Japan)
2	Centrifuge	Tuneup (Italy)
12	Cooled centrifuge	Eppendorf (Germany)
26	Disposable Petri dishes	Al-Hani (Lebanon)
20	Disposable syringe 10, 5, & 3ml	Medeco (Germany)
10	Distillator	GFL (Germany)
15	ELISA plates 96 wells	BioTek (USA)
13	ELISA Reader and Washer	BioTek (USA)
27	Ependroff tubes	Sigma(England)
4	Hood	Tips how (USA)
8	Incubator	Memmert (Germany)
16	Light microscope	Olympus/Japan
11	Microcentrifuge\	Eppendorf (Germany)
29	Micropipettes 5-50, 0.5-10, 100-1000 µl	SIAMED (Germany)
28	Multichannel micropipette reservoir	SIAMED (Germany)
25	PCR tubes	Sterellin Ltd. / UK
17	PH meter	LKB (Sweden)
14	Printer	LX -400-UK Epson/UK
6	Sensitive Balance	Sartorius (Germany)
9	Shaker	TKA (Italy)
3	Sysmex	Sysmex (Japan)
23	Ultraviolet transelemented	Optima (Italy)
19	Vitek 2 system apparatus	Biomerieux (France)
22	Vortex	IKA (USA)
18	Water Bath	Kottermann (Germany)

3.1.2 Chemical and Biological materials

Table (3-2): Chemical and Biological materials used in the study.

No.	Chemical & biological materials	Company and Origin
3	Agarose	BioBasic (Canada)
11	DEPC water	Bioneer (Korea)
24	Dimethyl sulfoxide (DMSO)	BDH (England)
14	DMEM media	Sigma
1	Ethanol	BDH (England)
6	Ethidium Bromide	BioBasic (Canada)
16	Gram's stains	AL-Hilal(K.S.A)
19	Hydrogen peroxide (H ₂ O ₂)	Oxoid (UK)
2	Isopropanol	BDH (England)
21	Kovac's reagent	BDH(UK)
5	Loading dye	Bioneer (Korea)
13	Lymphocyte separation medium 1073	HiMedia (India)
15	Methanol	BDH (England)
22	Methyl red reagent	BDH(UK)
23	Oil Immersion	BDH(UK)
10	PCR water	Bioneer (Korea)
17	Physiological Normal Saline	Alfaiha(Jordan)
9	Proteinase k	BioBasic (Canada)
12	RPMI Medium 1640 (1X) + GlutaMax -1	Gibco (UK)
4	TBE buffer	BioBasic (Canada)
18	Tetramethyl-p-phenylene-diaminedihydrochloride (Oxidase test)	Fluke (UK)
20	Thionin and Basic fuchsin stain	BDH(UK)

3.1.3. Antibacterial disks

Table (3-3): The antimicrobial disks used for detection the susceptibility of *Brucella* isolate to 19 selected antibiotics.

Antibacterial disks	Disk potency (Mcg/disk)	Inhibition zones of antibiotics (mm)			Company / (origin
		Resistant	Intermediate	Sensitive	
Clindamycin (CD)	2	≤ 15	16-18	≥ 19	HiMedia (India)
Ampicillin (P)	10	≤ 13	14-16	≥ 17	HiMedia
Erythromycin (E)	15	≤ 15	16-20	≥ 21	HiMedia
Tobramycin (TOB)	10	≤ 12	13-14	≥ 15	HiMedia
Piperacillin (PRI)	100	≤ 17	13-14	≥ 18	HiMedia
Vancomycin (VA)	30	≤ 25	26-27	≥ 17	HiMedia
Aztreonam (AZT)	30	≤ 15	16-21	≥ 22	HiMedia
Cifotaxime (CTX)	30	≤ 14	15-22	≥ 22	HiMedia
Chloramphenicol (C)	30	≤ 12	13-17	≥ 18	HiMedia
Ciprofloxacin (CIP)	5	≤ 15	16-20	≥ 21	HiMedia
Ceftriaxone (CRO)	30	≤ 13	14-19	≥ 21	HiMedia
Amoxycylav (AMC)	30	≤ 13	14-17	≥ 18	HiMedia
Netillin (NET)	30	≤ 12	13-14	≥ 15	HiMedia
Gentamycin (GEN)	10	≤ 12	13-14	≥ 15	HiMedia
Ticarcillin(TIC)	75	≤ 14	15-19	≥ 20	HiMedia
Nitrofurantoin (F)	300	≤ 14	15-18	≥ 19	HiMedia
Amikacin(AK)	30	≤ 14	15-16	≥ 17	HiMedia
Tetracycline (TE)	30	≤ 11	12-14	≥ 15	HiMedia
Imipenem (IPM)	10	≤ 13	14-15	≥ 16	HiMedia

3.1.4 Commercial kits

Table (3-4): The commercial kits used in our study.

No.	Kit	Company	Country
1	Human Brucellosis IgG-ELISA kit	NovaTec.	Germany
2	Genomic DNA Extraction Kit	GeneAid	USA
3	AccuPrep [®] 2X GreenStar qPCR Master Mix kit	Bioneer	Korea
4	MTT Cell Viability Assay Kit	Abnova	USA
5	<i>Brucella</i> Rose Bengal antigen kit	Thermo Fisher	United kingdom
6	2-mercaptoethanol kit	Central Drug House	India
7	Nitroblue tetrazolium kit	Bangalore Genei	India
8	API 20E kit	Biomerieux	France
9	Vitek 2 System Kit	Biomerieux	France
10	Trepan blue kit	Bangalore Genei	India
11	Monospecific antiserum <i>Brucella abortus</i>	Difico	USA
12	Monospecific antiserum <i>Brucella melitensis</i>	Difico	USA

3.2 Methods**3.2.1 Preparation the Culture Media**

The culture media is prepared according to the instruction company devices on containers and it is autoclaved at 121C° for 15 minutes, in 15 bar/in², and incubated at 37 C° for 24 hours to be sure from their sterility and then kept at 4°C until they used, as shown in the Table (3-5):

Table (3-5): The culture media and purpose from their used

Medium	Purpose
Brain Heart Infusion broth	Cultivation of a wide variety of fastidious organisms
Trypticase soya broth	Cultivation of a wide variety of microorganism
Blood Agar Base	Determining the ability of bacteria to hemolysis human blood cell
MacConkey agar	To cultivation of Gram- negative bacteria.
<i>Brucella</i> Agar	Selective media for Brucellaceae
Muller- Hinton agar	Antimicrobial susceptibility testing.
Trypticase Soya Agar	It used for keeping and maintaining the bacterial samples (short-term and long-term preservation)
Nutrient agar broth	Growth and subculture of bacteria isolate.
Kligler iron agar	Determining glucose and lactose fermentation and possible hydrogen sulfide H ₂ S production for identification of Gram- negative bacteria.
Urea Agar	Differentiation of microorganism on the basis of urease production

3.2.2. Preparation of Solutions**I. Normal saline**

Normal saline prepared by dissolving 8.5 g NaCl in 1 liter distiller water. Autoclave 15 min at 121C°. Cool to room temperature (Florin *et al.* 2014).

II. Phosphate Buffered Saline (PBS)

Phosphate Buffered Saline (PBS) prepared according to (Du *et al.* 2012).

1. Dissolve the following in 800ml distiller water.

8g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, 0.24g of KH₂PO₄

2. Adjust pH to 7.4 with HCl.

3. Adjust volume to 1liter with additional distiller H₂O.

4. Sterilize by autoclaving.

III. Roswell Park Memorial Institute (RPMI) -1640 medium

1 liter of RPMI-1640 base medium prepared as follows:

1 bottle RPMI (fluid - ice chest) verify that the fluid contains glutamine and bicarbonate, if not, add 2.1g NaHCO₃ (Sodium Bicarbonate) and 10 ml Glutamate (100X) and 10ml of 100 x Pen/Strep (cooler) (Arora, 2013).

IV. Dulbecco's Modified Eagle's Medium (DMEM)

1 liter of DMEM Base Medium with Glutamine readied as follows:

1 bottle DMEM (sigma powder - cooler), 2.1g NaHCO₃ (Sodium Bicarbonate), 10ml of 100 x Glutamine (cooler), 10ml of 100 x Pen/Strep (cooler) (Can utilize mix of Pen/Strep/Glutamine) conform the pH to 7.2 and complete the volume to 1 liter utilizing box water (Note: channel ought to be put on and taken off in refined hood (Arora, 2013).

V- Nitro blue tetrazolium (NBT) dye

10 mg NBTdye dissolved in 10 ml physiological saline supported with phosphate particle at pH 7.4 (Choi *et al.* 2006).

VI- MTT Solution

It prepared by adding 1 mL of sterile PBS (ph=7.4) to 5 mg vial of MTT then mix by vortexing or sonication until dissolved (Stockert *et al.* 2012).

VII- Agarose

One gram of agarose boiled in 100 ml 1X TBE buffer and left to cool at 50 C° (Sambrook and Russell, 2001).

VIII- Trypan Blue Solution:

It prepared by adding 4 mg of trypan blue powder to 1 mL of water or PBS (Louis and Siegel, 2011).

X- Thionin and Basic fuchsin stain

It has been prepared by dissolving 1.0g of Thionin and Basic fuchsin (provided by BDH company, UK) in 1000 ml of distilled water and put in a water bath at 100 °C for 1hr.

3.2.3 Collection of specimens

One hundred blood samples collected from positive rose Bengal test patients depended on age, gender, region, taking medication, and smoking according to manifestations of patients, including Fever, headache, drowsiness, sweating, and joint pain, and 50 healthy individuals as a control group during the extended period from December/ 2015 to July/ 2016 in Al-Hussein Teaching Hospital of Muthanna Province.

Firstly, It is withdrawn two ml of the whole blood patient, to be centrifuged at a speed of 3000 RPM/min for 15 minutes, then the partition of the serum to a spotless tube to execution Rose Bengal test, If the result of the test was a positive, we withdraw 10 ml from patients and divided as follows:

4ml of whole blood were injected into screw-capped bottles contain sterile trypticase soya broth incubated for 48 hours at 37°C, and afterward sub-cultured on duplicate agar plates and incubated in an air and in an atmosphere containing 5-10 % carbon dioxide (OIE, 2009).

4 ml are directly used to measure the WBC count, phagocytic activity, and lymphocyte transformation index.

1 ml is sent directly to the laboratory to extract of DNA to molecular detection.

1ml is centrifuged at speed 3000 RPM/ min for 15 minutes, then the serum transfer to a perfect tube to done ELISA.

3.2.4 Study design

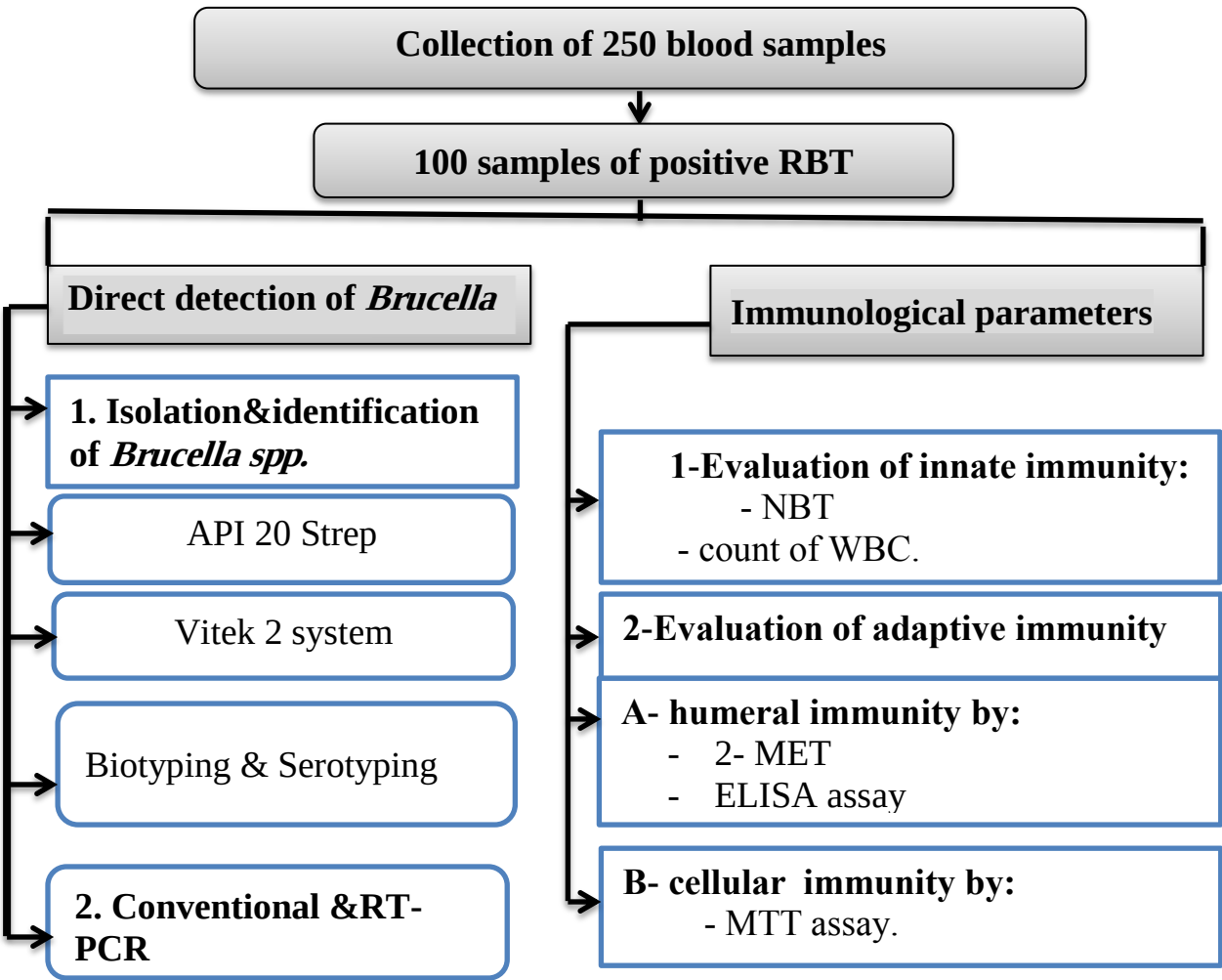


Figure (3-1): Scheme of study design in this thesis.

3.2.5 Isolation

The methods used for isolation of *Brucella* spp are:

3.2.5.1 Blood culture

Five ml of whole blood withdraws from vein of patients after sterilization by iodine 70%, then replace the needle of the syringe and injected directly in screw-capped bottles contain sterile trypticase soya broth incubated for 48 hours at 37C°, and afterward sub-cultured on blood and macConkey agar plates and incubated in an air and in an atmosphere containing 5-10 % carbon dioxide. The screw-capped bottles remain incubated for one month and sub-cultured return every two days.

3.2.5.2 Lysis- centrifugation method

The whole blood samples collected in a sodium citrate tube, and centrifuged at speed 3000 RPM/min for 15 minutes, then collected the serum in clean tube, after that cultured the serum on blood and macConkey agar plates and incubated in an air and in an atmosphere containing 5-10 % carbon dioxide (Mantur and Mangalgi, 2004).

3.2.6 Identification of isolates

Isolates identified according to (Murray *et al.*,2003) as the followings:

3.2.6.1 Cultural characteristics

The fastidious organism grey, shiny, circular, convex and punctate colonies (0.6 μm - 1.5 μm) in 48 hours, inability to ferment lactose and Clear or white colonies on MacConkey (MAC) agar at 48 hours. Growth seen on Blood Agar (BA) and Chocolate Agar (CA). Non-hemolytic, non-pigmented small gray colonies, may require 48-72 hours for discrete colonies to become evident, CO₂ enhances growth.

