Sudan University of Science and Technology
College of Graduate Studies

Development of National Diagnostic Standard Operating Procedures (SOPs) for Isolation, Identification and Typing of Brucella Isolates

تطوير طرق التشخيص القومية القياسية المرجعية لعزل وتعريف وتصنيف معزولات البروسيلا

By
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DEDICATION

TO

DEAR HUSBAND,

MY COMPATINATE PARENTS,

LOVELY SISTERS, BROTHERS, AND FRIENDS

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First of all, I would like to thank **ALLAH** who gave me the ability to finish this thesis.

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Abstract

Brucellosis is primarily a disease of animals which is transmitted to man either directly or indirectly, and it continues to be a zoonosis of worldwide public health and economic importance. It is caused by 10 known species of the genus *Brucella*. The causative bacterium was named in honor of Sir David Bruce the discoverer of *Brucella melitensis.*

This research done at Department of *Brucella* in Central Veterinary Research Laboratories (CVRL). The main objective of this research was to develop a national Standard Operating Procedures (SOPs) for isolation and characterization in the Brucella Laboratory. SOPs are sets of step-by-step instructions compiled by the laboratory scientists to help workers carry out routine operations. SOPs aim to achieve efficiency, quality output and uniformity of performance and regulations which should be in alignment with the international standards. This research focused on studying the methods of diagnosis of *Brucella* in the laboratory by isolation, classification and characterization using different culture media. Typing of *Brucella* isolates was carried out by both classical biochemical identification and advanced Polymerase Chain Reaction (PCR) test. In this study two isolates were used successfully isolated from suspected samples which were knee joint hygromal fluid from a bovine and blood sample of a febrile patient who did not respond

to antibiotic or anti malarial therapy. In colony counts, cultured the isolates in the enriched liquid media, which were Brain-Heart Infusionbroth, potato infusion broth, nutrient and Laurel Treptose under anaerobic incubation condition, the minimal number of the bacterial colonies was calculated as one colony per one ml of media. The ecometric tested Six solid nutrient culture media, which were potato agar, Tryptose, Tryptose soya agar, Thyr Martin agar, Serum dextrose agar and blood agar for growth. The Biochemical methods were used including, anaeropic incubation, production of H_2S, growth in media which contain Thionine 2%, SafraninO 5% and Basic fuchsin 2% , testing the colonies with mono specific anti sera A and M (for *B. abortus* and *Brucella melitensis*). Finally the growth of the two isolates was tested in the presence of five *brucella* phages. The two isolates were typed by both conventional methods and PCR were *Brucella abortus* biovar 6 from the knee hygromal and *Brucella melitensis* biovar 1 from the human blood sample..

In Ecometric technique the isolates were 100% growth in all nutrient media under anaerobic incubator of 37^{o}C for 3-5 days. In aerobic incubation the growth of *Brucella abortus* was 55%-85%, the best media was blood agar and Tryptose soya agar followed by Tryptose agar. The growth of *Brucella melitensis* in agar medium at 37^{o}C for 3-5 days was lower and to varying degrees from 25%-30%.

. In colonies count, the best medium for growth of *Brucella abortus* was potato broth followed by Brain heart infusion, the best medium for growth of *Brucella melitensis* was Brain heart infusion followed by followed by potato broth The results showed that all media supported the propagation of the *brucella* cells . In biochemical tests the anaeropic incubation helped the rapid growth of two *Brucella* isolates sulfur dioxide was found to be produced from *B.abortus* and not from *Brucella melitensis*. In dyes showed that *Brucella*

abortus did not grew in the solid media containing the three dyes where as *brucella melitensis* grew in all media containing the three dyes. In mono specific anti sera A and M gave the classical picture of the two isolates. *B. abortus* positive result with mono specificanti sera(A) and negative result with anti-sera (M), *B. melitensis* was positive result with mono specific (M) anti sera and negative result with (A) anti sera. In bacterio phages lysed the colonies of the *B. abortus* isolate except the phage rough canis which gave negative result.All five phages fail to lyse the *Brucella melitensis* colonies. In DNAs extracted from the isolates were amplified using a species specific AMOS cocktail. each isolate was amplified alone, the use of the multiplex primer PCR as a rapid and differentiating screening test was illustrated. When PCR technique was used using AMOS primers (*Abortus*, *Melitensis*, *Ovis*, *Suis*)، the result of isolated samples was *Brucella abortus* from animal isolate and *brucella melitensis* from human isolate. On conclusion the type of solid in ecometric test and broth media support and improved the best growth of two isolates proved to be of value in isolation and identification and typing of *brucella* species.

ملخص الأطروحة

الحمى المالطية هو مرض يصيب الحيوانات وينتقل إلى الإنسان بشكل مباشر أو غير مباشر ، ولا يزال من الأمراض الحيوانية المنشأ ذات الأهمية الاقتصادية والصحية العامة في جميع أنحاء العالم وهو ناتج عن 10 أنواع معروفة من جنس البروسيلا. تم تسمية البكتيريا المسببة تكريما للسير ديفيد بروس مكتشف البروسيلا المالطية.تم إجراء هذا البحث في قسم البروسيلا فيمركز المعامل والبحوث البيطرية المركزي. الهدف الرئيسي من هذا البحث هو تطوير إجراءات تشغيل معيارية وطنية للعزل والتوصيف في معمل البروسيلا. إجراءات التشغيل الموحدة هي مجموعات من التعليمات خطوة بخطوة التي جمعها علماء المختبر لمساعدة ال عاملين على تنفيذ العمليات الروتينية. تهدف إجراءات التشغيل الموحدة إلى تحقيق الكفاءة وجودة المخرجات وتوحيد الأداء واللوائح التي يجب أن تتماشى مع المعايير الدولية. ركز هذا البحث على دراسة طرق تشخيص مرض البروسيلا في المختبر عن طريق العزل والتصنيف والتوصيف باستخدام وسائط الاستزراع المختلفة. تم إجراء تصنيف عزلات البروسيلا عن طريق كل من التعرف البيوكيميائي الكلاسيكي واختبار تفاعل البلمرة المتسلسل المتقدم.

في هذه الدراسة تم استخدام معزولتين تم عزلهما بنجاح من العينات المشتبه بها وهي سائل مفصل الركبة مأخوذ من عينة بقرية وعينة دم لمريض مصاب بالحمى لم يستجيب للعلاج للمضادات الحيوية أو العلاج المضاد للملاريا. تم تصنيف المعزولات بالطرق التقليدية و بتفاعل البلمرة المتسلسل على أنها البروسيلا المجهضة العترة 6 من سائل الركبة والبروسيلا المالطية العترة 1 من العينة البشرية. تم استخدام ستة وسائط لزراعة المغذيات الصلبة ، وهيآجار البطاطس، التربتوز آجار ، آجار الصويا التربتوز، آجار ثير مارتن ، آجار سكر الديكستروز والمصل وآجار الدم. تم إخضاع جميع الوسائط الصلبة للاختبار القياسي البيئي. تم حساب المستعمرات المعزولات في الوسط السائل الم غذي، والتي كانت عبارة عن مستخلص أغشية الدماغ والقلب، ومرق البطاطس، وخلاصة الحساء ولوريل تريبتوز وذلك تحت ظروف الحضانة اللاهوائية، تم حساب أقل عدد من المستعمرات البكتيرية على أنها مستعمرة واحدة لكل 1 مل من الوسائط الغذائية وأظهرت النتائج أن عدد المستعمرات البكتيرية في جميع الوسائط تدعم تكاثر خلايا البروسيلا. بناءً على عدد الطوائف

تم استخدام الطرق البيوكيميائية وهي إنتاج غاز كبريتيد الهايدروجين والنمو في الوسط الذي يحتوي على الثايونين بنسبة 2% و سفرانين O 5٪ و الفوكسين القاعدي 2٪ ، واختبار المستعمرات بمضاد أحادي النوع A و M (للبروسيلا المجهضة و البروسيلا المالطية). أخيرًا تم اختبار نمو هاتين المعزولتين في وجود خمس عاثيات للبروسيلا. عند استخدام تقنية الإختبارالقياسي البيئي كانت النتيجة

نمواً بنسبة 100٪ للعزولتين في جميع أوساط المغذيات تحت حاضنة لاهوائية بدرجة حرارة 37 درجة مئوية لمدة 3-5 أيام. في الحضانة الهوائية كان نمو البروسيلا المجهضة 55٪ - 85٪، أفضل وسط هو أجار الدم وأجار التربتوز الصويا يليه أجار التربتوز. كان نمو البروسيلا المالطية في وسط أجار عند 37 درجة مئوية لمدة 3-5 أيام أقل وبدرجات متفاوتة من 25٪ إلى 30٪. عند حساب مستعمرات المعزولات في الوسط السائل المغذي أفضل وسط لنمو البروسيلا أبورتس هو مرق البطاطس يليه مرق القلب والدماغ ، وأفضل وسط لنمو البروسيلا المالطية هو مستخلص أغشية القلب والدماغ يليه مرق البطاطس.

في الفحوصات البيوكيميائية وجد ان للحضانة اللاهوائية ساعدت على النمو السريع للعزولتين من البروسيلا. ووجد أن ثاني أكسيد الكبريت ينتج من البروسيلا المجهضة وليس من البروسيلا المالطية. عند استخدام الأصباغ الثلاثة، البروسيلا المجهضة لم تنمو في الوسط الصلب المحتوي على الأصباغ الثلاثة حيث نمت البروسيلا المالطية في جميع الأوساط المحتوية على هذه الأصباغ. أعطت نتائج مضادات المصل أحادية النوع A و M الصورة الكلاسيكية للمعزولتين، أعطت البروسيلا المجهضةنتيجة موجبة بمضاد المصل أحادي النوع (A) ونتائج سلبية بمضاد مصل (M)، وكانت نتيجة البروسيلا المالطية موجبة بمضاد المصل أحادي النوع (M) وسلبية مع مضاد المصل احادي النوع (A). قامت العاثيات الجرثومية بتحليل مستعمرات معزولة البروسيلا المجهضة ماعدا العاثية الكلبية الخشنة التي أعطت نتيجة سلبية. فشلت جميع العاثيات الخمس في تحليل مستعمرات البروسيلا المالطية. عند استخدام الحمض النووي المستخلص من المعزولات باستخدام بادئات التفاعل في كوكتيل AMOS الخاص ب أنواع البروسيلا المجهضة، المالطية، الغنمية والخنزيرية . كل معزولة بمفردها.وضح أن إستخدام تفاعل البلمرة المتسلسل التمهيدي المتعدد كاختبار فحص سريع و تفريقي. عند إستخدام تقنية الحمض النووي باستخدام بادئات AMOS ، كانت نتيجة العينات المعزولا ت هي البروسيلا المجهضة من المعزولة الحيوانية والبروسيلا المالطية من المعزولة البشرية. وفي الختام استخدام أنواع الأوساط الزراعية الصلبة في تقنية الإخبار القياسي البيئي (الايكومترك) والأوساط الغذائية السائلة دعمت وحسنت النمو الأمثل المعزولتين وكانت ذات قيمة في عزل ووصف العينتان. الاختبارات البيوكيميائية وتقنية الحمض النووى، حسنت تحديد نوع البروسبلا .

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CHAPTER ONE

1.1 INTRODUCTION

Brucellosis is endemic in many developing countries and is caused by many species of the genus *Brucella* that affect man, domestic and some wild animals, and marine mammals (Geresu *et al*., 2016). It is estimated at 500 000 new human cases of brucellosis annually worldwide (Pappas *et al*., 2006). Brucellosis is the most important zoonosis and has gained prominence over the years since its discovery on the island of Malta (Abubakar *et al.*, 2012). Brucella species are gram negative cocci bacilli, which are classified into species by various techniques such as growth patterns on media and phage susceptibility. There are six "classical" recognized species; *B.abortus, B. melitensis, B. suis, B. ovis, B. canis,* and *B.neotomae* (Hadush and Pal, 2013). lately, four new Brucella species have been recognized and classified, namely, *B. pinnipedialis, B. ceti, B. microti , B. inopinataand B.papionis* (Foster *et al*., 2007; Scholz *et al.*, 2009 and Whatmore *et al*., 2014).The disease in humans is also known as "undulant fever" or "Malta fever," since the first isolation of the causative bacterium was made in the Mediterranean island of Malta from spleens of British soldiers who became infected as a consequence of drinking contaminated caprine milk. The most common Brucella species infecting humans are *B. melitensis, B. abortus and B. suis*. Among these, *B. melitensis* and *B. suis* are the most aggressive species for humans. These bacteria cause a severe syndrome, which if not treated may lead to death. *B. canis* and the marine Brucella strains have been reported sporadically to infect humans. *B. ovis* and *B. neotomae* have not been detected in humans. The marine strains may represent, however, a real hazard for human communities that hunt and gain continuation from whales and seals and for workers that are in close contact with marine mammals (Higgins, 2000).

Standard Operating Procedures (SOPs) provide employees with a reference to common practices, activities, or tasks. New employees use a SOP to answer questions without having to interrupt supervisors to ask how an operation is performed. The international quality standard ISO 9001 essentially requires the determination of processes (documented as standard operating procedures) used in any manufacturing process that could affect the quality of the product, Raytheon (2006).

Diagnostic techniques including visualization of the organism in samples, culturing and conduction of different tests always important in discovering the presence of infection in animals and could be confirmed by isolation the organism from aborted fetuses, vaginal secretions, blood, hygroma fluid and milk of infected animals (Godfroid *et al.*, 2010; Alton *et al.*, 1975). Vaginal swab taken immediately after abortion is the ideal source of Brucella. Also, fetal membrane, stomach contents and tissues are ideal materials of isolation. The infected animals secrete organisms in milk; hence, it can also be used for isolation. Selective enrichment is usually performed for body fluid samples. Aborted material is usually a rich source of bacteria thus can be directly inoculated on selective agar for isolation.

1.2 The main Objectives were:

The general objective of this research was to carryout simple applicable diagnostic Standard Operating Procedures for isolation and identification of Brucella organisms from field samples in Sudan.

1.3 The specific objective

• Isolation of *Brucella* sp in different type of culture media and selective media.

• Identification and typing of *Brucella* using different tests macroscopic appearance (phase ,crystal violet and characteristic of *Brucella* colonies),

microscopic appearance (Smear staining by Gram and Zeihl-Neelsen stain) ,growth in dyes (Thionin, Basic Fuchsine and Safranine0),Production of H_2S, growth in partial CO_2, Phage lysis and Agglutination in Mono-specific Anti sera.

• Maintenance of *Brucella* isolates, by different procedure short term and long term .

• Application of Nucleic Acid Recognition method for Identification of Brucella species (PCR).

CHAPTER TOW

1.LITERATURE REVIEW

2-1 Definition:

Brucellosis is a term, which is used for bacterial infection by any species of the genus *Brucella* .it most commonly affected cattle, sheep, goats and camel in sexually mature animals. Brucellosis is largely a reproductive disease with economic losses associated with abortion, infertility, decreased milk production and impairment of livestock international trade(EuropeanCommission,1999and international Air transport Association,2003), contact with animals, low awareness, and poor hygienic conditions which favor infections (Schelling *et al*., 2003).

Brucella melitensis strain Rev.1 has the normal properties of a biovar 1 strain of *B. melitensis*, but develops smaller colonies on agar media, does not grow in the presence of basic fuchsine, Thionin (20µg/ml) or benzyl penicillin (3µg/ml) (final concentrations), but does grow in the presence of streptomycin at 2.5 or 5 µg/ml (5 IU/ml). (OIE 2009).

Brucellosis is an infectious, contagious, and worldwide spread form of an important zoonosis disease caused by bacteria of the genus *Brucella*. In animals, the disease primarily affects cattle, sheep, goats, swine, and dogs, and is characterized by abortion or infertility and affects people and other animal species (Ray and Steele, 1979). In human-beings, the disease is characterized by intermittent fever, chills, sweating, headache, myalgia, arthralgia, and a diversity of nonspecific symptoms (Young and Corbel, 1989).

2-2 The genus *Brucella*:

Members of the genus *Brucella* are classically defined as facultative intracellular pathogens, which cause infectious disease of the genitourinary tract of sheep, goats, pigs, cattle, dogs and other animals. Human beings

usually acquire the disease through contact with infected animals or their product .This definition does not honor their true nature, which is better understood as facultative extra cellular intracellular parasite. Which means that the *Brucella* preferred place is the intracellular environment of host cells. This environment sustains extensive replication, allowing bacterial expansion and the subsequence transmission to new host cells (Moreno and Morayon, 2002).

The ability of *Brucella* spp. to successfully survive and replicate within different host cell explains their Pathogenicity .Extensive in replication of Brucella spp .In placental trophoblasts is associated with abortion in there animal preferential hosts ,and persistence in macrophages lead to chronic infections that are at hallmark of brucellosis in both natural animal host and humans (Roop *et al.*,2009).

2.3 Taxonomy:

Based on the sequence of processes in establishing the systematic the genus i.e. strain description, identification key and hierarchical ordering of the organisms into a classification scheme according to know and observed relationships, taxonomy is a dynamic and ongoing rather than static process. Verger (1985) proposed that the nomenclature of Brucella to be changed to reflect the closeness or DNA-DNA homology, and the genus be considered as one species, *B.melitensis*. Thus *B.abortus* would become *B.melitensis* biovar *abortus*. DNA polymorphism, as determined electrophoretically, was done on the genus *Brucella*. All the five species examined (*B.abortus, B.melitensis, B.suis, B.ovis* and *B.canis*) had a species specific DNA fingerprint. The electrophoretic DNA fingerprints of polymorphism unexpectedly confirm the natural host as phenotypic characteristic for classifying *Brucell*. Four of the five species arose from acommon ancestor, and that *B.canis* can be considered an authentic strain recently evolved from this species (Bellis and Roizes, 1988).

Brucellosis has been recognized as a febrile illness of the Mediterranean region since antiquity. *B. melitensis*, the main etiologic agent of brucellosis in small ruminants, was the first species in the genus brucella described. It was first isolated by Bruce in 9 July 1887. He discovered the microbial cause of the disease in the island of Malta and named the causative bacterium Micrococcus melitensis Bruce (1888).