3.2.6.2 Microscopic examination

Brucella appears on microscopic examination as a tiny, often faintly stained gram-negative coccobacilli (0.4 μm x 1.0 μm). *Brucellae* are coccobacilli or short rods, usually arranged singly, but sometimes in pairs or small groups. They are not truly acid fast. However, they are resistant to staining by weak acids, and stain red against a blue background with the Stamp's modification of the Ziehl-Neelsen method. Other organisms such as *Coxiella burnetii* can resemble *Brucella*.

3.2.6.3 Biochemical tests

I- Oxidase test

Isolated colonies of culture were scraped by a sterile wooden applicator stick and smeared across a wet filter paper with 1 % oxidase reagent. A reaction was considered to be positive when the color of the growth turned to dark purple within 10 second. All *Brucella* strains have the oxidase action (American Society for Microbiology, 2013).

II- Catalase test

A scrap of culture, using a platinum loop or a sterile wooden stick, was mixed with a drop of hydrogen peroxide 3% solution and placed on a slide in a Petri dish. The oxygen bubble indicates that reaction is positive (Tp, 2010).

III- Nitrate reduction

The reagent A (sulphanilic solution) and reagent (B α – naphtholamine solution) have been added drop by drop to 5 ml of trypticase nitrate broth culture until the appearance of red pink or maroon color which used as an indication of nitrate reduction positive. *Brucellae* should be positive to this test (Affi *et al.*, 2011).

IV- Indol test

Producing indol rings from the destruction of the amino acid tryptophan has been used to identify *Brucella* species. Kovac's reagent (0.5 ml) was added to 5 ml of peptone water cultured with *Brucella*. A positive result indicated by the appearance of Brilliant red ring (González *et al.*, 2006). *Brucella* should be negative to this test.

V- Methyl Red – Voges-Proskauer (MR-VP)

According to the manufacture instructions, 37°C, 2 – 3 drops of methyl Red reagents 1-2 drop of VP reagents has been added to two tubes containing *Brucella* cultured MR-VP broth. Red color should be appeared after 1 minutes, which indicate positive result to MR. If the red color appear after 10 minutes, it is an indication of VP positive (Dash *et al.*, 2012).

VI- Citrate Utilization test

Brucella colonies has been streaked on Citrate medium and incubated for 72 hours at 37 C°, if the medium changes in blue color indicate that the result is positive (Polen *et al.*, 2007). *Brucella* should be negative to this test.

3.2.6.4 APi-20 system (Analytical profile index)

The API-20Strep system assay is used to confirm the identification of *Brucella spp.*, this system was performed according to (Forbes *et al.*, 2007).

3.2.6.5 Vitek 2 System Diagnosis

The Vitek 2 System used to confirm the biochemical test; the principle of the system is a fully automated system for evaluation of microbial biochemical reaction assay is performed according to the (Gherardi *et al.*, 2012).

3.2.6.6 Antibiotic sensitivity test

The susceptibility of *Brucella spp.* isolates were determined by the disk diffusion method, using the standardized single disk method was carried out (CLSI, 2015).

3.2.6.7 Stock culture

Slants of nutrient agar with bacterial growth of *Brucella spp.* isolates were incubated at 37 C° for 18-24 hours, then it stored for one month in refrigerator which can be used for a short term maintaining, while long term maintaining was done by inoculating isolates using 5ml brain heart infusion broth with 1ml of sterile glycerol by autoclave and vortex vigorously then stored at - 20C° (Collee *et al.*, 1996).

3.2.6.8 Serotyping and Biotyping

The serotype&biotype of the *Brucella spp.* performed according to (Agriculture (USDA) and Service (APHIS), 2013). The strain of *Brucella species* diagnosed in laboratories department and veterinary researchers in the province of Baghdad.

I- CO₂ required for growth

Some strains required CO₂ for primary isolation, therefore incubate in present 5-10% CO₂ in primary isolation to assist the growth and differentiate species of *Brucella*.

II- H₂S production

The slope trypticase soy agar streaked with isolated *Brucella* colony and examined by the strip of lead acetate paper which has been placed in the tube so that does not come in contact with the medium and was held in a place between the plug and the side of the tube. Blackened lead acetate paper is an indicative of

hydrogen sulfide production. Almost all *Brucella abortus* biotypes should be positive to this test.

III- growth on dyes (Thionin and Basic Fuschin)

Each isolate was inoculated to a set of dye tolerance media. Each set contains 7 tubes of prepared medium. Three tubes contained Heart Infusion Agar (HIA) with dilutions of Thionin (1:100000, 1:50000, and 1:25000). Three tubes contained HIA with dilutions of Basic Fuschin (1:100000, 1:50000 and 1:25000). The growth control tube contained only Heart Infusion Agar. The tubes were liquefied by boiling and pour plates were prepared. The plates were allowed to cool and solidify at room temperature. All plates were used the day of preparation a suspension of a 72-hour culture was made with phosphate buffered saline equivalent to the density of a 0.5 McFarland standard. Each test plate was inoculated with a 1 loopful of the organism suspension inoculated in a single streak down the middle of the plate. Each set of plates was incubated in 5-7% CO at 35-37 C for 96 hours. The plates were examined for growth each day.

IV- Urease production

Urea agar base has been prepared depending on the manufacture's instructions, by dissolving (2gm) in 95 ml of distilled water and sterilized by autoclaving at 121C° for 15 minutes. After cooling at room temperature, urea solution (40%) was added to the urea agar base. Then, the agar was divided in test tubes (5 ml per each tube). *Brucella* isolates have been cultured on urea agar base at 37C° and monitored every hour for 5 hours, in order to differentiate different species of *Brucella*.

V- Agglutination by monospecific antisera

A slide placed in a Petri dish, then a small portion of growth picked up by a sterile loop and emulsified in 3 drops of saline. A drop of anti-*Brucella* serum was added then mixed and examined for agglutination.

3.2.7 Molecular detection

3.2.7.1 Bacterial Genomic DNA

The bacterial genomic DNA of *Brucella species* isolates were extracted by using (Presto™ Mini gDNA Bacteria Kit, Geneaid. The USA), and done according to Geneaid company instructions.

3.2.7.2 Blood genomic DNA extraction

The DNA of whole blood is extracted by using (ZymoBead™ Genomic DNA Kit, Zymo research, USA).

3.2.7.3 Gel electrophoresis

PCR products of each gene were analyzed by using agarose gel electrophoresis method as following steps (Sharareh *et al.*, 2016).

1. Agarose gel 1% prepared in using 1X TBE and dissolving in water bath at 100 C° for 15 minutes, after that, left to cool 50C°.
2. Then 3μL of ethidium bromide stain were added into the 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 into each comb well and 5ul by (10000bp Ladder) in one well. The gel plate was settled in electrophoresis chamber and fill by 1X TBE cradle. At that point, an electric current was performed at 100 volts and 80 AM for 1hour.
5. PCR products were visualized by using an ultraviolet transilluminator.

3.2.7.4 Quantitative Realtime- Polymerase chain reaction (qPCR)

RT-PCR technique was performed for detection *Brucella species*, The method was carried out according to the method described by (Wang *et al.*, 2014). As following steps:

3.2.7.4.1 PCR Primers

The PCR primers used in the study are shown in Table (3-6), (Bioneer, Korea).

Table (3-6): The PCR primers used in the study to diagnosis human brucellosis.Cite from "Wang *et al.* 2014"

No.	Primer	Sequence		Target gene
1	B4	F	'5-TGGCTCGGTTGCCAATATCAA -3'	bcsp31
	B5	R	'5-CGCGCTTGCCTTTCAGGTCTG-3'	
2	JPF	F	'5-GCGCTCAGGCTGCCGACGCAA -3'	omp2
	JPR	R	'5-ACCAGCCATTGCGGTCGGTA-3'	

3.2.7.4.2 Quantitative Real-Time PCR master mix preparation

qPCR master mix was prepared by using AccuPower™ Green Star Real-Time PCR kit that using SYBER green dye detection of gene amplification in Real-Time PCR system for target and incorporate the accompanying as shown in Table (3-7):

Table (3-7): The components of (qPCR) master mix preparation.

qPCR master mix	Volume
DNA template	2.5µL
Forward primer (10pmol)	1 µL
Reverse primer (10pmol)	1 µL
Nuclease-free water	15.5 µL
Total	20 µL

After that, these qPCR master mix components that mentioned above AccuPower Green star qPCR premix standard plate tubes that contain the SYBER green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in the Minopticon Real-Time PCR system as shown in Table (3-8).

Table (3-8): The thermal cycling conditions within the Minopticon Real-Time PCR system.

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

3.2.8 Nitro blue Tetrazolium (NBT) index

The assay was carried out in relation to a method offered by (Zakaria *et al.*, 2011) for all patients. NBT was used for evaluating of phagocytic activity of percentage. The assay accomplished in the subsequent steps:

- 1- In a microtiter plate of 96 wells, 175 µl DMEM media only were added to wells A1, A2, A3, A4 as negative control groups.
- 2- 100 µl of fresh blood from healthy individuals as a positive control groups were added to wells A5, A6, A7, A8 and 100 µl of positive rose Bengal test blood samples were added to others wells.
- 3- Then 175 µl DMEM media were added to each well, after that added 25 µl of NBT 1g/liter.
- 4- The microtiter plate was incubated two hours at 28 c°, then removed supernatant carefully.
- 5- The cell is fixed with methanol 100% (v/v) for 5 min.
- 6- Each well is washed twice with 125 µl methanol 70%.
- 7- The cell is allowed to dry in the air overnight and reduced NBT by dissolving 125 µl Potassium hydroxide (2N) and 150 µl dimethyl sulfoxide (DMSO) add to each well.
- 8- After that read by the ELISA reader at 650-wavelength.
- 9- The phagocytic activity percentage calculated according to the following equation (Perez-Serrano *et al.*, 1997).

Phagocytic activity percentage (%) = $\left\{ \frac{A-B}{B} \right\} \times 100$

A= Treated group (a positive control groups and positive rose Bengal test blood samples ; B= Negative control groups.

3.2.9 Total and differential count of WBC

WBC measured by Sysmex apparatus (Sysmex/ Japan) (Kim *et al.*, 2015).

3.2.10 Serological evaluation of humoral immune response

3.2.10.1 Rose Bengal Test (RBT)

According to (OIE, 2009) RB test has been performed on human serum samples in the following steps:

1. Serum samples and antigen have been brought to room temperature.
2. Each serum sample (25–30 µl) was transferred into white tile or plastic plate.
3. Antigen bottle has been shaken gently and an equal volume has been placed near each serum spot.
4. Immediately after the addition of the last drop of antigen, serum and antigen were mixed thoroughly using a clean glass or plastic rod. A circular or oval zone (2 cm in diameter) has been appearing.
5. The mixture was agitated gently for 4 minutes at room temperature.
6. Agglutination was read immediately. Any visible reaction is considered to be positive.

3.2.10.2 The 2-Mercaptoethanol test

Procedure For 2-Mercaptoethanol-Rapid Slide Agglutination Test (2ME-RSAT) according to (Brucellosis Antibody Test Kit)

1. Adding 2 drops of Reagent A, 2-Mercaptoethanol (Yellow Cap) to a tube containing 2 drops of the serum to be tested, and mix well.
2. Placing 1 drop of this mixture in a new reaction circle. If event agglutination means IgM and the infection is acute. If not agglutination goes to the third step.
3. Adding 1 drop of Reagent B, Antigen to the serum solution and mix with stir stick, spreading to fill the circular area. Do not allow the positive control test to touch the unknown serum sample.

4-Gentling rock the card for 10-15 seconds. Place on a flat surface and observe for agglutination for no longer than 2 minutes. If event the agglutination means IgG and the infection is chronic and if no agglutination means not found infected with *Brucella*.

3.2.10.3 ELISA Assay

The assay is carried out according to (Human Brucellosis IgG-ELISA kit, NovaTec., Germany).

3.2.11 Preparation of lymphocytes

The procedure according to the method of (Fuss *et al.*, 2009).

- 1- 2 ml of blood is collected from the patient's vein.
- 2- The collected blood is transferred to 4 ml heparinized green top tubes.
- 3- Made 1:1 dilution of whole blood with 1X PBS
- 4- Carefully underlay 3 ml of lymphoprep (density gradient separating medium). 4 ml of diluted blood is transferred to a 15 ml conical tube.
- 5- Immediately centrifuge at 1800rpm [700g] for 30 min in a cooling centrifuge (4C°).
- 6- Carefully remove the tube from the centrifuge, observing the three layers: top is clear supernatant, middle is an opaque fluid containing the PBMC, and bottom is RBC.
- 7- Carefully remove and discard the supernatant within of the opaque layer of the PBMC. Quickly transfer the buffy coat layer of peripheral blood mononuclear cell (PBMC) (around 1.5 ml) into a new 15ml conical tube.
- 8- Add RBMI 1640 media to the PBMC suspension to obtain a final volume of 10 ml. Mix well and centrifuge at 1800rpm for 10 min in a cooling centrifuge (4C°).
- 9- Pour off and discard the supernatant and combine RBMI 1640 media to obtain the final volume of 7 ml. Mix well and centrifuge at 1500rpm (500g) for 10 min in a cooling centrifuge (4C°) to remove platelets.
- 10- Pour off and discard the supernatant. Resuspend the pellet in 2ml of the freezing medium (RBMI 1640 media)

11- The cell count was adjusted to 7000 lymphocytes/ml using a hemocytometer. At the same time, the cell viability was also assessed using a dye exclusion method, and the dye was trypan blue. The lymphocyte viability had been always greater than 95%.

12- All these steps were carried out under aseptic condition.

3.2.12 MTT Assay

The MTT assay performed according to (MTT cell viability kit, Abnova, USA).

Examine Procedure

1. The plate and culture cells (80 μ L per well) in a clear bottom 96-well tissue culture plates. Assays can be performed on either adherent cells or cells in suspension. The number of cells can vary from 1,000 to 80,000 per well. The volume can vary from 50 to 150 μ L, although 80 μ L is used in this example. In addition to the test samples, one must include control wells of culture medium containing no cells or cells treated with a toxic reagent such as 0.1% saponin.
2. Adding test compounds and controls and incubate cells for the desired period of time (typically overnight). It is recommended that assays be run in duplicate or triplicate. A volume of 20 μ L in phosphate buffered saline (PBS) or culture medium is recommended for the test compounds and controls. The Control Reagent can be conveniently reconstituted with 5 mL PBS (1% saponin).
3. Warming Reagent and to room temperature. Add 15 μ L (per 80 μ L cell cultures) of Reagent per well and incubate for 4 hours at 37°C. The volume of the reagent should be adjusted depending on the volume of cell culture.
4. Adding 100 μ L of the Solubilizer to each well. Mix gently on an orbital shaker for one hour at room temperature. The volume of the Solubilizer should be adjusted depending on the volume of cell culture. If precipitation occurs in the Solubilizer, place the bottle in a warm water bath or at 37°C and shake to dissolve precipitates.
5. Measuring OD570nm for each well on an absorbance plate reader. The Maximum absorbance of the formazan dye lies between 560 and 590 nm. If desired, the OD measurement can be performed the following day. In this case, it is recommended to seal the plate to minimize evaporate.

The lymphocyte transformation index percentage calculated according to the following equation (Perez-Serrano *et al.* 1997).

$$\text{Lymphocytes transformation index (\%)} = \left\{ \frac{A-B}{B} \right\} \times 100$$

A= Treated group (positive control group and patients { positive rose Bengal test blood samples}) ; B= Negative control group.

3.2.13 Statistical analysis

Data subjected to analysis using the SAS program (Statistical Analysis System) version 9.1 (2010). Comparisons among proportions performed using Chi-Square test. $P < 0.05$ was considered as significant. Criteria used in the evaluation of the traditional test in the present study were, according to (Parikh *et al.*, 2008). Two-way ANOVA with Interaction and Least significant differences (LSD) post hoc test performed (multiple comparisons), to assess significant difference among means. $P < 0.05$ was considered statistically significant.