The organism infected goats, and transmission to humans occurred by drinking raw goat milk. Bang (1897) described the "agent of contagious abortion" in cattle in Denmark. In the United States, Evans (1918) demonstrated the similarity between the bacterium of Bang and M. melitensis. Meyer and Shaw (1920) proposed the genus *Brucella* containing two species, *B. abortus* and *B. melitensis*.

The third agent of brucellosis was isolated by Traum (1914) in the United States, and Huddleson (1929) named the organism *B. suis* after its natural host, the pig. Buddle (1956) reported *B. ovis* as the cause of reproductive disease in sheep. Stoenner and Lackman, (1957) isolated *B. neotomae* from the desert wood rat in Utah. Carmichael and Bruner, (1968) first recognized canine infections caused by *B. canis*, and human infections caused by this organism were reported soon afterwards.

The isolation of distinctive strains of *Brucella* from marine mammals has extended its ecologic range. *Brucella ceti* and *Brucella pinnipedialis* were found to infect a wide range of sea mammals namely seals, sea lions, walruses, dolphins, porpoises, whales and an otter (Foster *et al*, 2007). This organism appears to be widespread in marine mammals, and has probably been endemic in these populations for a long time. Afew infections have been associated with placentit is, abortions, neonatal mortality, meningoencephalitis, abscesses or other syndromes, but *Brucella* has also been isolated from normal tissues and asymptomatic animals. Marine mammal isolates of *Brucella* can infect terrestrial mammals, but the

frequency of this event is unknown. Some polar bears, which feed on marine mammals, are seropositive for Brucella, and there are concerns about possible impacts on this species. Experimental infections in cattle and sheep have been described. Rare human infections have also been documented. One marine mammal isolate caused acute brucellosis in a researcher. Three other infected people had no occupational exposure to marine mammals; two individuals had neurological signs, and the third developed spinal osteomyelitis. (The centre of food security and public health, 2009)

Another newly emerged novel species of the genus *Brucella* named *Brucella microti*, which had been originally isolated from clinical specimens of diseased wild common voles (Microtusarvalis) during a recent epizootic in the Czech Republic and subsequent isolation of the same species from soil (Scholz *et al*., 2008 a and b).

2.4 Structure of *Brucella*:

Brucella are gram-negative bacteria coco bacilli measuring about 0.6 to1.5 µm by 0.5 to 0.7µm they are non-spore forming, lack capsules or flagella and therefore, are non-motile. The outer cell membrane closely resembles that of other gram-negative bacilli with the dominant lipoly-saccharide (LPS) component and three main groups of proteins. The guanine-plus-cytosine content of the DNA IS 55-58 moles/cm .no Brucella species has been found to harbor plasmids naturally although they readily accept broad-host-range plasmids (young *et a*l 1989).

2.5 Characteristics of *Brucella*:

Brucella cultures have a tendency to undergo variation during growth. Changes in the colonial morphology of a culture are associated with changes in infectivity and antigenicity. Smooth colonies of smooth Brucella species are usually pathogenic, whereas rough colonies of same

have less infectivity and lack the antigenic characteristics of smooth colonies (Alton *et al.*, 1988)

2.5.1 Smooth colonies

Appear round, convex, shiny colonies with an entire margin, honey colored and transparent when viewed by transmitted light (Alton *et al.*, 1988).

2.5.2 Rough colonies:

Have a dry, dull, granular surface and reddish-yellow to yellowish-white in color (Alton *et al.*, 1988).

2.6 *Brucella. abortus*:

It is an intracellular bacteria, which means that it does not replicate outside the host organism. This bacterium, as an intracellular pathogen, enters phagocytes, such as macrophages, in humans and in cows. It attaches to the endoplasmic reticulum of these cells. Edgardo Moreno Dagger and Ignasio (2002) These smooth bacteria enter macrophages and then live in compartments of vacuolar space along the ER. The few cells that make it to these vacuolar spaces down regulate apoptosis genes within the macrophage and therefore cause the cell to resist self-death and these pathogens become resistant within these cells of the immune system. These resistant bacterium are what go on to cause chronic disease in human hosts. In bovine species the bacteria also infects the trophoblast epithelial cells, which are the cells that provide nutrition to the embryo . Yongqun He, (2006)

After a number of rounds of cellular replication in the trophoblast the cells lyse, causing more bacteria cells to enter the blood stream of the developing embryo . Detilleux, Philippe G., (1990). "These cells in the blood stream go on to colonize the placenta and fetus in pregnant female cows, and will go on to induce abortion of the fetus . Félix J. Sangari (2000).

Though *Brucella abortus* is an intracellular bacterium it can remain alive outside the host without replicating. This bacterium can remain in the excrement of cattle and the aborted fetuses of the cattle for quite some time depending on the exact conditions; thought the average time is around 30 days. Outside the host the bacteria cells are affected by direct sunlight; the pathogen can be eliminated by pasteurization, and can be killed by disinfectants. Edgardo Moriyón Dagger and Ignasio (2002).

2.7 *Brucella melitensis*:

It is usually more severe. Brucellosis is a zoonotic disease and its spread in man is affected by its prevalence in animals (Elzer, 2007). Information about the prevalence of the disease in man in the Sudan is scarce as it is usually misdiagnosed and even diagnosed cases are not usually recorded.

The (OIE) is mandated to develop the standards, guidelines and recommendations to facilitate trade in animals and their products. In the case of a azoonotic disease such as *B. melitensis* infection in small ruminants, OIE believes that protection of human health must be achieved through the control of the disease in the animal population. Vaccination of the population has to be considered as the best option for the control of the disease (FAO, 2009).

Brucella melitensis strain Rev.1 vaccine has been used worldwide and its significant value in protecting sheep and goats in endemic areas is recognized (OIE, 2009), according to the OIE standards it is the reference vaccine for control of brucellosis in small ruminants. Accordingly *B. melitensis* Rev1 vaccine is a critically needed in the Sudan for the control of brucellosis in small ruminants and even in camels where the disease is caused by *Brucella melitensis.*

2.8 Transmission:

The disease could seriously impair socio-economic development for livestock owners, which represent a vulnerable sector in rural populations in general and pastoral communities in particular. It has a significant public health implication for a pastoral community in consequence of lifestyles, feeding habits, close contact with animals, low awareness, and poor hygienic conditions which favor infections (Schelling *et al*., 2003).

From public health viewpoint, brucellosis is considered an occupational disease that mainly affects slaughterhouse workers, butchers, and veterinarians. Transmission typically occurs through contact with infected animals or materials with skin abrasions. Symptoms in human brucellosis can be highly variable, ranging from non–specific, flu-like symptoms (acute form) to undulant fever, arthritis, orchitis and epididymitis (Gul and Khan, 2007).

2.9 Pathogenicity of Brucellosis:

Brucella enter the body through abrasions of the skin and directly through the mucosa or conjunctiva. Following entry, Brucellae invade the mucosa, after which phagocytes ingest the organisms. They are ingested by neutrophiles where they reproduce, lyses of the host cell, and are liberated into the blood. They are then phagocytized by the reticulo endothelial cells of the spleen, liver, and bone marrow. Pathogenesis depends upon strain virulence and the status of the host immune response (Elberg, 1973). Intracellular Brucellae are protected from serum antibody and from some antibiotics. This aspect of their life cycle results in a latent phase of disease with gradual release of endotoxin into the blood producing relapsing fever and eventually resulting in the hypersensitivity of chronic disease. Brucellae survive in macrophages for weeks or months, contributing to their hematogenous spread throughout the body and to subsequent

production of granulomas of liver, spleen, lymph nodes, bone marrow, and lung. Abscesses may persist for years but small granulomas usually resolve or calcify. Granulomas contain epithelioid cells, lymphocytes, and multinucleated giant cells. Chronic brucellosis may result in abscesses of subcutaneous tissue, testes, epididymis, ovary, gall bladder, kidney, and brain. Meningitis and endocarditis are markedly frequent complications (Mousa *et al.*, 1986 and Al-Kasab *et al*., 1988).

Infection of the supraspinatus bursa, also known as fistulous withers, is the most common manifestation of brucellosis in horses. The withers are swollen and painful, and purulent discharge may occur. Osteomyelitis (bone infection) of the spinous processes of the thoracic vertebrae may occur, requiring removal of infected bone and tissue. Infection of the supraatlantal bursa, also called poll evil, is a less common syndrome of brucellosis. Brucella has also been reported as a cause of recurrent uveitis, abortion, and orchitis, but horses appear to be more resistant to infection than cattle, swine, and goats (MacMillan and Cockrem, 1986).

Little is known about the pathological changes in camels. Gross lesion may be found in the predilection sites uterus, udder, testicles, lymph nodes, joint bursa and placenta. Hydrobursitis was often observed in brucellosis positive dromedaries causing swelling of the bursa (Werney and Kaaden(2002)

Clinical features can be diverse and the course of the disease is variable. There may be an acute or insidious onset.(Agab 1997) found that the most animal products (WHO, 1997).clinical signs associated with brucellosis in cattle were carpal hygroma, fever, septicemia, abortion, mastitis and weakness accompanied by weight loss. The predominant symptoms in natural infected sheep and goats are abortion, stillbirth, and the birth of weak off springs (OIE, 2009). The incubation period varies from 15 days to

several months depending on the infection site, dose, age, vaccination, sex, stage of pregnancy and natural resistance (Nicoletti, 1980).