4. Results and Discussion

4.1 Collection of specimens and isolation of *Brucella species*

One hundred positive rose Bengal test samples were collected from peripheral human blood as a serodiagnostic method for human brucellosis infection in Al-Hussein teaches Hospital of Muthanna Province, to the period from December/ 2015 to July/ 2016. The samples divided according to age groups from 0-80 years, the females (55%) higher than males (45%) and rural areas (61%) higher than urban areas (39%). The taking of therapy was (28%) and the smoking percentage (11%) as shown in Table (4-1). Only one *Brucella species* isolate (1%) was isolated by Lysis-centrifugation method as shown in Figure (4-1). This strain used as control in real-time PCR, while blood culture method failed to give a positive result 0 (0%).

Table (4-1): The ages, gender, region, medication, and smoking of the patients, according to a positive results for rose Bengal test.

No. of patients		0	10	31	25	16	14	2	2	Percent
Ages average/ years		0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	-
Gender	Male	0	4	18	8	6	6	1	2	45 (45%)
	Female	0	6	13	17	10	8	1	0	55(55%)
Region	Rural	61								61%
	Urban	39								39%
Taking medication	Therapy	28								28%
	No therapy	72								72%
Smoking	Smokers	11								11%
	No smokers	89								89%

The samples taken from peripheral blood to culture because it is difficult to obtain samples from the lymph nodes or bone marrow or synovial fluid as well as the spinal cord. Lysis-centrifugation method gives the best result 1 (1%), compared with blood culture method 0 (0%). Also it is less time (2 days) compared to the time it used to process blood cultivation (may be 1 a month). Perhaps the reason for that is due to the site of *Brucella* be intracellular and when the centrifuge process liberated cells into an extracellular site and thus be ready for cultivation, unlike blood culture directly that need to be enough time to liberate cells into an extracellular site. This is identical with researchers Mantur & Mangalgi found that the best method to isolate *Brucella* is lysis centrifugation method (Mantur & Mangalgi, 2004). In this method, the average detection time had been decreased(Çetin, *et al.*, 2007).

The results in isolate the bacteria from the human blood in the current study may be belonged to most samples taken during the winter where the presence of few numbers from bacteria in the blood and the use of antibiotics during the sampling, where found that 28% of patients handlers for treatment. Many patients also neglecting initial diagnosis to bacteria that turn into chronic phase and then after that is difficult to isolate. the sensitivity of isolation in the acute form had been reported as lower than 90% and in the chronic form, to be as low as 20% (Yagupsky, 1999). (Azar *et al.*, 2006) were not conforming to current study where they found 8 (26.6) blood culture cases were positive from 30 (100%) cases collecting from peripheral blood, all 8 strain diagnoses as *Brucella melitensis*. (Cekovska *et al.*, 2010) were not compatible to present study where they found 16 (14.2%) blood culture cases were positive from 113 (100%) cases collecting from peripheral blood.

A total of 50 blood culture bottles were processed during the evaluation period, out of which, seven (14%) were positive by BACTEC system with a mean detection time of six days(Alikhani *et al.*, 2012).

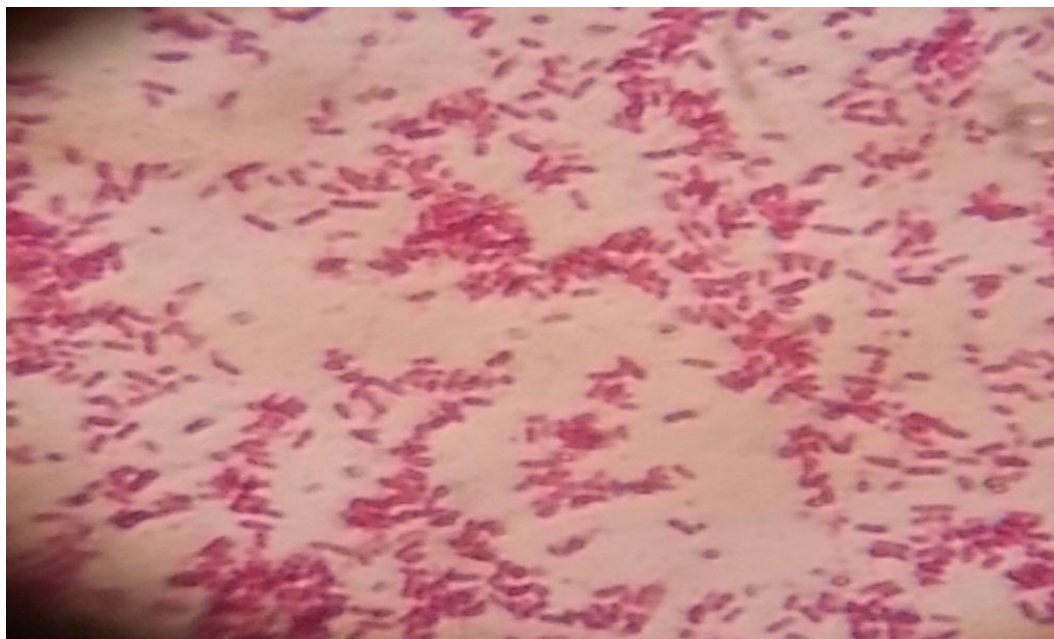


Figure (4-1): Gram staining of *Brucella* appear gram-negative coccobacillus or small rods, lone or small groups under microscopic examination.

4.2 Api-20E system

These biochemical tests confirm the diagnosis of *Brucella spp.* which has the numerical profiles (0200004) or (0220004) as it is shown in the Figure (4-2). Api20 E Strep result for *Brucella* isolate shown in (Appendix A).

API 20 E is a standardized identification system for Enterobacteriaceae and other non-fastidious, Gram negative rods which uses 21 miniaturized biochemical tests and a database.

In the evaluation by (Washington *et al.*, 19971) approximately 93.0% of the 129 *Enterobacteriaceae* and five *Aeromonas* strains were correctly identified to species level. (Smith *et al.*, 1972) showed an overall accuracy rate of 96.4% in testing 366 enteric strains. Over the next 20 years, the API 20E strip was compared to many other identification systems and, because of its large acceptance by the clinical microbiology laboratory market, became somewhat of a “gold standard” among commercial systems.

Because of the continued expansion of the database while the original substrate pattern in the strip was maintained, (O'Hara *et al.*, 1992) reevaluated the strip for its accuracy in the identification of *Enterobacteriaceae*. For those organisms routinely isolated in clinical laboratories (e.g., *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*), the strip accurately identified 87.7% at 24 h and 96.3% at 48 h. For organisms less routinely isolated, e.g., *Providencia stuartii* or *Escherichia vulneris*, the API strip identified only 78.7% at 24 h. There have been several studies of accuracy aimed at individual genera.

(Archer *et al.*, 1987) reported accuracies of 66 and 51% in the identification of *Yersinia* spp. When incubation was at 28 and 37C°, respectively. They also reported greater accuracy in identification of *Yersinia enterocolitica* biogroups 1 and 2 as opposed to biogroups 3 and 4 (97 to 100% as opposed to 27 to 47% at either temperature). This report indicated that many of the misidentifications were due to the inability of organisms to ferment melibiose and rhamnose at 37C°.

(Sharmer *et al.*, 1990) reported accuracies of 97% for all biogroups of *Y. enterocolitica* when incubated at 28C° and of 90% for *Yersinia* spp. Overall. They also reported problems with the fermentation of melibiose and rhamnose, as well as inositol. Identifications of *Vibrio cholerae* were only 50% accurate, while 9 of 10 identifications each of *Vibrio fluvialis* and *Vibrio hollisae* were correct, but all at the “good likelihood, low selectivity” level of probability.



Figure (4-2): Diagnosis of *Brucella species* by using Api-20E system.

4.3 Vitek 2 System Diagnosis

The principle of the Vitek 2 system is fully automated system for evaluation of microbial biochemical reaction, The analytical profile guide of this system displays possibility 98% identification percentage. The results which are obtained from this system are shown in Table (4-2).

The identification of bacteria with the VITEK 2 system is mainly done within several hours, from 3–18 hours, faster for the Enterobacteriaceae family than other fastidious and non-fermentative bacterial genera (Petrovska *et al.*, 1997).

The VITEK 2 system identifies only *Brucella melitensis* strains and not the other bacteria from the *Brucella* spp. Nevertheless, all the oxidase positive strains isolated from blood cultures which were not pseudomonades were identified by VITEK 2 in the examined period, so we are sure that other *Brucella* strains were not isolated from the blood cultures. The identification based on 48 biochemical and physiological test reactions is useful for determining the differences in the isolated strains. There were four types of *Brucella melitensis* identified by the VITEK 2 system. Minor differences

were detected in Glycine Arylamidase, Alpha-Glucosidase and L-Lactate alkalisation (GlyA+, AGAL+ and ILATk+) reactions (Cekovska *et al.*, 2010).

Strains were isolated from blood cultures of two patients suffering from fever without apparent cause and from a bone marrow culture of a patient with a left tibial lesion. They were initially identified as *Bordetella bronchiseptica* (1 case) and *Ochrobactrum anthropi* (2 cases) through the Vitek 2 Compact microbial identification system and Gram-negative (GN) identification card. As the first case (misidentified as *Bordetella bronchiseptica*) had brucellosis symptoms of splenomegaly, mildly elevated transaminase, typical undulant fever, and a significantly elevated lymphocyte level in his white blood cell count, 16S rRNA PCR amplification and sequencing were performed and results were analyzed using GenBank (accession numbers. The results indicated that the highest level of identity was to *Brucella abortus*, *Brucella melitensis*, and *Brucella microti* among the *Brucella* spp., as well as *Ochrobactrum anthropi* ATCC 49188 (query coverage, 100%; maximum identity, 99%) (Yang *et al.*, 2013).

Table (4-2): The Vitek 2 system result

No	Test	Mnemonic	Result
1	D – Amygdalin	AMY	-
2	Ala- Phe – Pro –Arylamidase	APPA	+
3	Leucine Arylamidase	LeuA	-
4	Alanine Arylamidase	AlaA	-
5	D – Ribose	dRIB	-
6	H ₂ S production	H ₂ S	-
7	D – Raffinose	dRAF	-
8	Adonitol	ADO	-
9	Phosphatidylinositol Phospholipase C	PIPLC	-
10	Cyclodextrin	CDEX	-
11	L – Proline Arylamidase	ProA	+
12	Tyrosine Arylamidase	TyrA	+
13	L – Lactate alkalization	ILATK	+

14	Glycine ARYLAMIDASE	GlyA	+
15	O/129 resistance (comp.vibrio)	O129R	-
16	D – Xylose	dXYL	-
17	L – Aspartate Arylamidase	AspA	-
18	Beta – Clucronidase	BGURr	-
19	D-sorbitol	dSOR	-
20	Lactose	LAC	-
21	D – mannose	dMAN	-
22	Salicin	SAL	-
23	Arginine Dihydrolase 1	ADH1	-
24	Beta Galactopyranosidase	BGAR	-
25	Alpha – Galactosidase	AGAL	+
26	Urease	URE	-
27	N – Acetyl – D - Glucosamine	NAG	-
28	D – Mannose	dMNE	-
29	Saccharose / Sucrose	SAC	-
30	Beta – Galactosidase	BGAL	-
31	Alpha – Mannosidase	AMAN	-
32	L – Pyrrolidonyl - Arylamidase	PyrA	-
33	Polymixin B Resistance	POLYB	-
34	D – Maltose	dMAL	-
35	Methyl – B – D – Glucopyranoside	MBdG	-
36	D – Trehalose	dTRE	-
37	Alpha – Glucosidase	AGLU	-
38	Phosphatase	PHOS	-
39	Beta Glucuronidase	BGUR	-
40	D- Galactose	dGAL	-
41	Bacitracin Resistance	BACI	-
42	Pullulan	PUL	-
43	Arginine Dihydrolase 2	ADH2S	-

4.4 Antibiotic sensitivity test for *Brucella melitensis* Rev1

The principle of antibiotic sensitivity test depends on the measurement of inhibition zone of bacterial growth, the antimicrobial disks were used for detection the susceptibility of *Brucella species* isolates to 19 selected antibiotics,. The result of antibacterial agent activities on *Brucella melitensis* Rev 1. The strain was resistant to

Clindamycin (CD), Ampicillin (P), Erythromycin (E), Vancomycin (VA), Aztreonam (AZT), Cefotaxime (CTX), Ceftriaxone (CRO), Amoxyclav (AMC), Nitrofurantoin (F).

Some antibiotics have intermediate inhibition zones against this bacteria, such as Tetracycline (TE), Amoxyclav (AMC), and Ticarcillin (TIC). The most useful antibacterial agents were aminoglycosides (Gentamycin (GEN) (39mm), Tobramycin (TOB) (36 mm), Netillin (NET) (32mm), Amikacin (AK) 30mm), Ciprofloxacin (CIP) 31mm, Imipenem (IPM) (28mm), Piperacillin (PRI) 25mm, Chloramphenicol (C) 20mm as it is shown in Figure (5-3). Compared with standard Inhibition zones of antibiotics (mm), Inhibition zones (mm) to antibacterial agents shown in (Appendix D).

Brucella melitensis is the commonest etiological agent causing human brucellosis worldwide. Intracellular localization of *Brucella* species within monocytes and macrophages of the reticulo-endothelial system limits the choices of antimicrobial agents for effective treatment of systemic and localized brucellosis. Although a variety of antimicrobial agents appear to be active in vitro, the results of susceptibility testing do not always correlate with clinical efficacy (Corbel, 2006). Amongst tetracyclines, doxycycline is the most common antimicrobial drug used for treatment of uncomplicated brucellosis in adults and children for more than 8 years (Corbel, 2006). The World Health Organization (WHO) recommends doxycycline (6 weeks) in combination with aminoglycosides (streptomycin or gentamicin for 2–3 weeks) or rifampicin (6 weeks) (Corbel, 2006).

The most preferred and effective aminoglycoside in treatment of brucellosis is streptomycin (Corbel, 2006; Ariza *et al.*, 2007; Solís & Solera, 2012). Combination with streptomycin was found to be superior as compared to rifampicin, in terms of relapse or treatment failure (Solís & Solera, 2012). Even though gentamicin is more active in vitro and is associated with fewer side effects than streptomycin, there are not enough studies, which justify the replacement of streptomycin (Corbel, 2006; Ariza *et*

al., 2007). In the present study, the strain was susceptible to gentamicin (39mm) and Tobramycin (TOB) (36 mm) and found to be in the range described previously (Abdel-Maksoud *et al.*, 2012; Hashim *et al.*, 2014; Deshmukh *et al.*, 2015).

Ciprofloxacin is a potential alternative therapeutic option in the treatment of brucellosis with excellent oral bioavailability and reaching high concentrations in phagocytic cells (Kaya *et al.*, 2012). However, ciprofloxacin is not recommended for monotherapy due to the lack of bactericidal activity at the intracellular acidic pH (Abdel-Maksoud *et al.*, 2012), high rate of relapse and the risk of development of overall fluoroquinolone resistance in the community (Pappas *et al.*, 2006b). Therefore, its use in combination with doxycycline is recommended as an acceptable alternative but not as first line regimen (Falagas, 2006). We found ciprofloxacin (31mm) in the susceptible range when compared to MIC breakpoint criteria of slow growing bacteria and these findings are consistent with previous studies (Abdel-Maksoud *et al.*, 2012; Kaya *et al.*, 2012).

Trimethoprim/sulfamethoxazole (TMP/SMX) is an alternative agent recommended for treatment of brucellosis in pregnancy and in children under 8 years old. It has also been recommended for treatment of osteo-articular complications, neurobrucellosis and endocarditis, in combination with doxycycline, aminoglycoside and rifampicin (Corbel, 2006).