2.10 Synonyms:

The historical synonyms of the disease in animals and human-beings:

In domestic animals, brucellosis has been commonly known as enzootic abortion or bovine contagious infection, epizootic abortion, infectious abortion, contagious abortion, slinking of claves, Bang's disease, and ram epididymitis.

In the case of human brucellosis, it has been described by various names including undulant fever, Malta fever, Mediterranean fever, gastric fever, Mediterranean gastric fever, Gibraltar-Rock fever, Cyprus fever, Neapolitan fever, intermittent gastric fever or intermittent typhoid fever, pseudo typhus, febristypho-malariae, and fever sudorale (Ray and Steele, 1979).

2.11 Diagnostic Methods

2-11-1 Bacteriological Methods

Great care should be employed during handling any material containing *Brucella* organisms. Generally, precautions to be taken include use of safety cabinet in laboratory; wearing gloves, protective cloth and facemask, autoclaving materials in contact with the organism and disinfecting contaminated surfaces (Alton *et al.*, 1975). Commonly used basal media include:

Serum dextrose (Agab et al., 1994), serum tryptose agar, glycerol dextrose agar, trypticase, and soya agar (Alton *et al.*, 1975). Terzolo *et al.*, (1991) suggested that Skirrow agar is a satisfactory medium for both Brucella species and Campylobacter fetus. Contamination is prevented by use of selective media containing actidione (30 mg/l), bacitracin (25mg/l), polymixin B (5mg/l) and vancomycin (20mg/l) (Walker, 1999).

Milk samples, vaginal swabs, semen and aborted fetus are useful for recovering the organisms at ante mortem. Samples collected at necropsy include multiple lymph nodes, spleen, udder, pieces of uterus and testicular tissue (Agab et al., 1994). Tissue specimens are directly cultured on solid media whereas milk cultures are performed by centrifuging milk at 5900 to 7700 x g for 15 minutes (Walker, 1999). Cultures then,

incubated at 37^0C with 5-10 % CO_2 enrichment for three days and above (Alton *et al.*, 1975; Gameel *et al.*, (199); Agab *et al.*, (1994). Characteristics colonies have small convex, smooth translucent appearance (Gameel *et al.*, (1993); Agab *et al.*, 1994). Demonstration of the bacteria is by staining with Gram-negative stain or modified-Zeihl-Neelsen staining. Animal inoculation (an old method) can also reveal characteristics lesion in liver, spleen and epididymis of a guinea pig (Walker, 1999). Further characterization is based on stereotyping, phage typing, dye sensitivity, and biochemical tests.

Florescent antibody test and polymerase chain reaction methods have been described for Brucella species identification (Walker, 1999; Quinn *et al.*, 2002).

2.11.2 Serological Methods

Brucellosis was first diagnosed by a serological test by Wright and Smith, (1897) using a simple tube agglutination test. Subsequently, various modifications to the tube agglutination test and numerous other tests have been developed to increase test accuracy. The procedures are divided into two categories, the Conventional Tests and Primary Binding Assays. All conventional tests rely on the antibody performing a secondary function, for instance fixation of complement while in primary binding assays the only function of the antibody is attachment to its antigen. (Nielsen and Wu, 2010)

2.12 Basal medium:

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 recognized clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. 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 tryptocase)-soya agar (TSA). The addition of 2-5% bovine or equine serum is necessary for the growth of strain such as *B.abortus* biovar 2, and many laboratories systemically added serum to basal media, such as blood agar base (Oxoid) or Columbia agar (BioMe.ieux), with excellent result. 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 (OIE, 2009).

2.13 Selective media:

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress to growth of organisms other than *Brucella*. The most widely used selective medium is the Farrell's medium, which is prepared by the addition of six antibiotics to a basal medium (OIE, 2009). However, nalidxic acid and bactracin, at the concentration used in Farrell's medium, have inhibitory effects on some *B.abortus* and *B.melitensis* strains. Therefore the sensitivity of culture increases significantly by the simultaneous use of both Farrell's and the modified Thayer-Martin medium (Marin *et al*., 1996). Contrary to several biovars of *B.abortus*, growth of *B. melitensis* is not dependent on an atmosphere of CO_2 (OIE, 2009). Bacterial colonies may be provisionally identified as *Brucella* on the basis of their cultural properties and

appearance. Definition identification of suspect colonies can only be made using technique available at *Brucella* Reference centers (Corbel, 2006).

2.14 The situation of brucellosis in the Sudan:

Increase in the prevalence rate of brucellosis in the Sudan which ranged between 3-70% in cattle, 3-50% in sheep and goats and 3-40% in camels in different States. Biovars 1, 3, 6, 7 and 9 were isolated) for *B.abortus* and biovars 1, 2 and 3 for *B. melitensis* from different farm animals (Shigidi, 2010).

A serosurveillance of brucellosis conducted by the (FAO, 1998) showed that brucellosis was widespread among dairy herds in Khartoum and Gazira states with prevalence of 54% and 55%, respectively.

Brucellosis in the Sudan was first reported in a dairy farm in Khartoum, where B. *abortus* was isolated from an aborted Friesian cow (Bennet, 1943) after that many investigators isolated the organism from cattle in many parts of the country. The disease was also diagnosed serologically in many parts of the country (Shallali *et al*., 1982)

Serological investigation on the prevalence of the disease was carried by some workers and the disease was diagnosed in many areas, the disease was reported widely spread in domestic animals in Upper Nile Province (Nasri, 1960), in Wadi Halfa, Northern Sudan (Abdalla, 1966), in Khartoum state (Fayza et al., 1990), in Senar state (Ehsan, 2011), in Kasala State (Omer, 2007) and in Kurdufan State (Suliman, 2011).

In Sudan; according to recent researches the disease was found endemic throughout the country. The first isolation of *B.abortus* biovar 3 from camel (*C. dromedarius*) in the Sudan was by Agab *et al.* (1994). Three isolates of *B.abortus* biovar 3 were recovered out of 38 different samples obtained from free ranging camels (C. dromedarius) in eastern Sudan (Montasser, 1999)

2.15 *Brucella* isolated:

Isolation of *Brucella* was conducted on modified Farrell's serum dextrose agar according to standard procedures Alton *et al.* (1988). Modified Farrell's serum dextrose agar with 5 % horse serum, 1 % dextrose, and the following antibiotics (added to 1-l medium): cycloheximide (100 mg), bacitracin (25,000 IU), polymyxin B sulfate (5,000 IU), vancomycin (20 mg), nalidixic acid (5 mg), and nystatin (100,000 IU) were used for primary selective isolation of brucellae. Plates were inoculated with sample material and incubated aerobically and in the presence of 5–10 % carbon dioxide at 37 °C. These plates were examined 3–7 days post-inoculation for bacterial growth..

Suspected material such as placenta, cotyledon, Isolation and identification of the causative agent is sometime necessary for confirmatory or epidemiological vaginal discharge, fetal lung, liver, abomasal contents, hygroma fluid and samples of tissues collected at postmortem such as mammary glands, and internal iliac lymph nodes from female, testis, epididymis, seminal vesicles, accessory glands, external inguinal and internal iliac lymph nodes from males are used for isolation of Brucella organisms Alton, (1988).

2.*16 Brucella* Isolated in Sudan:

Brucella melitensis was first isolated from a human case in Sudan in 1904 (Hasseb, 1950). In 1943 Bennett reported the disease in a dairy herd in Khartoum where *B. abortus* was isolated for the first time. In 1953 *B. melitensis* was isolated from milk in the Gazira area, (Dafalla and Khan, 1958). *B. melitensis* was isolated from cattle, sheep and goat's milk in Gazira, Central Sudan (Dafalla, 1962). Also Habiballa, *et al.*, (1977) isolated the same organism from a placenta of an aborted cow. Many other studies had reported the isolation of *B. abortus* in different parts of the

country (Shigidi and Razig, 1971-1973, Khalafalla *et al.,* 1987 and Musa *et al.*, 1990).

Other investigators isolated *B.abortus* from cattle in Fung area in the Blue Nile and Juba in Southern Sudan (Dafalla, 1962) and from cattle in Kordofan (Ibrahim and Hebiballa, 1975). *B.abortus* biovar 3 was also isolated from camels in Eastern Sudan. (Agab *et al.*, 1995).*B. abortus* was isolated from human patient in Khartoum State, (Erwa 1966). In Western Sudan, *B. melitensis* was isolated from an infected mixed flock of sheep and goats (Musa and Jahans, 1990). Another study in Sudan recorded the isolation of *B.abortus* biovars 3 and 7 from Cattle in Khartoum State and *B. melitensis* biovar 2, which was isolated from a placenta of a 7 month aborted fetus and another from fetal stomach contents from an 8 month aborted calf in Khartoum State. The isolation of biovars from exogenous breeds highlights their role in introduction of new biovars to the country, (El Sanousi, Enaam, 2006).

Brucella species isolated from animals and humans in the Sudan Modified from Musa *et al*,(2008), as illustrated in Table (1).

Table 1: *Brucella* species isolated from animals and humans in the Sudan.

Brucella species	No. of isolates	Biovar	Source	Locality	Author
B. abortus	1	-	Dairy cattle	Khartoum	Bennett (1943)
B. abortus	1	-	Human	Khartoum	Erwa (1966)
B.melitensis	2	3	Cows & sheep milk	El Gazira	Dafalla (1953)
B. abortus	1	-	Dairy cattle	Juba	Dafalla (1962)
B. abortus	1	6	Aborted cow	Fung district	Dafalla (1962)
B. abortus	1	-	Hyproma of a bull	Khartoum	Shigidi and Razig (1971)
B. abortus	1	-	Cow's milk	Baggara Cattle, Kordofan	Ibrahim (1974)
B. abortus	1	6	Aborted cow	S. Darfur	Musa & Mitchell (1985)
B. abortus	22	6	Hypromas of cattle	Abassya S. Kordofan	Khalafalla *et al*. (1987)
B. abortus	41	6	Cattle	South and West Darfur State	Musa *et al.* (1990)
B.melitensis	1	3	Sheep	SouthDarfur State	Musa *et al* (1990)
B. abortus	7	6	Cattle	West Darfur	Musa (1995)
B. abortus	1	1	Cow	Kartoum	Selma (2006)
B. abortus	2	1	Cow's milk	Khartoum	Ismail (2007)

B. abortus	8	6	Cow's milk	Khartoum	Ismail (2007)
B. abortus	3	6	Cattle	Khartoum	El Sanousi Enaam (2006)
B. abortus	3	6	Camel	Darfur	El Sanousi Enaam (2006)
B. abortus	1	7	Cow	Khartoum	El Sanousi Enaam (2006)
B. abortus	1	3	Cow	Khartoum	El Sanousi Enaam (2006)
B. abortus	1	1	Cow	Khartoum	El Sanousi Enaam (2006)
B.melitensis	2	2	Cows	Khartoum	El Sanousi Enaam (2006)
B. abortus	2	6	cattle	Portsudan	El Sanousi Enaam *et al*, (2011)
B. abortus	2	6	Human	Portsudan	ElSanousi Enaam *et al*, (2011)
B. abortus	1	1	Cow	Kartoum	Selma (2006)

Modified from Musa *het al*,(2008).

Table 2: Diseases and principle host of the Brucella species.

Species	Host	Diseases	Geographical distribution
B.abortus	Cattle Sheep, goat and Pigs Horses Human	Abortion and orchitis Sporadic abortion Associated with bursitis (poll evil and fistulous withers) Undulant fever	Biotype 1.world wide(common) 2.worldwide(not common) 3. India, Eygept, East Africa. 4-Britain and Germany. Other biotypes are frequently isolated.
B.melitensis	Goat, sheep Cattle Human	Abortion. Occasional abortion and excretion in milk. Malta fever	Many sheep and goat raising regions except New Zealand Australia and North Am,

Quinn *et al* ,(1994)

2.17 Epidemiology of the disease:

The survival of the organism in the environment may play a role in the epidemiology of the disease. Many studies reviewed the ability of *Brucella* organisms to survive under various experimental and environmental conditions. Temperature, humidity and pH influence the organism ability to survive in the environment. Brucellae are sensitive to direct sunlight, disinfectants and pasteurization. In dry conditions they survive only if embedded in protein (Davies and Casey, 1973). *Brucella* can survive in tap water for several months at 4-8^{o}C, 2.5 years at 0^{o}C and several years in frozen tissues or media. Also they can survive up to 60 days in damp soil, and up to 144 days at 20^{o}C and 40% relative humidity, 30 days in urine, 75 days in aborted fetuses and more than 200 days in uterine exudates. (El Sanousi, Enaam 1999).Important role in the prevention and maintenance of the disease among farm animals. The role and magnitude of risk factors varied ,but the presence of good sanitary measurers in farms are considered as protective factor, where relative risk was less than 1 and the attributable risk was -0.01 (kaoude *et al*.,2010).

Economic losses from *B.melitensis* infections are very significant and include decrease productivity as a result of abortion, weak off spring and decreased milk production, as well as loosing trade opportunities.

B. melitensis is very contagious for humans and the disease, unless diagnosed and treated both promptly and effectively, can become chronic, affecting multiple body systems. The infection is acquired by humans following ingestion of contaminated dairy foods and from occupational exposure to infected live animals or car cases during slaughter. While sheep and goats are the major reservoir of *B.melitensis* infection, there is increasing evidence of emergence in cattle and camels. (FAO, 2010).). The disease has considerable impact on the economy through loss of milk, meat

and by diminished animal working power (Unger, 2003).signs varies depending on the affected organ system (cutler *et al*, 2005).

In man, transmission occurs as a result of ingestion of milk, contact via skin abrasion, mucous membranes and inhalation (Radostits *et al*., 1994; Seifert, 1996). Masoumi *et al.*, (1992) recorded higher prevalence rate among butchers and people who habitually consume raw milk. Camel keepers consume camel milk as well as liver without heat treatment. This is even considered as delicacy (Gameel *et al.*, 1993). There is also a close contact between herdsmen and the animal during watering, grooming, riding, nursing sick ones and delivery assistance (Abbas and Yassin, 1987).

The disease could seriously impair socio-economic development for livestock owners, which represent a vulnerable sector in rural populations in general and pastoral communities in particular. It has a significant public health implication for a pastoral community in consequence of lifestyles, feeding habits, close contact with animals, low awareness, and poor hygienic conditions which favor infections (Schelling *et al.*, 2003).

The bacteria are found in tissues and fluids associated with pregnancy the udder and the lymph nodes which drain the relevant areas. Most infections either result from ingestion of bacteria from diseased animals or contaminated feedstuff s however; infection may also be acquired by respiratory route and by contamination of abraded skin and mucosal surfaces .natural breeding transits infection in swine, dogs and to lesser extent, sheep and goats (FAO, OIE, 2006). Contact with animals, low awareness, and poor hygienic conditions, which favor infections (Schelling *et al.*, 2003).

Food and Agriculture Organization of the United Nations (FAO) and the Office International des Epizooties, (OIE) emphasize the importance of this disease, as brucellosis has not only direct public health implications but also poses a potential barrier to international trade of animals and

2.18 Host Factors:

Infection may occur in animals of all age groups, but persists commonly in sexually mature animals (Radostits *et al.*, 1994). Generally, infection is acquired after three years of age with increase in the subsequent age groups (Majid *et al.*, 1999; Abou- Eisha, 2000).

Some studies revealed the equal distribution of Brucella antibodies among males and females (Abu Damir *et al.*, 1984; Abbas *et al.*, 1987; Radwan *et al.*, 1992). In other findings it appeared that females are more susceptible to the disease than males (Agab *et al.*, 1997; Ajogi and Adamu, 1998). Higher susceptibility in female animals is attributed to physiological stresses (Walker, 1999). Female animals have essential epidemiological importance not only in susceptibility but also in disseminating the disease via uterine discharge and milk. The role of males in the spread of disease under natural condition is not important (Radostits *et al.*, 1994).

The extent to which infection rate varies due to breed difference is not well known. (Wernery and Wernery, 1990) reported that breeding camels had lower brucellosis infection rate than racing animals. This was justified as due to racing camels (but not breeding animals) utilizing unpasteurized cow milk.

2.19 Historical Prospective

It is known from written resources that sheep and goats were the primary domestic animals in the Roman Empire. Small ruminant's milk was used to make cheese, one of the primary ingredients in Roman cuisine. It was therefore hypothesized that milk and milk products were important sources of an infectious food-borne disease that was later know as the "Maltese fever" (i.e. brucellosis due to *Brucella melitensis*). The Roman town of Herculaneum was destroyed by the tremendous volcanic eruption of Mount Vesuvius in August 79

(A.D. Recently; L.Capasso) found bone lesions typical of brucellosis in adult skeletal remains of people killed during the first volcanic surge of Mount Vesuvius (Capasso, 2002) He also demonstrated by scanning electron microscopy analysis of a buried carbonized cheese, the presence of cocco-like forms that were morphologically consistent with *Brucella* spp. (Capasso, 2002).

Sir David Bruce isolated in 1887 the organism (Micrococcus melitensis) responsible for Maltese fever from a British soldier who died from the disease in Malta (Bruce, 1887). This bacterium was renamed *Brucella melitensis* in his honor. In 1905, Zammit demonstrated, again in Malta, the zoonotic nature of *B.melitensis* by isolating it from goat's milk (Zammit, 1905).

2.20 Geographical distribution:

Livestock in the Sudan satisfies the internal demand and leaves substantial excess for export, which represents about 22% of the country's total exports and contributes about 19% of GDP. (Babiker *et al.* 2009).

Sheep and goats exist in different localities all over the country. Sheep are reared throughout the country separately or often mixed with goats and share pasture and water with cattle and camels. They contribute effectively to the economy of the country by exportation to the Arabian Peninsula. In Sudan, goats are a major source of meat. They also supply their owners with milk. In arid and semi-arid zones of the country, goat's milk is one of the major sources of food. Goat trade to the Arabian Peninsula and exportation of their skins to some Asian and European country constitute an important component of Sudanese foreign trade (Suliman 2011).

2.21 Identification of *Brucella* species:

Brucella can be performed after isolation in pure culture by conventional bacteriological methods, including antigenic and phage typing, and also using molecular biology tools.