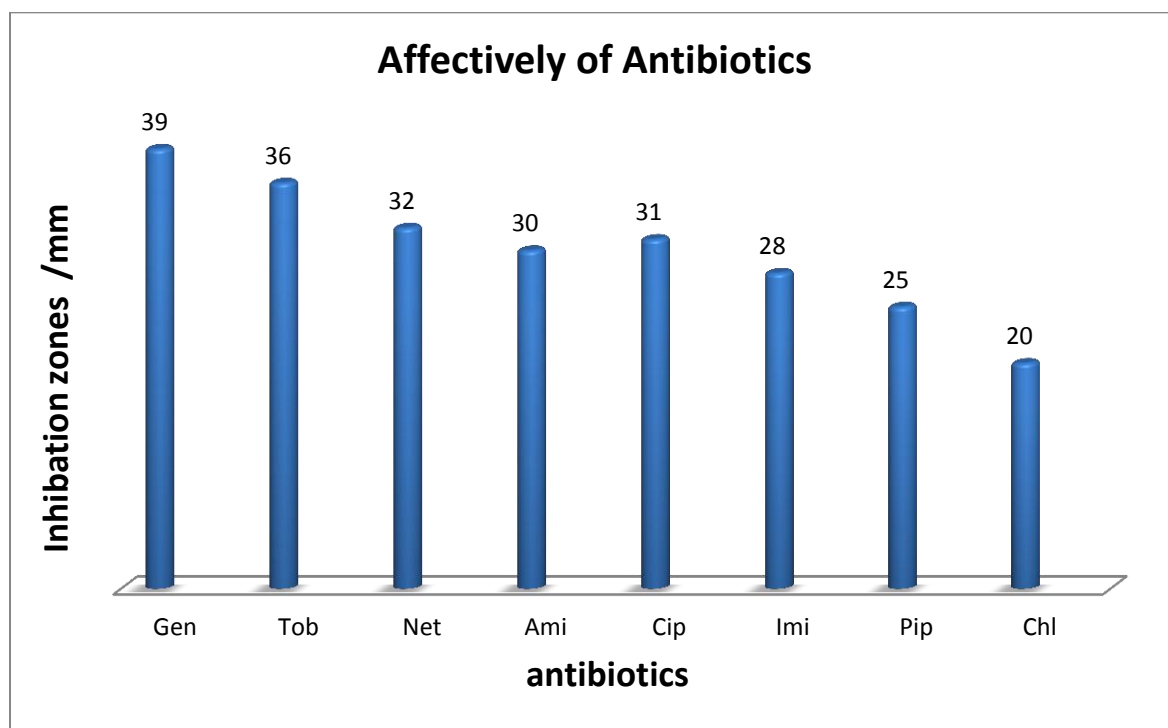


Figure (4-3): Sensitivity of *Brucella melitensis* Rev1 to antibiotics.

4.5 Serotyping & Biotyping

These groups of tests were used to detect the species and strain of *Brucella*. The Result of the monospecific antisera agglutination test confirmed that the *Brucella* isolate previously are *Brucella melitensis* as it is shown in Figure (4-4). Then biotyping test results confirmed which the isolated strain is *Brucella melitensis* Rev1 as shown in Table (4-3).

Serotyping is the set of antigens that characterizes the group, and classical bacteriological biotyping techniques to identify phenotypes characteristics have traditionally been used to classify *Brucella* strains. Although these techniques have proved useful, they are subjective and it is well recognised that some strains may give unexpected or anomalous results to some or all of the tests (Dawson *et al.*, 2008). All *Brucella* strains were identified as *Brucella melitensis* by biochemical characteristics such as positive oxidase and Urease, negative H₂S, no CO₂ requirement and growth in

the present of basic fuchsin and thionin (20 mg mL^{-1}) (Al-Garadi *et al.*, 2011). Fifty two *B.melitensis* isolates were serotype 3, and 10 isolates were serotype 1 according to A and B monspesific antisera agglutination test (Sayan *et al.*, 2009).

Table (4-3): Serotyping & Biotyping results of *Brucella* isolate.

CO2 require- ment	H2S production	Bacterial growth on dyes						Urease	Agglutination by monospecific antisera	
		Thionin			Basic Fuschin					
-	-	A*	B	C	A	B	C	-	A	M
		-	-	-	-	-	-		-	+

***Concentration of deys:**

A- 1:25.000

B- 1:50.000

C- 1:100,000

Agglutination by monospecific antisera:

A- Monospecific antisera of *Brucella abortus*

M- Monospecific antisera of *Brucella melitensis*

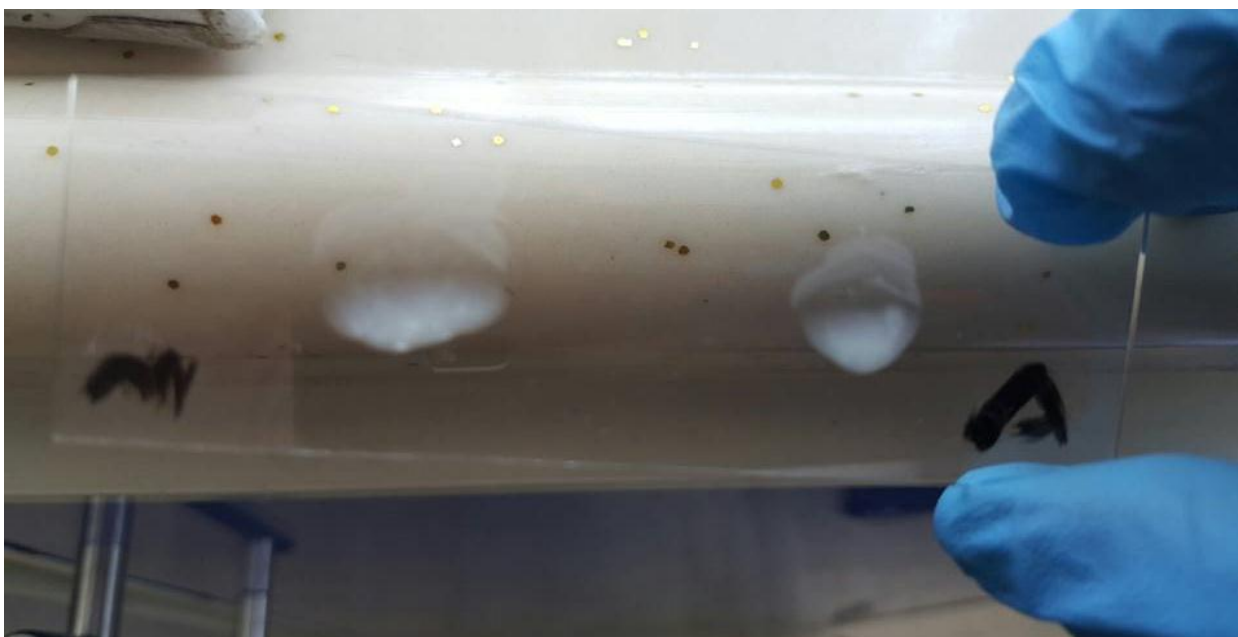


Figure (4-4): Agglutination by monospecific antisera (A- *B.abortus* = negative, m- *B. melitensis*= positive).

4.6 Molecular detection

4.6.1 Extraction of DNA

The DNA of 100 samples of whole blood and DNA of control sample extracted and purified using bacterial genome DNA purification Kits. The results were detected by electrophoresis on 1% agarose gel and exposed to U.V light in which the DNA appeared as compact bands.

4-6.2 Conventional PCR

This technique is used to confirm the diagnosis of a control sample which is used in RT-PCR by using omp2 (JPF/JPR) and bcsp31 (B4/B5), as diagnostic genes as shown in Figures (4-5), and (4-6), respectively.

For the diagnosis of human brucellosis, a PCR assay with one pair of primers was developed, which amplifies the target genomic sequence of *Brucella species*. Primer pairs include the primers for sequences encoding 16S rRNA (Nimri, 2003), outer

membrane protein (omp2a, omp2b) (Bardenstein *et al.*, 2002), 31-kDa immunogenic *Brucella abortus* protein (BCSP 31) (Wang *et al.*, 2014).

Studies showed that standard PCR appeared to be a more sensitive technique than microbiological methods, not only for the diagnosis of a first episode of infection, but also for the early detection of relapses (Briones-Lara *et al.*, 2007). Some research groups also assessed the performances of standard PCR as diagnostic tools for human brucellosis with respect to conventional methods. Their results showed that standard PCR is a promising diagnostic tool for patients with clinical signs and symptoms, and negative serological results, allowing an accurate and early diagnosis of human brucellosis (Asaad and Alqahtani, 2012; Gemechu *et al.*, 2011).

The standard PCR is simple and efficient. However, the efficiency of this method is dependent on the specificity of the primers. Different primer pairs have previously been published for *Brucella species* detection, and only a few of them have been used in human samples. Baddour & Alkhalifa, 2008 comparative sensitivity of 3 pairs of primers amplify 3 different fragments, including a gene encoding BCSP 31 (B4/B5), a sequence 16S rRNA of *B. abortus* (F4/R2), and a gene encoding omp2 (JPF/JPR). The results showed that the sensitivity of the B4/B5 primer pair, JPF/JPR primer pair and the F4/R2 primer pair was 98%, 88.4% and 53.1%, respectively. Navarro *et al.*, 2002 also, compared PCR methods using these 3 pairs of primers as described above. Their results further indicated that the three primers assayed showed a difference in sensitivity by the presence of human genomic DNA.

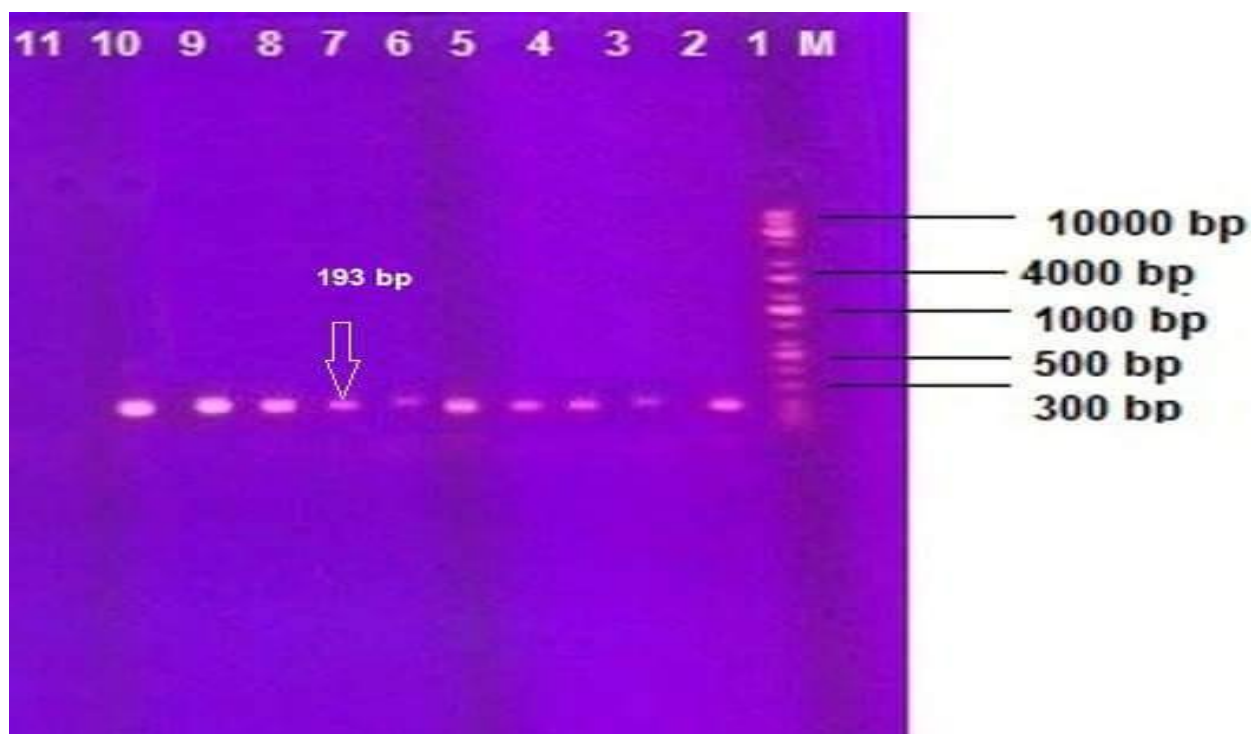


Figure (4-5): Diagnosis of an *omp2* gene on agarose gel electrophoresis 1% at 100 Volt for 1hour. M: Molecular weight ladder (100-10000 bp), n: 1-11: (193 bp).



Figure (4-6): Diagnosis of a *bcs31* gene on Agarose gel electrophoresis 1% at 100 Volt for 1hour. M: Molecular weight ladder (100-10000 bp), n: 1-11: (223 bp).

4.6.3 Quantitative Real-Time PCR (qPCR)

The RT-PCR technique was performed for detection *Brucella* in whole blood samples. One shared approach used in quantification is that the customary curve technique. During this technique, customary curve is used as a location standard for final threshold cycle (CT) standards from DNA amplification of specimens with indefinite concentrations. The RT-PCR technique was achieved to find of microorganism DNA duplicates quantity in one hundred blood samples were positive for rose Bengal test takes a look at to diagnosis of brucellosis. These samples are subjected to RT-PCR for confirming the diagnosis by using SYBR Green I and bcsp31 and omp2 as diagnostic genes.

Results showed that 38 (38%) samples were positive and 62 (62%) were negative for both genes omp2 and bcsp31 (Figures 4-8 and 4-9). The results, according to age groups/ years, which are shown in Table (4-4), The positive cases shown in Figure (4-7) were as follows (0 (0%), 5 (50%), 13 (41.9), 9 (36%), 5 (31.25), 4 (28.57), 1 (50%), 1 (50%), respectively). The negative cases were as follows (0 (0%), 5 (50%), 18 (58.1%), 16 (64%), 11 (68.75%), 10 (71.43), 1 (50%), 1 (50%), respectively).

The major advantages of real-time PCR are that it can be performed in a very short time, does not require electrophoretic analysis, and avoids contamination (Redkar *et al.*, 2001). In presnt study, we used blood samples to diagnose human brucellosis by RT-PCR, The samples that can be tested by real-time PCR include cultured *Brucella* cells (Redkar *et al.*, 2001). Serum (Queipo-Ortuño *et al.*, 2005b). Blood, and paraffin-embedded tissues (Kattar *et al.*, 2007).

Because microorganisms of the *Brucella* are facultative intracellular pathogens and the inoculum found in patients is normally small (Queipo-Ortuño *et al.*, 2005b). Most studies of PCR assays involving human brucellosis have been undertaken with whole blood samples. However, Zerva *et al.*, 2001 reported a greater sensitivity in serum samples than in whole blood samples, even though the amount of circulating DNA in serum is presumably lower.

The current study used SYBR Green I, bcp31 and omp2 as diagnostic genes. SYBR Green I, as one of the most commonly used DNA binding dyes, binds to total amounts of DNA generated during PCR, so it can induce specific and non-specific amplification (Navarro *et al.*, 2015). there are many studies that used this genes to diagnosis of *Brucella* (Redkar *et al.*, 2001; Queipo-Ortuño *et al.*, 2005b; Falguni Mukherjee & Jainendra Jain, 2007; Bounaadjia *et al.*, 2009).

In the present study, of the 100 samples, only 38 (38) samples were positive to this test. In the similar study 50 patients divided into age groups by mean was 37.7 ± 18.3 (range; 12-80 years), the total positive cases were 44 (88%) (Surucuoglu *et al.*, 2009). In the another study 59 patients included in the study, 41 (69.5%) were men, and 18 (30.5%) were women. The mean age ($\pm$ SD) was 43.2 ± 15.7 years (range, 15–75 years), 55 (93%) patients were positive for brucellosis (Queipo-Ortuño *et al.*, 2005b).

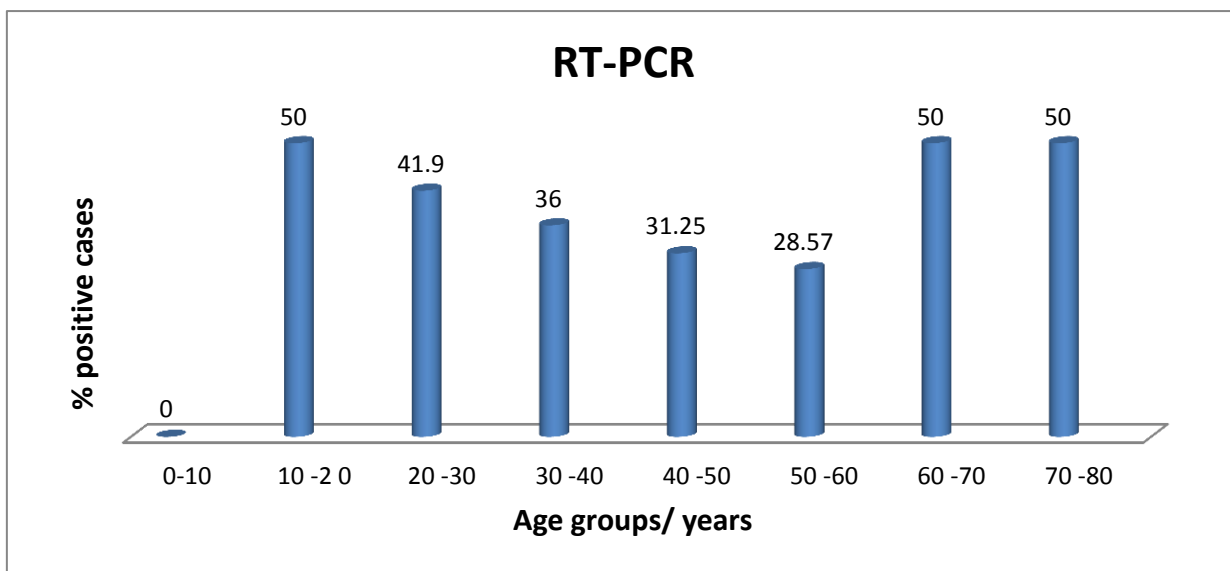


Figure (4-7): The positive cases percentage which diagnosed by RT-PCR, according to age groups/ years.

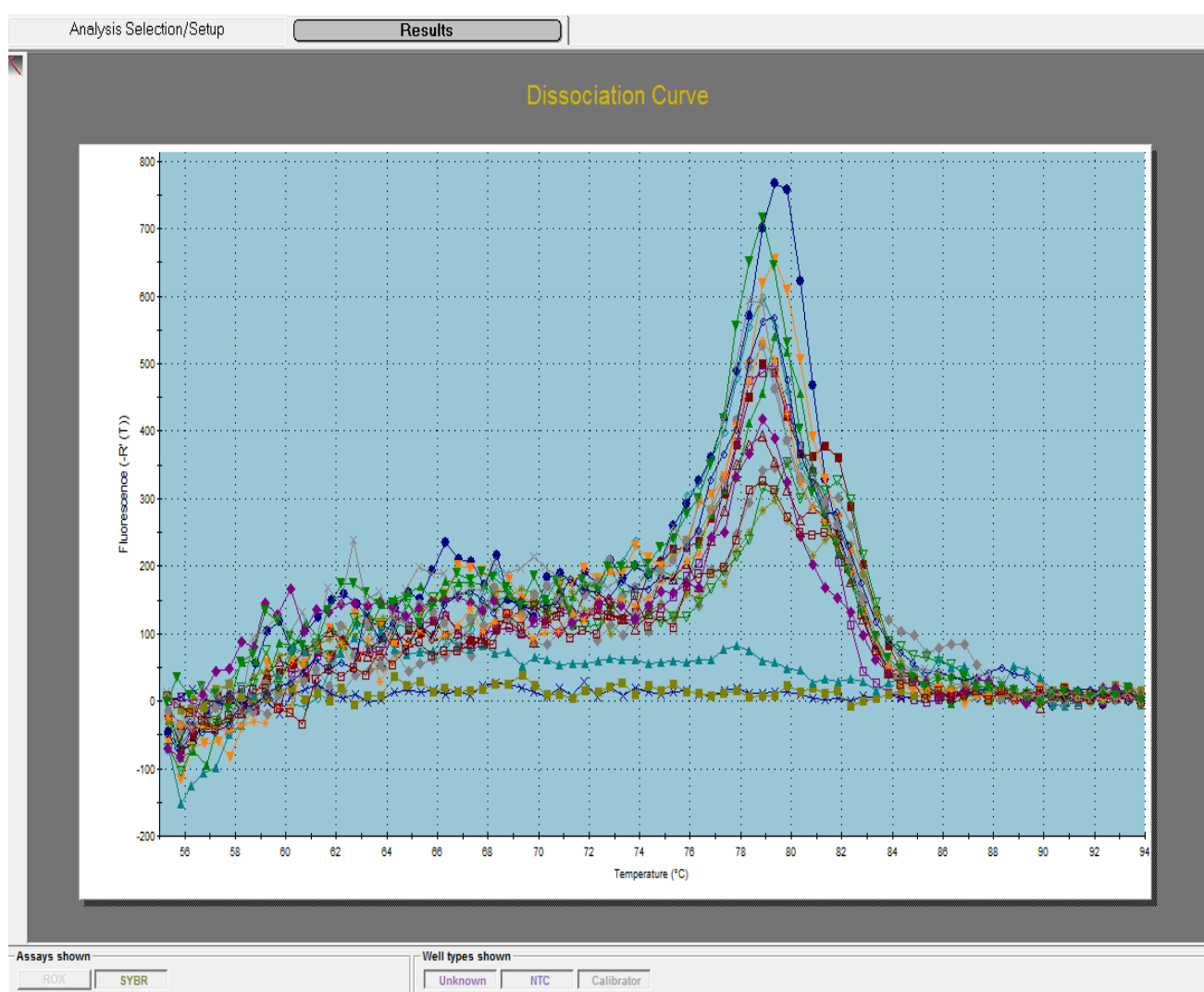


Figure (4-8): Dissociation curve for bcs31Gene in human brucellosis, that display a positive and negative DNA sample from human blood, By using SYBER green 1 RT-PCR amplification.

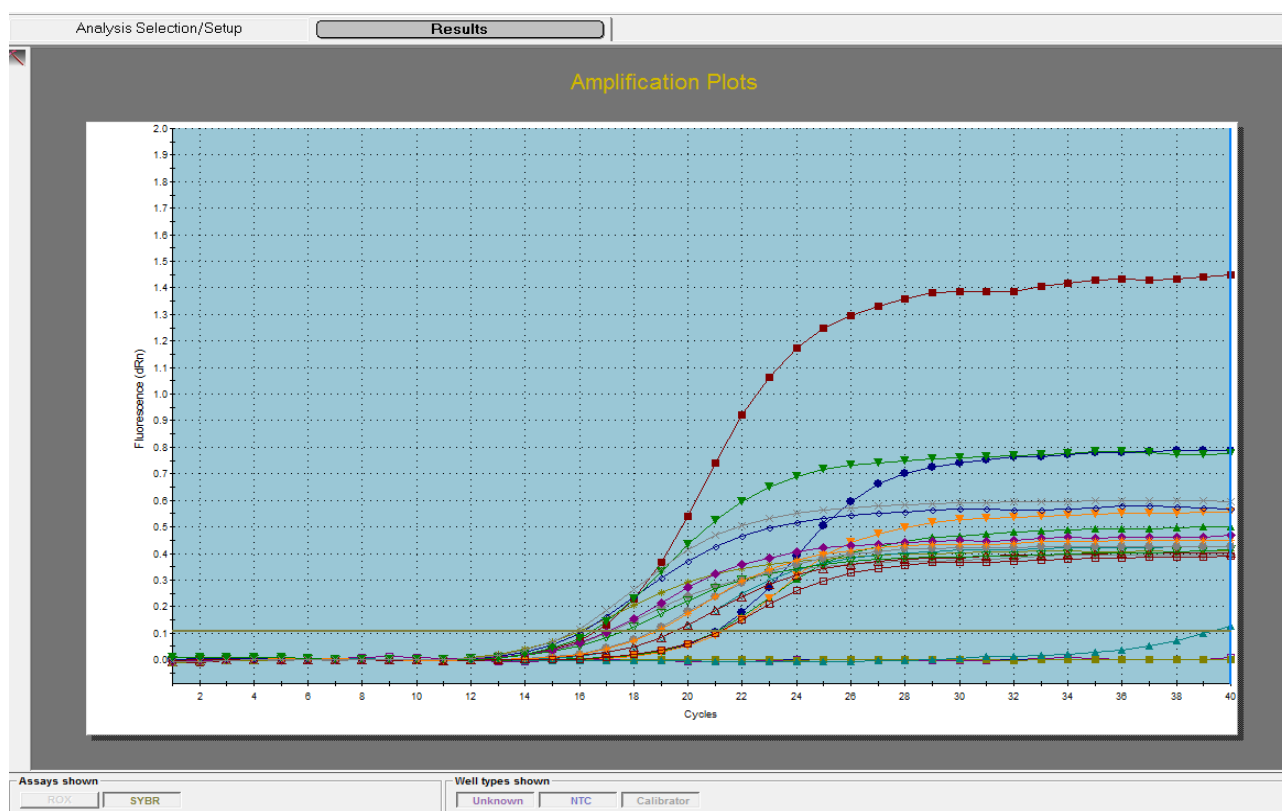


Figure (4-9): Real-Time PCR amplification design for bcs31 Gene in human brucellosis, that display a positive and negative DNA samples from human blood, By using SYBER green 1 RT-PCR amplification.

4.7 Nitro Blue Tetrazolium (NBT) index

The Nitro blue Tetrazolium test is used for evaluating the phagocytic activity percentage of peripheral blood leukocytes. The results of current study show that there is a significant increase ($P < 0.05$) in phagocytic activity (266%) in the leukocytes of patients positive rose Bengal test, compared with deionized detailed water as a negative control (0%) and (112%) in the leukocytes of healthy individuals as positive control as shown in Table (4.5). The results, according to age groups indicate that dissimilar noteworthy rises in the NBT index, which signifies the phagocytic activity % (-, 58%, 375%, 608%, 350%, 308%, 204%, and -29%, respectively). The greatest NBT index

was noted in group IV (608%), and worse NBT index noted in group VIII (-29%). As shown in Table (4-6) and Figure (4-10).

Table (4-5): Nitro Blue Tetrazolium (NBT) index results to control and patient groups.

Group	Mean±SE	Phagocytic activity percentage	P value
Negative control	0.024±0.0007 ^b	0%	<0.05
Healthy individuals (Positive control)	0.051±0.005 ^{cd}	112%	
Patients	0.088±0.006 ^a	266%	

Table (4-6): Nitro Blue Tetrazolium (NBT) index results according to age groups.

Groups	Age groups/ years	Mean±SE	Phagocytic activity percentage
I	0-10	-	-
II	10-20	0.038±0.006 ^d	58%
III	20-30	0.114±0.012 ^b	375%
IV	30-40	0.170±0.010 ^a	608%
V	40-50	0.108±0.005 ^b	350%
VI	50-60	0.098±0.007 ^b	308%
VII	60-70	0.073±0.008 ^c	204%
VIII	70-80	0.017±0.003 ^d	-29%
LSD		0.0236	

♣ LSD=Least significant difference.

♣ Means with different subscript letter differ significantly ($P < 0.05$)

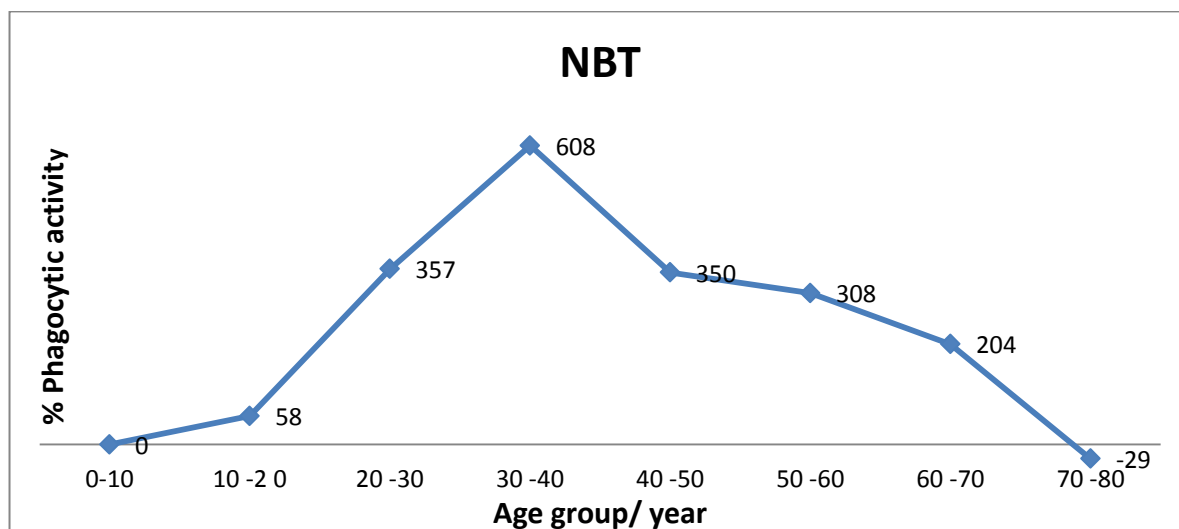


Figure (4-10): Phagocytic activity (%) measured by using NBT, in correlation of the age group/ year.

The NBT test is an indirect marker of the oxygen-depend bactericidal activity of the phagocytes (Dubaniewicz & Hoppe, 2004). NBT is a dye with low reduction potential and performs intensively stained products—formazanes. NBT is easily phagotized by the cells and is reduced to formazane inside mitochondria. The spontaneous NBT test use for screening of metabolic activity of granulocytes and/or monocytes. The induced NBT test assesses the functional abilities of phagocytizing cells. The positive results of NBT reduction test show the percentage of stimulating the cells by bacterial products phagocytes and occurrence of infection. The false positive results of NBT test exist during bacterial infection with the presence of large necrosis or in lymphoblastic leukemia. False negative are present in myeloma (Esfandiari *et al.*, 2003). The NBT reduction test and its modifications are useful for detection and differentiation of bacterial diseases. The sensitivity and specificity in diagnosing the diseases by neutrophilic count and specificity count are observed at 69.8% and 56.2% respectively, and the sensitivity and specificity of the cases by NBT positive cells is observed 72.3%

and 68.4% respectively. Hence the NBT test has high sensitivity and specificity when compared to neutrophilic count (Mohammed and Anunayi , 2014). The study shows in murine macrophages highest ROS induction by *Brucella* rough LPS, lower inductions by *Brucella* combined LPSs and lowest induction with *Brucella* smooth LPS (Akhtar *et al.*, 2012).

4.8 Total and differential count of WBC

The results of total and differential WBC are shown in the Table (4-7). The results between age groups showed significantly ($P<0.05$) different. The total leukocyte count among eight groups as follows (-, 7800, 5300, 6300, 5000, 8500, 5100, and 3600, respectively.) Leukocytosis is not shown in all groups while leukocytopenia showed in group VIII (3600). The total neutrophils count as follows (-, 5800, 1700, 2500, 3500, 4200, 2400, and 1100, respectively). Leukocytosis is not shown in all groups while neutropenia shows in groups III (1700), IV (2500), VII (2400), and VIII (1100). The differential neutrophils count % as follows (-, 68.42, 31.74, 39.38, 71.00, 49.50, 47.00, and 28.60, respectively). Leukocytosis shows in groups II (68.42) and group V (71.00), while neutropenia shows in groups III (31.74), IV (39.38), VIII (28.60).