2.22 Typing of *Brucella* species

Identification of *Brucella* at genus level is simple and can be achieved in most laboratories, most conveniently by serological methods. Accurate identification to species and biovar level is only possible in reference laboratories. The most common differential characteristics of the classical *Brucella* species and biovars (alton 1988) are illustrates the characteristic of the *Brucella* isolates from marine mammals (Jahans *et al.*,1997 and Claraveau *et al.*,1998)

2.23 Molecular methods and Molecular Tests for Animals:

A number of probes have been used for the detection by polymerase chain reaction (PCR)of Brucella DNA in samples of human or animal origin. Identification at genus level is easily achieved under laboratory. Condition by PCR with primers taken from a variely of available sequences, such as those of the 16s rRNA, heat shock proteins, outer-membrane protein, insertion sequence 1S711(also known as 1S6501). Cross-identification or detection of phylogenetically related bacteria with some of the primers is possible,. In addition, gene polymorphism has been examined for the differentiation at species and biovar levels with partial success(Vizcaino et al., 2000). Tests detecting Brucella cells or antigens by molecular methods have been described but they have not found a practical use in the diagnosis of brucellosis, and this is also true of tests detecting directly Brucella DNA with labeled probes (Mayfield *et al.*, 1990).

The PCR is fast, can be performed on any body tissue, and can yield positive results as soon as 10 days after inoculation. It was first developed

for brucellosis in 1990, using a 635-bp fragment of B. abortus strain 19, (Fekete, *et al.*, 1990).Subsequently, two major gene sequences have been used as targets: the 16S rRNA gene sequence, (Romero et al., 1995),which represents total genus-specific homology and has been satisfactory in clinical settings (Nimri, 2003) and the BCSP31 gene, which encodes an immunogenic protein of the external membrane of *B. abortus* (Baily *et al.*, 1992) and has been extensively studied in clinical practice (Morata *et al.*, 1999).Cross-reactivity with Ochrobactrum is noticed sporadically with both techniques. A comparison of the two techniques showed superiority of the 16S rRNA target in terms of sensitivity (Navarro *et al.*, 2002).

Nested PCR: it is proved to have superior specificity and sensitivity, although it is more prone to contamination (Matar *et al.*, 1996).

Real-time PCR: Most likely the diagnostic tool of the future, offering the possibility of results in 30 minutes, (Queipo-Ortuno *et al.*, 2005).

PCR ELISA: New promising variation, (Morata *et al.*, 2003). Other variations of PCR exist, such as arbitrarily primed PCR, PCR with random amplification of polymorphic DNA, and a specific multiplex PCR that can concomitantly diagnose brucellosis, Q fever, plague, and anthrax and was developed for purposes of biowarfare defense, (McDonald *et al.*, 2001).

An assessment of the possible practical value of the existing PCR protocols is complicated by the fact that an efficient DNA extraction from samples is critical for optimal diagnostic sensitivity. It has been noted that even in culture, Brucella cells are resistant to conventional DNA extraction protocols (Romero and López-Goñi, 1999).

The few studies that have compared the diagnostic performance of PCR with that of standard bacteriological or serological tests suggest that PCR could be a valuable diagnostic test in animal brucellosis. In the examination of tissues, organs and stomach contents of *B. abortus* infected

cattle, a PCR protocol with primers taken from a putative *B. abortus* Omp showed 98% sensitivity and 96% specificity as compared to bacteriological culture (Fekete *et al*., 1992).

In a study carried out to assess the ability to detect *Brucella* in the stomach contents of aborted sheep fetuses, PCR with 16S rRNA primers performed closely to bacterial cultures, although the number of samples studied was low (Cetinkaya *et al*., 1999). For obvious reasons, milk would be one of the samples of choice, and in one study, conventional PCR with 16S rRNA primers was found to have 100% specificity and 87.5% sensitivity with respect to bacteriological culture (ELISA showed 100% specificity and 98.2% sensitivity in the same samples; (Romero *et al*., 1995a).

2.24 *Abortus Melitensis Ovis Suis* (AMOS) PCR:

AMOS PCR is one of the first PCR assays to differentiate among Brucella species and was the so-called (AMOS) PCR. AMOS PCR is used for differentiation of brucella species (*abortus, melitensis, ovis, suis*). This assay comprises cocktail of five oligonucleotide primers which can identify selected biovars of four species of *Brucella* using the polymorphism arising from species-specific localization of the genetic element IS711 in *Brucella* DNA. (Bricker and Halling, 1994).

2-25 Treatment of brucellosis in human and animal:

The ability of *Brucella* organisms to replicate within cell vacuoles is a determining factor in the chronic course of the disease and in the tendency to relapse.

Within the intracellular position, *Brucella* organisms are protected from a large number of antibiotics that cannot reach the intracellular environment.

An appropriate therapy must use antibiotics capable of killing both extracellular and intracellular *Brucella* organisms (Ariza, 1999).

Since no single antibiotic therapy achieves complete eradication of intracellular infection. Additive effect by the use of two or more antibiotics for prolonged periods is required to cure and to reduce the frequency of relapses. The possibility of enhancing the effectiveness of antibiotics showing good invitro activity by including them into liposomes, it has been studied by several authors (Vitas *et al.*, 1997), but attempts to use some of these formulations in animals have been discouraging (Nicoletti *et al.*, 1989). The absence of plasmids should be an important factor explaining, at least in part, the observation that the in vitro susceptibility of *Brucella* strains to antibiotics which remains stable over time (Bosch *et al.*, 1986).

Antibiotics have been rarely employed in animal brucellosis but they have been applied in some cases to treat animals of high genetic value, or to prevent spreading of the disease in small dairy herds of highly valuable animals. In cattle, mono therapy with either oxy tetracycline or free or liposomal streptomycin (Nicoletti *et al.*, 1985; Nicoletti *et al.*, 1989) is very unsatisfactory. A more prolonged treatment (14 oxytetracycline and 8 streptomycin doses) accompanied by intramammary infusion with oxytetracycline for 4 days is reported to have a 100% of success (Radwan *et al.*, 1993). Combinations of aureomycin plus streptomycin or tetracyclines plus streptomycin have produced satisfactory results (Giauffret and Sanchis, 1974). Successful antibiotic therapy does not result in a clearance of the lesions characteristic of this disease (Marín *et al.*, 1989).Brucellosis in horses is quite severe and prolonged treatments are required. A mixture of oxytetracycline (20 mg/kg) and streptomycin (15 mg/kg) for at least 21 days is recommended, (Pappas, 2005).

2.26 Control, Eradication and Prevention

Control and subsequent eradication of brucellosis can be achieved by the simple, costly and sometimes impractical procedure of totally eliminating the primary hosts from the selected area, with or without replacement of the population with clean animals. Although this method has been attempted by some, most countries that have achieved eradication of brucellosis from bovine, ovine and caprine herds have followed a stepwise strategy (Flowers, 1977). The first step towards the control of brucellosis in a specific population usually consists of the serological examination of the animals. The second step is the vaccination of the population at risk followed by removal and slaughtering of the serologically positive animals. During this phase, brucellosis-free farms and regions with or without vaccination could be declared. The final step is to stop vaccination. Constant serological surveys are necessary, mainly when potential foci of contamination are conflicting with brucellosis-free areas. Only after several years of continuous surveillance, brucellosis may be considered eradicated from these non vaccinated serologically negative herds. Up to now, only strain 19 and Rev. 1 vaccines have demonstrated to control and eradicate brucellosis under conditions where control programs have been implemented (Blasco, 1997).

2.27 Quality control of culture media in a microbiology laboratory:

The nature of reporting of a microbiology laboratory depends upon the quality of the culture media used. Quality of the media directly affects the observations and inferences described from the cultural characteristics of microorganisms. Examining of different parameters of media such as growth supporting characteristics, physical characteristics, gel strength and

batch contamination can help to assess their quality. There are different methods to check all these parameters systematically. The meticulous performance of quality control of culture media can assure precision in reporting (Basu *et al.*, 2005).

2.28 Standard Operating Procedures (SOPs)

In research, SOPs should be defined as "detailed, written instructions to achieve uniformity of the performance of a specific function". SOPs usually get applied in microbiological and molecular processing for diagnostic studies. There the focus is always set on repeated application of unchanged processes and procedures and its documentation. Study director is mainly responsible for SOPs. The Quality Assurance Unit are individuals who are responsible for monitoring whether the study report and tests are meeting the SOP. SOPs can also provide employees with a reference to common practices, activities, or tasks. New employees use a SOPs to answer questions without having to interrupt supervisors to ask how an operation is performed. The international quality standard ISO 9001 essentially requires the determination of processes (documented as standard operating procedures) used in any manufacturing process that could affect the quality of the product, Raytheon (2006).

2.29 Economic impact:

Economic losses from *B. melitensis* infections are very significant and include decreased productivity as a result of abortion, weak offspring and decreased milk production, as well as loosing trade opportunities.

B. melitensis is very contagious for humans and the disease, unless diagnosed and treated both promptly and effectively, can become chronic, affecting multiple body systems. The infection is acquired by humans following ingestion of contaminated dairy foods and from occupational exposure to infected live animals or carcasses during slaughter. While

sheep and goats are the major reservoir of *B. melitensis* infection, there is increasing evidence of emergence in cattle and camels. (FAO, 2010).

The disease is also associated with infertility and prolonged calving intervals, and has considerable impact on camel production. Chronic inflammation of epididymis, of the joints, tendon sheath and synovial bursae especially at the carpus may also occur in camels (Abbas *et al* 2002), Agriculture Organization of the United Nations (FAO) and the Office International des Epizooties, (OIE) emphasize the importance of brucellosis as not has only direct public health implications but also poses a potential barrier to international trade of animals and animal products. Such a barrier could seriously impair socio economic development, especially for a vulnerable sector in many rural populations of the livestock owner (WHO, 1997). The disease has considerable impact on the economy through loss of milk, meat and by diminished animal working power (Unger, 2003).signs vary depending on the affected organ system (Cutler *et. al*,2005).

CHAPTER THREE

MATERIALS AND METHODS

3-1 Asepsis and Sterilization

The laboratory work that required aseptic conditions was performed in the laminar flow (FASTER BH-EN 2003).The Sterilization of all glassware, Petri dishes, pipettes, Roux flasks and McCartney, ampoules and vials was carried out in an oven (Memmert OVN BRU .05-0.01) set at 160^0C for one hour. Sterilization of screw-capped tubes and rubber stoppers was done by autoclaving at 121^0C for 30 minutes (Alton 1975).. Dextrose and dyes was sterilized by steam, loop sterilized by flame.

All media were sterilized by autoclaving at 121^0C for 15 minutes, except media that were affected by heat, dextrose by steaming for 20minute, dyes steaming for one hour in Autoclave (Eguitron-watt 3500). Serum was sterilized by filtration using Seitz filter. All the bacteriological laboratory work that required aseptic conditions was performed in a laminar flow (Faster BH EN 2003).

The sterilization of all glass ware: Petri dishes, Roux flasks, culture screw-capped tubes, tubes, MacCarteny bottles, Pasteur pipettes, ampoules, vials and microscope slides were sterilized in hot air oven at 160°C for 1hour.

All media were sterilized using heat by autoclaving at 121^oC for 30 minutes, components that were affected by heat (e.g. serum and dextrose), were sterilized by filtration, using Seitz filter.

3-2 Diluents for *Brucella* suspensions:

3-2-1 Normal saline:

This was done according to Alton (1988), 8.5g sodium chloride was added to litter of distilled water and sterilized by autoclave at 121^oC for 15 minutes.

3-2-2 Phosphate-buffered saline:

This was prepared according to Alton, (1988).Normal saline, adjusted to a pH of 6.4 by the addition of 6.67 g of potassium Di hydrogen phosphate (KH_2PO4) then added 3.0 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) mixed well then distributed to last container sterilized by autoclave this was used for harvesting and suspending *Brucella* cells, particularly in vaccine production The PH of each new lot always-checked before use.

3-2-3 Peptone water:

This was prepared according to manufactured company (oxoid) 15g of peptone was added to litter of distilled water. Mixed well and prepared into two sets of tube eight tube in each set with peptone 9 ml in each tube, covered by cotton and foil, sterilized by autoclave at 121^oC for 15 minutes store at 4^0C till were used.

3-2-4 Phenol saline:

This was performed according to Alton (1988), 5g of phenol was added to litter of normal saline and sterilized by autoclave 121^oC for 15 minute.

3.2.5 Distilled water (D.W.):

Tab water was distilled to PH 7, using water distiller (Elega, UK)

3.3.6 preparation of *Brucella* seed

3-2-6-1 Seed management:

A freeze-dried (lyophilized) original seed of *Brucella melitensis* strain Rev1 was obtained from the OIE Reference Laboratory for Brucellosis

Procedure:

The seed was reconstituted with 2 ml of Phosphate Buffer Saline (PBS) and the suspension was propagated in a number of Serum Dextrose Agar (SDA) slopes contained in tubes. They were incubated at 37^oC for 5 days and then examined as described by Corbel et al. (1983) and Alton (1975).

3-2-6-2 Propagation of *Brucella* seed:

Procedure:

Seed material stocked on Serum dextrose agar(SDA) slopes was selected and harvested by adding sufficient amount of sterile Phosphate Buffer Saline (PBS); pH 6.4 to cover the slope. Tthe culture was suspended by rolling the tube between the hands. This seed suspension was added to 150ml of PBS pH 6.4, then each Roux-flasks containing 150ml of SDA medium was cultured with 4ml of seed suspension, which was uniformly distributed over the surface of the agar by tilting the flasks. The flasks were then incubated at 37^{o}C for 5 days with the agar uppermost. After 5days two Roux-flasks with satisfactory growth were selected and carefully examined for any visible contamination. Excess liquid present in any of the Roux-flask was discarded into potent disinfectant, and then 30ml of PBS was added to each Roux-Flask. All bottles were left for about 30 minutes with the medium downwards, and then gently agitated to suspend the growth. The suspension from each Roux-flask was poured into a corresponding vial aseptically and control tests were then conducted according to Alton (1988) and OIE (2009).The suspension was stored at 4^{o}C till used.

3-3 culture media:

3-3-1 Potato agar:

This medium was prepared according to Alton et al., (1988).

The formula (g/l)

-Sodium chloride 5g

-Peptone 10g

Beef extract 5g

-Bacto agar 30g

-Potatoes 250g

Procedure:

Slights of Raw potatoes were washed and peeled and then 250gof potatoes were added to one liter of distilled water with minimum exposure to air. This medium was prepared by two ways, one by water bath the mixture was kept overnight in covered container at approximately 60^0C overnight then filtered through gauze. The second way by boiled the potatoes for 30 minute an then filtrated through gauze and then the following ingredients in formula were added, then heated the mixture to dissolve the agar, 20ml of glycerol was added to 1litter of medium, 1% sodium hydroxide solution was added to adjusted of pH 7.0± 0.2, then the medium was dispend to Petri-dishes and McCartney.

3-3-2 Serum dextrose agar (Farrell medium) basal medium:

This medium was prepared according to Alton (1988).

nutrient agar formula (CM3 OXOID)

Lablimco Powder 1.0 g/litter D.W.

Yeast extracts 2.5

Peptone 5.0

Sodium chloride 05.0

Agar 15g

Procedure:

This medium modified from Alton (1988) as by oxoid.

This medium was prepared by adding 14g of Nutrient agar to 360ml of DW then boiled to dissolve completely then sterilized by autoclave at 121^oC for 20 minutes and then cooled to 50-56^0C in water bath. Then dextrose 0.1% was prepared by adding 1g Dextrose to 10ml sterile DW steaming by autoclaved for 20 minutes. The Brucella Antibiotic Supplement (Oxoid) was prepared by added 10ml DW mixed well to dissolve incubated at 37^0C for 20 minutes, serum(5%) 25 ml to 500 ml of medium, mixed dextrose

supplement and serum to 360 ml of medium and immediately dispended to the final container Petri dishes and McCartney and stored till used.

3-3-3 Tryptose agar:

Both Difco tm REF 263400 and Oxoid lot 1108302 were used.

Dextrose g/l DW 01.0g

Treptose 20g

Sodium chloride 05.0g

Agar 15.o

Procedure:

This medium was prepared according to manufacture company by adding 40g of powder medium to one liter of distilled water boiled to dissolve completely for 1 minute, sterilized by autoclave 121^{o}C for 15minutes and immediately dispended to Petri dishes and McCartney, and stored at 4^{0}C till used.

3-3-4 Thayr-Martin agar (New Yorkcity medium)GCC mo 367 oxoid:-

Formula:-

Especial peptone g/l 15.0g

Corn starch 01.0

Sodium chloride 05.0 g

Di potassium hydrogen phosphate 04.0

potassium dihydrogen phosphate 01.0

Agar NO-1 15.0

Ph. 7.2±0.2

Procedure:

This medium was prepared according to the method of the manufactured company by adding 18g of Oxoid GC agar base to 235ml of DW then boiled to dissolve the agar, sterilized by autoclave at 121^{o}C for 15 minutes, and dispended to final container sterile Petri-dishes and McCartney. And stored at 4^{0}C till used.

3-3-5: Blood Agar base no .2 Oxoid code (M271)

Formula:

Propose peptone	g/L DW	15.0
Liver digested		2.5g
Yeast extracts		5.0g
Sodium chloride		5.0g
Agar		12.0g PH 7.4

Procedure:

This medium was prepared according to the formula and method of the manufactured company by adding 40g from powder medium to on litter of distilled water boiled to dissolve completely, mixed and sterilized by autoclave 121^{o}C for 20 minute and cool to 45-50^{0}C in water bath, 5% defebrenated blood was added mixed with gently rotation and poured into Petri-dishes let to be solid, stored at 4^{0}C till used.

3-3-6 Tryptone soya agar (Oxoid) code powder CM131)

Formula:

Triptone	g/l	15.0
Soya peptone		5.0
Sodium chloride		5.0
Agar		15.0

Ph 7.3+-.2

Procedure:

This medium was prepared according to formula of manufactured company(oxoid) By adding 40g of medium to litter of Distilled Water boiled to dissolve completely sterilized by autoclave 121^{o}C for 20 minutes then dispended to final container Petri dishes, and McCartney stored at 4^{0}C till used.