The total monocyte count is as follows (-, 100, 300, 400, 200, 1100, 500, and 500, respectively). Monocytosis only showed in group VI (1100), while monocytopenia only showed in group II (100). The differential monocyte count % as follows (-, 1.56, 2.50, 2.40, 8.90, 12.40, 14.60, and 14.67 respectively). Monocytosis showed in groups V (8.90), VI (12.40), VII (14.60), and VIII (14.67), while monocytopenia only showed in group II (1.56). The total lymphocyte count as follows (-, 2500, 3100 , 3000, 3000, 3200, 2200, and 2040, respectively). Both lymphocytosis and lymphocytopenia are not shown in all groups. The differential lymphocyte count % as follows (-, 30.28, 58.20, 55.00, 25.00, 38.00, 34.00, and 56.50, respectively). The lymphocytosis showed in

groups III (58.20), IV (55.00), and VIII (56.50), while the lymphocytopenia is not shown in any group.

Table (4-7): Total and differential WBC count correlated to age group/ year.

Groups	Age groups/years	WBC Total	Neutrophils		Monocytes		Lymphocytes	
			Total	Differential	Total	Differential	Total	Differential
		cells/ μ L	cells/ μ L	%	cells/ μ L	%	cells/ μ L	%
I	0-10	-	-	-	-	-	-	-
II	10-20	7800 $\pm$ 583 ^a	5800 $\pm$ 70 ^a	68.42 $\pm$ 0.08 ^b	100 $\pm$ 16 ^e	1.56 $\pm$ 0.05 ^e	2500 $\pm$ 170 ^a	30.28 $\pm$ 0.10 ^e
III	20-30	5300 $\pm$ 70 ^{bc}	1700 $\pm$ 70 ^e	31.74 $\pm$ 0.09 ^f	300 $\pm$ 70 ^{cd}	2.50 $\pm$ 0.07 ^d	3100 $\pm$ 44 ^a	58.20 $\pm$ 0.08 ^a
IV	30-40	6300 $\pm$ 114 ^b	2500 $\pm$ 70 ^d	39.38 $\pm$ 0.05 ^e	400 $\pm$ 35 ^{bc}	2.40 $\pm$ 0.50 ^d	3000 $\pm$ 836 ^a	55.00 $\pm$ 0.65 ^b
V	40-50	5000 $\pm$ 707 ^c	3500 $\pm$ 170 ^c	71.00 $\pm$ 0.70 ^a	200 $\pm$ 35 ^{de}	8.90 $\pm$ 0.06 ^c	3000 $\pm$ 707 ^a	25.00 $\pm$ 1.70 ^f
VI	50-60	8500 $\pm$ 170 ^a	4200 $\pm$ 35 ^b	49.50 $\pm$ 0.07 ^c	1100 $\pm$ 35 ^a	12.40 $\pm$ 0.07 ^b	3200 $\pm$ 70 ^a	38.00 $\pm$ 1.00 ^c
VII	60-70	5100 $\pm$ 35 ^c	2400 $\pm$ 30 ^d	47.00 $\pm$ 0.72 ^d	500 $\pm$ 71 ^b	14.60 $\pm$ 0.08 ^a	2200 $\pm$ 67 ^a	34.00 $\pm$ 0.60 ^d
VIII	70-80	3600 $\pm$ 40 ^d	1100 $\pm$ 32 ^f	28.60 $\pm$ 0.10 ^g	500 $\pm$ 66 ^b	14.67 $\pm$ 0.07 ^a	2040 $\pm$ 51 ^a	56.50 $\pm$ 0.07 ^{ab}
LSD		1032.7	239.26	1.1136	150.93	0.5872	1221	2.4109

Key: Means with different subscript letter in the same column differ significantly (P<0.05)

Hematological indicators in human brucellosis including anemia, leucopenia, neutropenia and thrombocytopenia (Solera, 2000).

The current results show decrease in WBC, neutrophils, and monocyte, increase in lymphocyte in most age groups with human brucellosis. This compatible with Olt et al. who show the total WBC in healthy group 6459.37 $\pm$ 1163.65 compared with brucellosis group 6187.50 $\pm$ 1968.42 at (p value 0.05), the total neutrophil in healthy group 3640.62 $\pm$ 1031.08 compared with brucellosis group 3106.25 $\pm$ 1356.21 at (p value 0.01),

the total lymphocyte in healthy group 2203.12 ± 545.64 compared with brucellosis group 2356.25 ± 664.26 at (p value 0.01) (Olt *et al.*, 2015).

Hematological complications such as anemia and leukopenia have most frequently seen in acute brucellosis cases. However, other hematological abnormalities such as severe thrombocytopenia, pancytopenia, acute hemolytic anemia, and disseminated intravascular coagulation are not infrequent (Buzgan *et al.*, 2010). In another study on 60 children with brucellosis showed anemia (43%), leukopenia (38%), leukocytosis (20%) and pancytopenia (18%) (Mohamad *et al.*, 2014).

The monocytes of patients with untreated brucellosis had significantly depressed phagocytic activities relative to the levels observed in healthy controls, The value for parameter of phagocytic activity of monocytes increased significantly after treatment but did not reach the values found in healthy donors. In the parallel study performed, they found no significant differences between the two study times in the phagocytic activities of monocytes from healthy controls (Rodríguez-Zapata *et al.*, 2010).

4.9 Rose Bengal test

The study is conducted on suspected patients with brucellosis infection in Al-Hussein teaching Hospital of Muthanna Province, 100 samples were all positive rose Bengal test collected from peripheral human blood, The number of positive rose Bengal patient was divided into eight groups according to age as shown in Table (4-8). The number and percentage of positive rose Bengal patient group from (I-VIII) was as follows (0 (0%), 10 (10%), 31 (31%), 25 (25%), 16 (16%), 14 (14%), 2 (2%), and 2 (2%) respectively). The most affected group is group III 31 (31%), while the least affected group is a group I 0 (0%) as see in Figure (4-11).

Table (4-8): The positive rose Bengal test, correlated with age group/ year.

Groups	Age group/ year	No. of samples	Positive Rose Bengal test	%
I	0-10	0	0	0
II	10-20	10	10	10
III	20-30	31	31	31
IV	30-40	25	25	25
V	40-50	16	16	16
VI	50-60	14	14	14
VII	60-70	2	2	2
VIII	70-80	2	2	2
Total		100	100	100

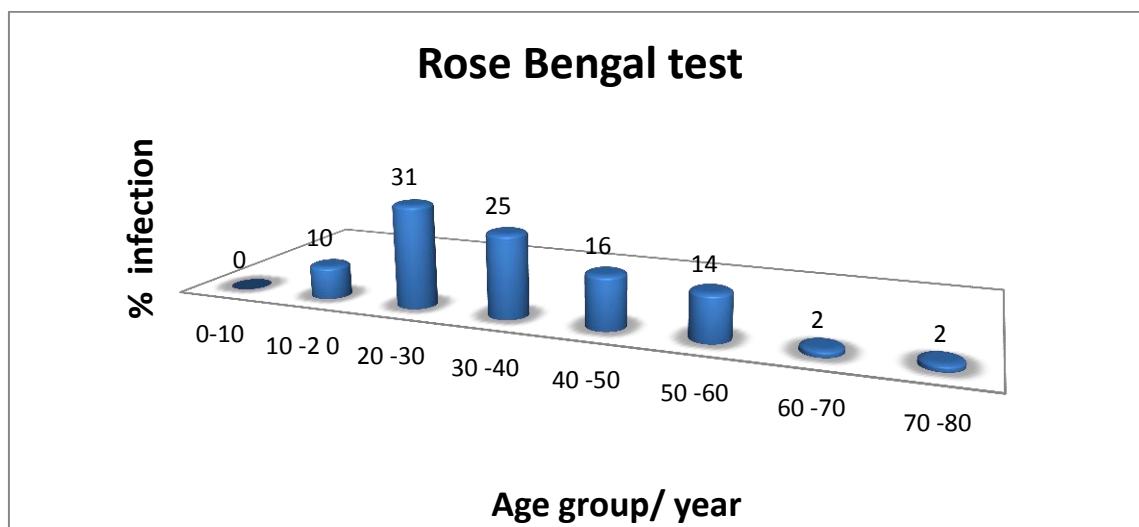


Figure (4-11): Rose Bengal test showed the percentage of positive cases, according to age group/ year.

In the current study used the Rose Bengal test as a first screening test, because it is the single test used to diagnose patients with *Brucella* in Muthanna province. So relied

on the positive samples for this test only and we confirmed the diagnosis by other tests and techniques. This is compatible with several researchers who have recommended using this test as a first screening test (Corbel, 2006; Diaz *et al.*, 2011; Franco *et al.*, 2007). The RB is usually used for brucellosis screening, yet experts propose that the result of this test must be approved by some special tests (Concepción Gómez *et al.*, 2008). This test is easily performed, is fast and has a high sensitivity, within 99% the diagnosis of acute patients. Specificity in RB is low, in the chronic form of infection, the probability of false negative results for this test is high because binding of antigen and antibody with refer to the subsequent effects, means the non-reactive Ag and Ab and the non-specific reaction and may be due to the cross reaction or prozone phenomena (Ruiz-Mesa *et al.*, 2005).

The Rose Bengal Plate (RBP) test in high risk rural areas, in that doing on the serum agglutination test (SAT) is not possible, has been introduced as a screening test and it is highly valuable. However, this test result must also be approved by another tests (Mantur & Amarnath, 2008). The study included 711 brucellosis patients, The mean age of the group was 38.2 ± 17.1 years (range, 14–91 years). The positive likelihood ratios of the rose Bengal test were 16.5, 10.4 and 4.2 for groups I, II and III, respectively (Ruiz-Mesa *et al.*, 2005).

4.10 The 2-Mercaptoethanol test

This test confirms the examination of Rose Bengal test addition, differentiate between chronic and acute infections. Positive 2-Mercaptoethanol test cases were 64 (64%) from 100 positive Rose Bengal test cases, as shown in the Table (4-9) and Figure (4-12). The acute infections were 17 (17%), and chronic infections were 48 (48%) as shown in the Table (4-10) and Figure (4-13). Negative cases were 35 (35%)

Statistical Analysis in Table (4-11) reveal that the 2- mercaptoethanol test has the highest sensitivity (92%), lowest specificity (53%), Positive Predictive Value (PPV) (54%), Negative Predictive Value (NPV) (91%), accuracy (68%), and Kappa coefficient (42%).

Table (4-9): The 2-Mercaptoethanol test results, according to age groups/ years.

Groups	Age group/ year	No. of samples	Positive cases	%	Negative cases	%
I	0-10	0	0	0	0	0
II	10-20	10	7	70	3	30
III	20-30	31	22	71	9	29
IV	30-40	25	15	60	10	40
V	40-50	16	10	62	6	38
VI	50-60	14	9	64.28	5	35.72
VII	60-70	2	1	50	1	50
VIII	70-80	2	1	50	1	50
Total		100	64	64	36	36

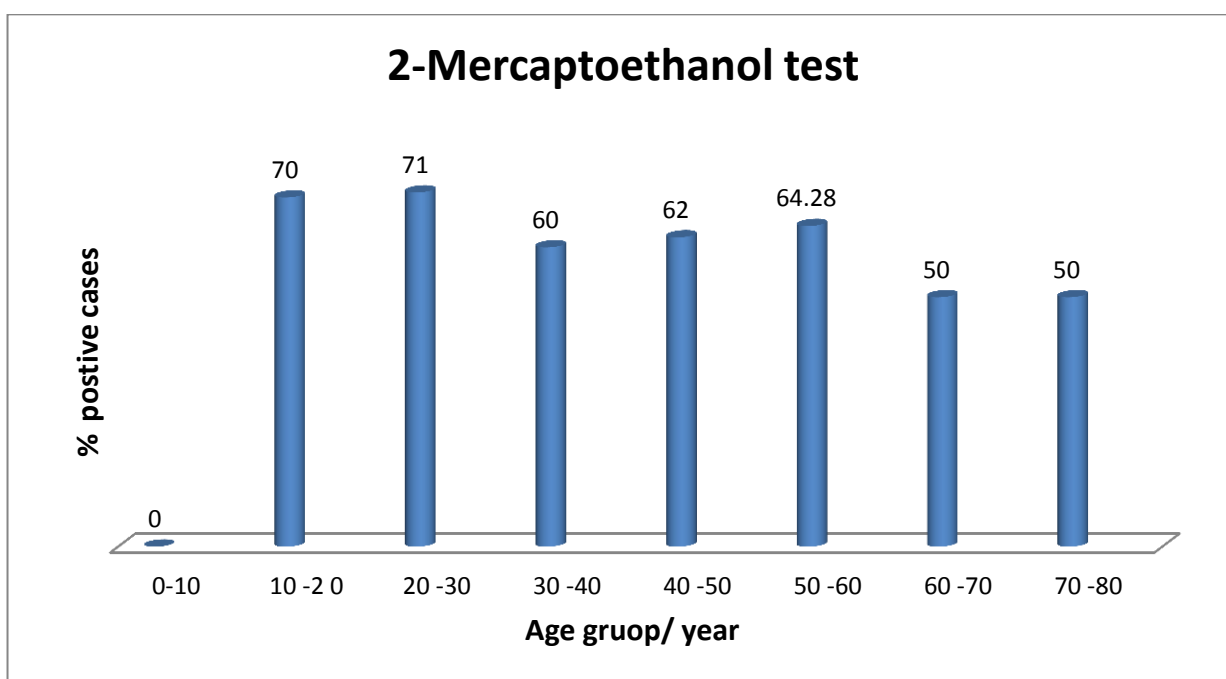


Figure (4-12): The positive cases percentage by 2-mercaptoethanol test according to age groups/ years.

Table (4-10): The acute and chronic infection by 2-Mercaptoethanol test, according to age groups/ years.

No. of groups	Age groups/ years	No. of positive samples	Acute	%	Chronic	%
I	0-10	0	0	0	0	0
II	10-20	7	1	14.29	6	85.71
III	20-30	22	6	27.28	16	72.72
IV	30-40	15	4	26.67	11	73.33
V	40-50	10	3	30	7	70
VI	50-60	9	3	33.33	6	66.67
VII	60-70	1	0	0	1	100
VIII	70-80	1	0	0	1	100

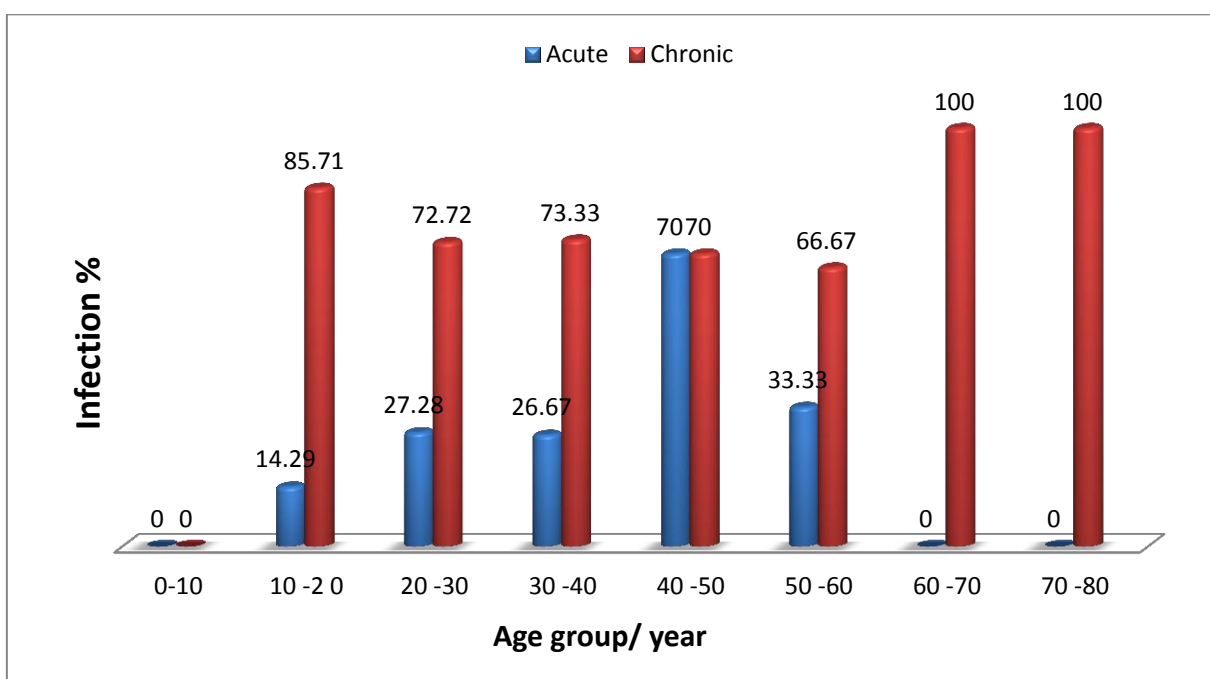


Figure (4-13): The percentage of acute and chronic infection by 2-Mercaptoethanol test, according to age groups/ years.

Table (4-11): Statistical analysis demonstrating a comparison between 2-Mercaptoethanol test and RT-PCR (golden test) results.

		Golden test RT-PCR		
2-ME	Test	+	-	Total
	+	35	29	64
	-	3	33	36
	Total	38	62	100

2-mercaptoethanol effectively denatures the IgM class of antibodies, so if a lessening in the titer is seen in the wake of utilizing this specialist, it implies that the commitment of IgM has been expelled leaving the IgG component. This separation of counteracting agent classes is essential; as it takes into account the qualification of a later from an old

infection (Sendil Kumar & Prakash*, 2016). Because of the killing impact of 2-mercaptoethanol on IgM, 2ME-MAT restrains the serological reaction to *Yersinia enterocolitica* O:9, and also numerous other false positive serological reaction producers (Dashti *et al.*, 2012).

This study revealed that the 2- mercaptoethanol test has the highest sensitivity (92%), lowest specificity (53%), Sensitivity of the 2-mercaptoethanol test varies from 88.4 and 99.6%, and its specificity from 91.5 and 99.8% (Gall & Nielsen, 2004). A positive result with this test is a strong indicator of brucellosis. However, this test has some drawbacks including the toxicity of mercaptoethanol, which requires a fume hood for its manipulation, and the possibility of IgG degradation caused by the 2-mercaptoethanol, which may result in false negative results (Poester & Nielsen, 2010).

4.11 Indirect Enzyme-linked immunosorbent assay (ELISA)

The qualitative immuno- enzymatic determination of IgG-class antibodies against *Brucella* is based on the Indirect ELISA IgG. The cut-off value of developing IELISA IgG was determined using formula mentioned in our materials and methods section, and it was OD value of less than (9) which mean those results negative, higher than (11) will be considered as a positive, and between (9-11) will be considered a suspected.

The results of an indirect ELISA assay exclude the suspected cases (ELISA_{ex}) were 26 (26%) positive cases and 74 (74%) negative cases from 100 positive rose Bengal test patients. The positive cases, according to age groups as follows (-, 3 (3%), 10 (10%), 6 (6%), 4 (4%), 2 (2%), 1 (1%), and 0 (0%), respectively), and negative cases as follows (-, 7 (7%), 21 (21%), 19 (19%), 12 (12%), 12 (12%), 1 (1%), and 2 (2%), respectively) as shown in the Table (4-12) and Figure (4-14).

Groups	Age groups/	No. of	Negative	%	Positive	%
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	years	samples	cases		cases	
I	0-10	0	0	0	0	0
II	10-20	10	7	70	3	30
III	20-30	31	21	69.77	10	30.23
IV	30-40	25	19	76	6	24
V	40-50	16	12	75	4	25
VI	50-60	14	12	85.72	2	14.28
VII	60-70	2	1	50	1	50
VIII	70-80	2	2	100	0	0
Total		100	74	74	26	26

Table (4-12): The results ELISA_{ex} according to age groups/years

Statistical analysis in Table (4-13) reveal that the ELISA_{ex} has the highest specificity (96%), lowest sensitivity (63%), PPV (92%), NPV (81%), accuracy (84%), and Kappa coefficient (26%).

Table (4-13): Statistical analysis demonstrating a comparison between ELISA_{ex} and RT-PCR (golden test) results.

		Golden test RT-PCR		
(ELISA _{ex})	Test	+	-	Total
	+	24	2	26
	-	14	60	74
	Total	38	62	100

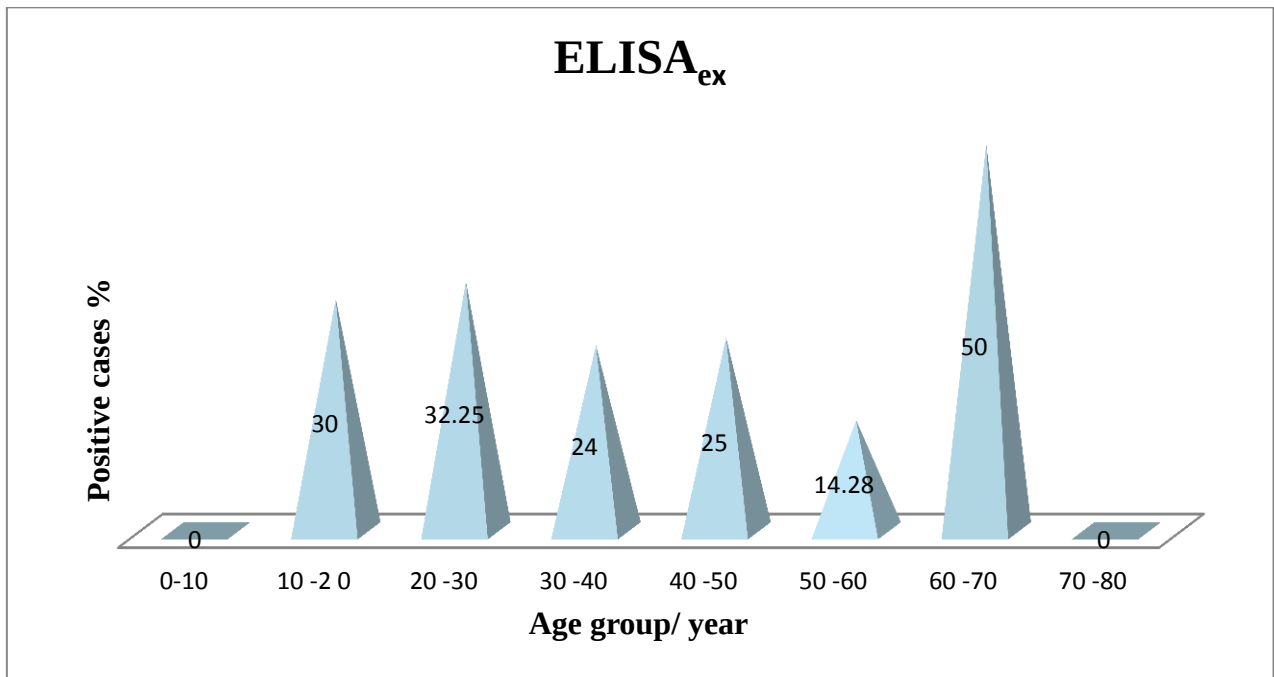


Figure (4-14): The percentage of positive cases by ELISA_{ex}' according to age groups/ years.

The results of an indirect ELISA include the suspected cases (ELISA_{in}) were 41 (41%) positive cases and 59 (59%) negative cases. The positive cases, according to age groups are as follows (-, 4 (4%), 13 (13%), 9 (9%), 7 (7%), 5 (5%), 2 (2%), and 1 (1%), respectively), and negative cases as follows (-, 6 (6%), 18 (18%), 16 (16%), 9 (9%), 9 (9%), 0 (0%), and 1 (1%), respectively). As shown in the Table (4-14) and Figure (4-15).

Table (4-14): The results ELISA_{in} according to age groups/years.

Groups	Age groups/ years	No. of samples	Negative cases	%	Positive cases	%
I	0-10	0	0	0	0	0
II	10-20	10	6	60	4	40
III	20-30	31	18	58.1	13	41.9
IV	30-40	25	16	64	9	36
V	40-50	16	9	56.25	7	43.75
VI	50-60	14	9	64.29	5	35.71
VII	60-70	2	0	0	2	100
VIII	70-80	2	1	50	1	50
Total		100	59	59	41	41

Statistical analysis in the Table (4-15) reveal that the ELISA_{in} has the intermediate estimations for sensitivity (73%) and specificity (79%), PPV (68%), NPV (83%), accuracy (77%), and Kappa coefficient (29%).

Table (4-15): Statistical analysis demonstrating a comparison between ELISA_{in} and RT-PCR (golden test) results.

		Golden test RT-PCR		
(ELISA _{in})	Test	+	-	Total
	+	28	13	41
	-	10	49	59
	Total	38	62	100

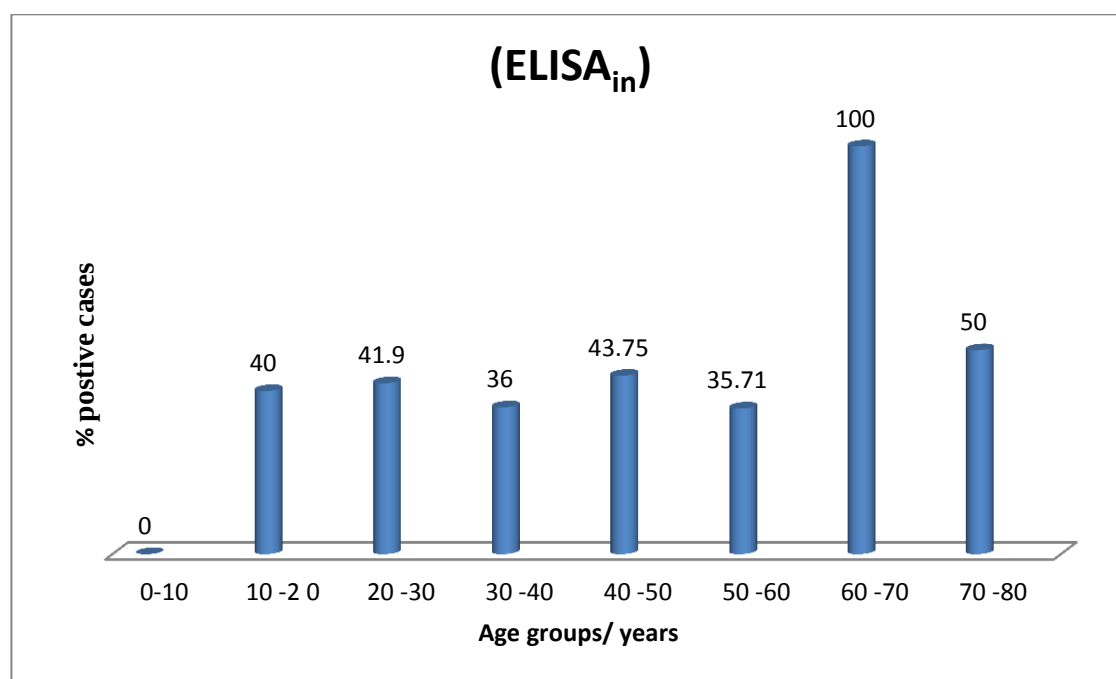


Figure (4-15): The percentage of positive cases by ELISA_{in} according to age groups/ years.

The diagnosis of brucellosis is based on the detection 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; Araj, 2010). Compared to the conventional agglutination methods, ELISA is more sensitive in acute and chronic cases of brucellosis and it offers a significant diagnostic advantage in the diagnosis of brucellosis in endemic areas (Mantur *et al.*, 2010).

In the current study, the indirect ELISA_{ex} IgG has the lowest sensitivity (63%) and highest specificity (96%), while the indirect ELISA_{in} IgG has the intermediate estimations for Sensitivity (73%) and specificity (79%). The sensitivities of ELISA IgG and IgM were 91% and 100%, respectively, while the specificity was 100% for both. These ELISAs are simple, rapid, and reliable for the diagnosis of human brucellosis (Araj *et al.*, 2005). The ELISA IgG test was more reliable than ELISA IgM test in the diagnosis of human brucellosis in Iran. Using a cut-off of 10 IU/ml and 50 IU/ml had a

most sensitivity (92.9%) and the most specificity (100%) for ELISA IgG test, respectively (Soudbakhsh *et al.*, 2009). Results also displayed the use of labeled recombinant protein A/G in IELISA, leads to specificity and sensitivity of 100% and 98.8% respectively which is conformed to other studies using the same labeled reagent (Ali mirjalili & Hesam lotfpuori, 2016).

Concerning ELISA, several studies have shown that it is the test of choice for the diagnosis of complicated and chronic cases, especially when other tests are negative (Cuzzubbo *et al.*, 2000; Niedrig *et al.*, 2001; Aranis *et al.*, 2008; Welch & Litwin, 2010; Fadeel *et al.*, 2011). Moreover, this essay reveals total and individual specific immunoglobulins rapidly (within 3 to 5 hours) and reliably. In addition, ELISA performance surpassed the other tests in the diagnosis of chronic and complicated cases such as patients with neurobrucellosis (Tuncel *et al.*, 2008). However, it cannot be advocated for routine use in the diagnosis of patients with acute brucellosis since the agglutination tests for this condition are as reliable and less expensive than ELISA (Araj *et al.*, 2005).

In the current study, the accuracy of ELISA_{ex} IgG (86%) and an accuracy of ELISA_{in} IgG (83%). This is not compatible with Welch & Litwin who show that the IgG assay tested had an accuracy of (56%) (Welch & Litwin, 2010). This is because of the high reproduction titer of IgG, which represents good opsonizing antibodies in *Brucella* infection.

4.12 Lymphocyte transformation index by MTT assay

The MTT assay is used for evaluating the lymphocyte transformation index % of peripheral blood leukocytes in positive rose Bengal test patients. The results of this study show that there is a significant increase ($P<0.01$) in lymphocyte transformation index % (209) in the lymphocytes of patients positive rose Bengal test, compared with deionized water as a negative control (0%) and (59%) in the lymphocytes of healthy individuals as positive control as it is shown in Table (4-16). According to age groups, results indicated dissimilar noteworthy rises in the lymphocyte transformation index %, (-, 288%, 335%, 307%, 216%, 181%, 105%, and 31%, respectively).

The higher lymphocyte transformation index % was recorded in group III (509.4), while the lowest lymphocyte transformation index % was recorded in group VIII (31) as shown in the Table (4-17) and Figure (4-16). Figure of isolation of lymphocyte from peripheral blood in positive rose Bengal test patients shown in (Appendices B).

Table (4-16): Lymphocyte transformation index for control and patients.

Groups	Mean $\pm$ SE	Lymphocyte transformation index %	P value
Negative control	0.054 $\pm$ 0.0007 ^b	0%	P<0.01
Healthy individuals (Positive control)	0.086 $\pm$ 0.0004 ^a	59%	
Patients	0.167 $\pm$ 0.007 ^a	209%	

Table (4-17): Lymphocyte transformation index for patients, according to age groups.

Groups	Age groups/ years	Mean±SE	Lymphocyte transformation index %
I	0-10	-	-
II	10-20	0.210±0.010 ^a	288%
III	20-30	0.235±0.015 ^a	335%
IV	30-40	0.220±0.011 ^a	307%
V	40-50	0.171±0.006 ^b	216%
VI	50-60	0.152±0.006 ^b	181%
VII	60-70	0.111±0.002 ^c	105%
VIII	70-80	0.071±0.006 ^d	31%
LSD		0.0265	

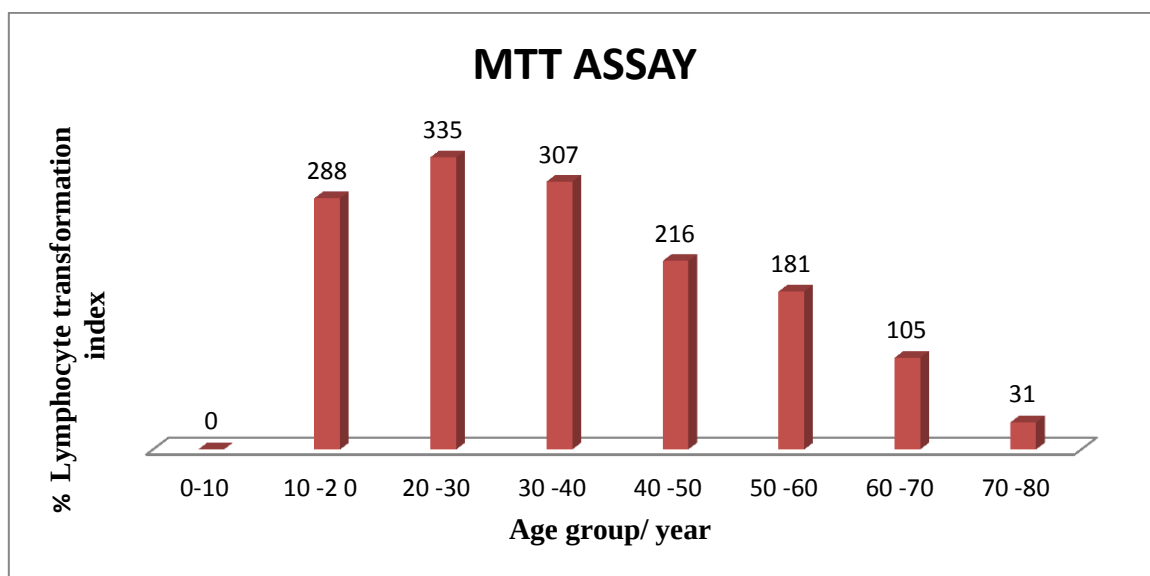


Figure (4-16): Lymphocyte transformation index %, which measured by MTT assay, according to age groups/ years.

The MTT test is a colorimetric test for surveying cell metabolic movement. NAD (P) H-subordinate cell oxidoreductase enzymes may under-characterized conditions, mirror the number of the suitable cells present. These catalysts are fit for decreasing the tetrazolium dye MTT to its insoluble formazan, which has a purple shading (Meerlo *et al.*, 2011).

This colorimetric microplate examines is practical as a result of the number of tests which can be performed at one time without the issue of radioisotopes and debased materials transfer. Uses of the MTT examine the utilization of the colorimetric measure for cell development and cell survival offers real favorable circumstances in velocity, effortlessness, expense, and wellbeing over traditional cell checking tests (Chapdelaine, 2011).

The absence of cytotoxicity was also observed by MTT assay. This technique correlates formazan production with cell viability, since a lower formation of this compound compared with particle-free media would be indicative of cell toxicity. For all formulations, formazan levels were always lower than negative control, indicating the absence of toxicity. Even higher amounts ($P < 0.01$) were detected for cells incubated with 502H microspheres (Lecároz *et al.*, 2006).

The result compatible with research which showed a significant increase in lymphocyte transformation index by MTT assay ($P \leq 0.05$ and $P \leq 0.01$) in Mannoproteins-treated vaccinated mice in comparison with negative and positive groups (Hanan Hassan Ali & Tareq Jaafar Al-Jindeel, 2013).

5. Conclusions and Recommendations

5.1 Conclusions

- 1- Brucellosis is considered an endemic disease in Muthanna city. All age groups are affected. The disease may persist as relapse, chronic localized infection or delayed convalescence. Complications may affect any organ system.
- 2- Transmission to humans occurs through occupational or environmental contact with infected animals or their products Foodborne transmission is a major source of infection, with cheese made from raw milk and unpasteurized milk presenting a high risk.
- 3- In acute brucellosis, isolation of *Brucella* from blood or other tissues is definitive, culture is often negative, especially in long-standing disease.
- 4- Real-time PCR assays are easy-to-use, produce results faster than conventional PCR systems, while reducing DNA contamination risks. The SYBR Green based real-time PCR assay is specific and highly sensitive and appears as an efficient and reproducible method for the rapid and safe detection of the genus *Brucella*.
- 5- The essential element in the treatment of all forms of human brucellosis is the administration of effective antibiotics for an adequate length of time.

5-2 Recommendations

- 1- *B. melitensis* Rev 1 is recommended for prevention of *B. melitensis* infection in sheep and goats.
- 2- The RBT is recommended for screening; ELISA or complement fixation are recommended for confirmation of infection in individual humans. Screening of milk samples by milk ring test or ELISA is useful for surveillance.
- 3- Few fragments are unique among the genomes. *B. melitensis* and *B. abortus* share more sequences than either does with *B. suis*. A comparison of the three genome sequences of *Brucella* gives us a foundation to further understanding of the *Brucella* genus and provides the groundwork to investigate the contribution of various pathways to the relative pathogenicity and virulence of these bacteria. The genome sequences allow construction of general *Brucella* microarrays to observe the dance between microbe and host in understanding the course of brucellosis infection.
- 4- Most of the assays revealed low detection limits and proved to be highly selective for the detection of the genus *Brucella* and the species that are most relevant for humans. Assays targeting the bcsp31 gene can be recommended to screen for *Brucella*. Species-specific assays should be consecutively applied confirming the primary diagnosis by a second gene target.
- 5- The key to effective surveillance is the case definition, reporting, analysis of data and dissemination of information for action. The surveillance program must be designed according to the adopted control strategy.
- 6- This study provides significant primary screening in a number of infections within Muthanna city, so open the way for further studies on the identification of human brucellosis in this city.

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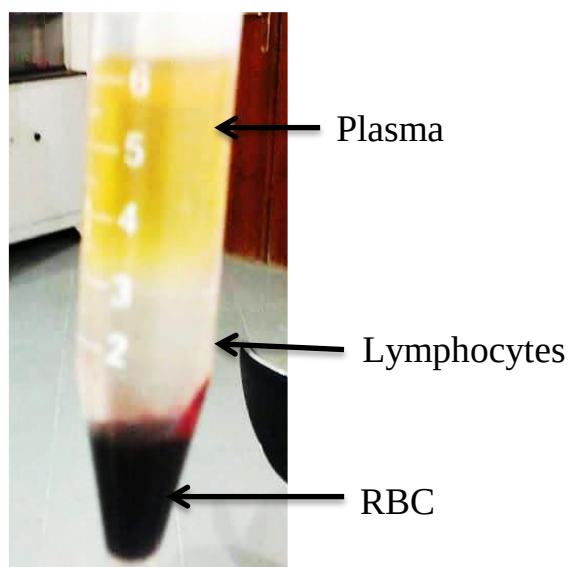
Appendices

Appendices

Appendix A: Api20 E Strep result for *Brucella* isolate

No	Test	Reactions/Enzymes	Result	Color
1	ONPG	β -galactosidase (Ortho NitroPhenyl- β DGalactopyranosidase)	-	Colorless
2	ADH	Arginine dihydrolase	-	Yellow
3	LDC	Lysine decarboxylase	-	Yellow
4	ODC	Ornithine decarboxylase	-	Yellow
5	CIT	utilization of citrate	+	Pale green / yellow
6	H ₂ S	Production of hydrogen sulfide	-	Colorless / greyish
7	URE	Urease	-	Yellow
8	TDA	Tryptophan deaminase	-	Yellow
9	IND	Indole Test	-	Colorless, pale green / yellow
10	VP	Voges Proskauer Test	-	Colorless
11	GEL	Gelatinase	-	No diffusion
12	GLU	Fermentation of Glucose	-	Blue / blue-green
13	MAN	Fermentation of Mannose	-	Blue / blue-green
14	INO	Fermentation of Inositol	-	Blue / blue-green
15	SOR	Fermentation of Sorbitol	-	Blue / blue-green
16	RHA	Fermentation of Rhamnose	-	Blue / blue-green
17	SAC	Fermentation of Sucrose	-	Blue / blue-green
18	MEL	Fermentation of Melibiose	-	Blue / blue-green
19	AMY	Fermentation of Amygdalin (Glycoside)	-	Blue / blue-green
20	ARA	Fermentation of arabinose	-	Blue / blue-green

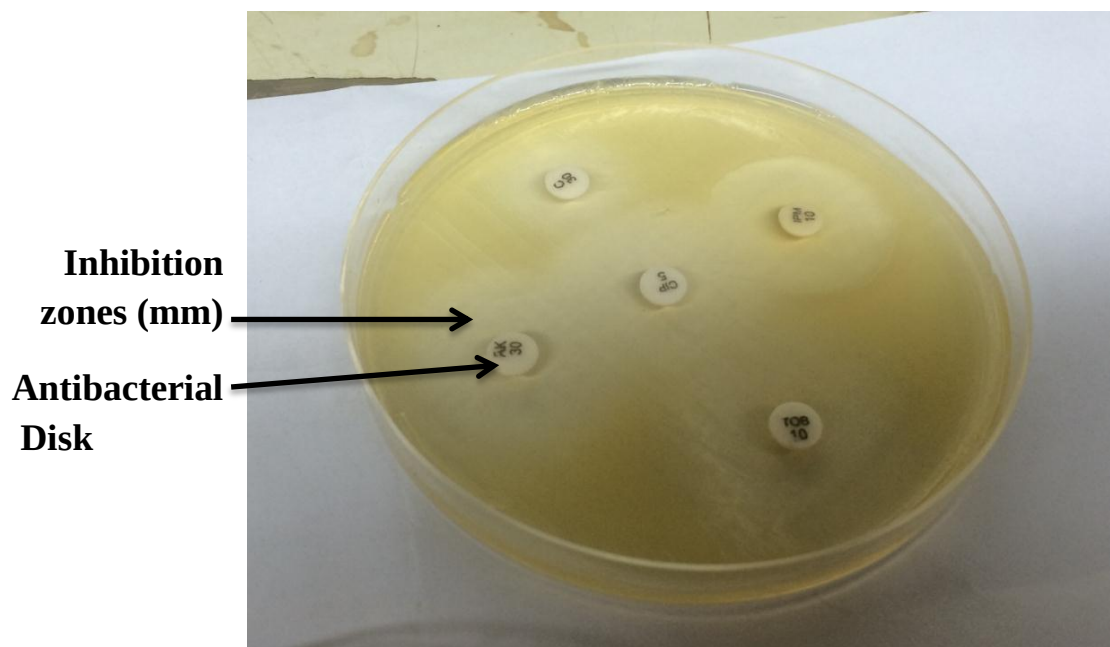
Appendix B: Isolation of lymphocyte from peripheral blood in positive rose Bengal test patients.



Appendix C: Screw-cap glass bottles during bacterial isolation period



Appendix D: Inhibition zones (mm) to antibacterial agents in *Brucella* colonies



Appendix E: Positive result in Catalase test (production of bubbles)



Appendices

الخلاصة

تضمنت الدراسة ١٠٠ عينة كانت موجبة لاختبار الـ روز بنكال (RBT) و ٥٠ عينة من اشخاص اصحاء جمعت من دم الانسان اعتمادا على العمر، الجنس، المنطقة، تناول العلاج والتدخين خلال الفترة الممتدة من كانون الاول/ ٢٠١٥ – تموز/ ٢٠١٦ في مستشفى الحسين التعليمي في محافظة المثنى. البروسيلا المألوية (*Brucella melitensis Rev1*) عزلت بطريقة تكسير الخلايا بالطرد المركزي (Lysis-centrifugation method)، تشخيص هذه العترة انجز بواسطة نظام (Api-20E)، جهاز (Vitek 2)، تفاعل انزيم البلمرة التسلسلي الاعتيادي (PCR)، التتميط المصلي والتتميط الحيوي، هذه العترة المعزولة استخدمت كسيطرة في تفاعل انزيم البلمرة التسلسلي ذات الوقت الحقيقي (RT-PCR). اختبار الحساسية للمضادات الحيوية انجز على المستعمرات النامية لهذه العترة بطريقة الانتشار القرصي، المضادات الحيوية الافضل لبكتريا البروسيلا المالطية عترة (Rev1) هي (Gentamicin and Tobramycin). تقنية تفاعل انزيم البلمرة ذات الوقت الحقيقي انجز على عينات الدنا المستخلصة من عينات دم حديثة موجبة لاختبار (RBT)، ذلك انجز باستخدام (bcsp31 and omp2) كجينات تشخيصية وصبغة (SYBR Green I). النتائج بينت ٣٨ (٣٨%) عينة كانت موجبة و ٦٢ (٦٢%) عينة كانت سالبة لكلا الجينين (bcsp31 and omp2)، لذلك تقنية تفاعل انزيم البلمرة ذات الوقت الحقيقي اعتبرت كاختبار ذهبي من ناحية الخصوصية والحساسية مقارنة بالاختبارات الاخرى في دراستنا. اختبار ٢-ميركاتوبوثانول انجز لتأكيد اختبار (RBT) إضافة الى التفريق بين الاصابات المزمنة والحادة. الحالات الموجبة لاختبار ٢-ميركاتوبوثانول كانت ٦٤ (٦٤%) والعينات السالبة كانت ٣٦ (٣٦%) من العينات الموجبة لاختبار (RBT)، عدد الاصابات الحادة كان ١٧ (١٧%) وعدد الاصابات المزمنة كان ٤٧ (٤٧%)، بحساسية عالية ٩٢% وخصوصية واطئة ٥٣%. اختبار الممتز المناعي المرتبط بالانزيم (ELISA) الغير مباشر للجسم المضاد (IgG) انجز لتشخيص المرضى المصابين بداء البروسيلات، نتائج اختبار الممتز المناعي الغير مباشر بدون الحالات المشكوك فيها ($ELISA_{ex}$) كانت ٢٦ (٢٦%) حالات موجبة و ٧٤ (٧٤%) حالات سالبة بخصوصية عالية ٩٦% وحساسية واطئة ٦٣%. بينما نتائج اختبار الممتز المناعي الغير مباشر مع الحالات المشكوك فيها ($ELISA_{in}$) كانت ٤١ (٤١%) حالات موجبة و ٥٩ (٥٩%) حالات سالبة بتقارير متوسطة من ناحية الحساسية ٧٣% والخصوصية ٧٩%. اختبار زرقة النتروتترازوليوم (NBT) انجز لتقدير فعالية البلعمة في المرضى الذين اعطوا نتائج موجبة لاختبار (RBT)، نتائج اختبار (NBT) اظهرت زيادة ملحوظة ($P<0.05$) في فعالية البلعمة (٢٦٦%) في دم المرضى الذين يملكون نتائج موجبة لاختبار (RBT)، مقارنة مع (١١٢%) في الاشخاص الاصحاء الذين استخدموا كمجموعة سيطرة موجبة. اختبار (MTT) انجز لتقدير نسبة استحالة الخلايا للمفاوية % في الخلايا للمفاوية للمرضى الذين اظهروا نتائج موجبة لاختبار (RBT)، النتائج بينت ان هناك زيادة ملحوظة ($P<0.01$) في نسبة استحالة الخلايا للمفاوية ٢٠٩% في

الخلايا اللمفاوية للمرضى الذين اظهروا نتائج موجبة لاختبار (RBT)، مقارنة مع (٥٩%) في الاشخاص الاصحاء الذين استخدموا كمجموعة سيطرة موجبة. العد الكلي والنسبي لكريات الدم البيضاء قدر بواسطة جهاز (Sysmex/japan)، النتائج اظهرت نقصان في كريات الدم البيضاء، الخلايا العدلة، الخلايا الوحيدة، وزيادة في الخلايا اللمفاوية في مستوى معنوية ($P<0.05$) في اغلب المرضى الذين اظهروا نتائج موجبة لاختبار (RBT).



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الكشف المناعي والجزئي للمرضى المصابين بالبروسيلة في محافظة المثنى

رسالة

مقدمة إلى مجلس كلية العلوم- جامعة المثنى كجزء من متطلبات
نيل درجة ماجستير/ علوم/ علوم حياة/ أحياء المجهرية

من قبل

أحمد شايح كريم الجياشي

بكالوريوس علوم الحياة ٢٠١٤

إشراف

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