3.4 Isolation of *Brucella*:

Isolation of *Brucella* organisms were tried from blood samples of humans and from bovine hygromas, one isolate of each was obtained and commenced by preparing smear from each sample, then stained by modified Zeihl-Neelsen (MZN). Any samples showed acid fast bacilli were cultured duplicate on Farrell medium (Serum dextrose agar) then incubated aerobically and anaerobically subculture was then incubated at 37^{o}C for 3-5days.then Subculture in Tryptose-agar medium in slant, incubated aerobically at 37^{o}C for 3-5 days then stored at 4^{o}C till use.

3.5 Preservation and Maintenance of *Brucella* Isolates:

Brucella cultures were maintained and preserved by two methods: short and long term methods.

3.5.1 Short-term Materials and Equipment:

Tryptose Agar slants, Brucella isolates pure cultures, Sterile inoculating loop , Para film or a re-sealable plastic bag , Refrigerator and Permanent marker.

Procedure:

Using a permanent marker, date and name of the *Brucella* isolate was written on the side of the agar slant then dip the sterile inoculating loop in the *Brucella* culture and followed the standard lab bacterial streaking protocol then, the culture was incubated at 37°C for 48 – 72 hours, then observed for growth on the agar, checked for purity, pure culture slant was wrapped in Para film, or place it in a re-sealable plastic bag to prevent the agar slant from dehydrating. The *Brucella* slants were stored at 4^{o}C.

3.5.2 Long-term preservation of *Brucella* isolates:

The *Brucella* isolates were harvested by phosphate buffer saline then baked in vials and lyophilized using the Laboratory Freeze Dryer machine and then stored at -20^{o}C till used.

3.6 Eco metric tests:-

3.6.1 Media:

Six media (Potatoes Agar, Tryptose Agar, Tryptone Soya Agar, Serum Dextrose Agar, Thyer.Martin and Blood Agar) were used to test their support to the growth of the Brucella isolates.

3.6.2 Brucella strains:

The study examined two fresh field isolates, *Brucella abortus* from animal isolate and *Brucella melitensis* as a human isolate. For each medium, a duplicate of test was done.

Procedure:

A suspension of 10^{10} organism's/ml of phosphate buffer saline for each organism under test was inoculated onto each of the media using the Ecometric technique (Mossel *et al.*, 1983). The Ecometric technique is based on streaking inoculums of bacteria in Petri dishes divided into four quarters . It gives a numerical measurement of media performance that can form the basis of records suitable for analysis. On duplicate plates, one 10µl loop of the suspension was streaked 20 times onto the medium, without refilling the loop, and the plates were incubated at 37°C for 4 days in an aerobic atmosphere containing 10% carbon dioxide (CO_2). The absolute growth index (AGI) was calculated as the mean percentage growth of the two plates and was determined by counting the numbers of continuous lines of growth for each medium type under test. Batches of media providing a mean value of greater than 50% were considered acceptable for use (with a minimum of 25% on an individual plate).The plates were d

ivided into 4 quarters (A, B, C and D) any quarter were represented 25% of the culture and it was cultured by made 5 lines (1, 2, 3,4 and 5) any line represent 5% of the culture. Schematic diagram (Fig1).

3-7 Preparation of Liquid Media (Enrich Media):

3.7.1 Brain-Heart Infusion (Oxoid code powder CM225

Formula:

Calf brain infusion solid g/l	12.5
Beef heart infusion solid	0.5
Propose peptone	10.0
Dextrose peptone	02.0
Sodium chloride	05.0
Disodium phosphate	2.5

Ph 7.4 +-0.2

Procedure

This medium was prepared according to manufactured company formula by adding 37g powder medium to litter of distilled water, mixed well and distributed into final containers (bijou vials), sterilized by autoclave 37^{o}C and then stored at 4^{o}C till used.

3.7.2 Laurel Treptose Broth (Oxoid Code Powder CM451:-

Formula:

Treptose g/l	20.0
Lactose	05.0
Sodium chloride	05.0
Di potassium hydrogen phosphate	02.75
Potassium hydrogen phosphate	02.75
Sodium lauryl sulphate	01.0

ph 6.8 +- 02.0

Procedure:

This medium was prepared by the formula and method of manufactured company. 35.6g of powder medium was added to litter of distilled water mixed well distributed into final container (bijou vials) sterilized by autoclave at 37^{o}C and then stored at 4^{0}C.

3.7.3 Nutrient Proth (Oxoid code powder CM1)

Formula:

Lab limco powder	g/l	01.0
Yeast extract		02.0
Peptone		05.0
Sodium chloride		05.0

PH7.2+-0.2

Procedure:

This medium was prepared by the formula of the manufactured company by adding 13g of medium powder to 1 litter of distilled water mixed well and distributed into final container (sterile bijou vials) sterilized by autoclave at 37^{o}C and then stored at 4^{0}C.

3.7.4 Potatoes Broth:-

Formula:-

Sodium chloride	g/l	5.0g
Peptone		10,0g
Beef extract		5.0
Potatoes		250g

Procedure:

This medium was prepared according to Alton *et al.*, (1988), washed slights of raw potatoes were washed three times by tab water and peeled and thinly sliced, 250 g/L was added in distilled water with minimum exposure to air. The mixture was kept overnight in water bath at 60^{0}C. The alternative method was by boiling of the potato for 30 minutes. Then for

the two methods, the infusion was filtered through gauze then made up to 1 litter of DW, 2% of glycerol and 1% sodium hydroxide were added and mixed well and distributed in bijou vials sterilized by autoclave at 37^{o}C then stored at 4^{0}C till used.

3.8 The viable Count:

This method according to (Miles and Mesra, 1938).

3.8.1 Ingredient:

Colonies of Brucella isolates (*abortus, melitensis*) and broth media.

Procedure:

Plates containing Tryptose agar were streaked with the culture so as to produce areas of dense growth. After five days of incubation on 37^{o}C the growth was examined and these brucella cultures were used for the test, a suspension was made. Duplicate eight test tubes of 9 ml peptone were placed in a raw in a rack, a dilution was made by place 1ml from either *Brucella*. *abortus* and *Brucella melitensis* suspensions in first tube mixed (vortex) hold 1 ml and placed in next tube and so till eight tubes, dilution will be 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640. In 10 dishes of tryptose agar medium made culture from dilution 1/160, 1/320, 1/640 incubated at anaerobic incubation at 37^{o}C for 3-5 days. *Brucella*. After the growth of colonies in sterile four tubes three colonies to thre ml of enriched media Brain-Heart Infusion potatoes, nutrient and Laurel Treptose), were added, to each (duplicated media for human and on for animal isolate were used) mixed well and incubated at atmosphere gar for 3-5 days and the results of the count were recorded (Fig 8).

3.9 Identification and typing of Brucella species:-

3.9.1 Identification of Brucella species:-

Identification of pure isolates was performed using:

3.9.1.1 Macroscopic Appearance

3.9.1.1.1 Characterization:

Two plates containing Tryptose agar were streaked with the culture of .human and animal isolates so as to produce areas of dense growth as well as individual colonies. After five days of incubation on 37^{o}C the growth was examined under the microscope and the results were recorded.

3.9.1.1.2 Phase:

Individual colonies obtained over the surface of solid media were observed by obliquely transmitted light on allow power stereoscopic microscope.

3.9.1.1.2.1 Crystal violet stain:

It was prepared according to White and Wilson(1951). two solutions were prepared (solution A and solution B). Solution (A) by dissolving 2g Crystal Violet in 20ml of 95% alcohol. Solution (B) by adding 0.8g Ammonium oxalate in 80ml of distilled water. Then mixed solution (A) with solution (B) to make the stock solution, then it was diluted 1:40 in distilled water. Four grown colonies of *Brucella* were : the human isolate, the animal isolate in addition to *Brucella melitensis* REV-1 and Brucella abortus Strain19 vaccine strains which were used as standard controls. All were flooded with crystal violet stain diluted 1:40 in distilled water just before use, and allowed to act for 15 to 20 seconds. Excess stain after which it is removed. The plate examined immediately with a stereoscopic microscope and the results were recorded.

3.9.1.2 Microscopic appearance:-

Smear from suspected samples or from pure culture isolates were fixed with heat and then stained by the two methods: Gram's and Zeihl-Neelson methods and examined microscopically under the oil immersion lens.

3.9.1.2.1 Smear:

3.9.1.2.1.1 Gram stain:

This was originally devised by Christian Gram in (1884).

Procedure:

Thin smear was made of two isolates culture to be examined on clean and dry slide. Allowed to dry in air. Smears were heat-fixed by flame, and flooded by crystal-violet for 30sc then were washed throatily with water. The lugol's' iodine was allowed to act for 30 seconds then it was decolorized by acid-alcohol 30sc and the slide washed throatily with water and it was then flooded with the carbol –fuchsine counter stain which was allowed to act for 30 sc. The stain washed off with water and was dried with air and view using oil immersion microscopy.

3.9.1.2.1.2 Ziehl-Neelsens, acid fast stain:

Procedure:

Air-dried heat –fixed smear over flame. Prepared as described for the Grams, stain then the slide flooded with dilute carbol-fuchsine and stained for 10minute and it was washed thoroughly in water. The smear was decolorized with o.5%v/v acetic acid for up to 30second taking care not to over-differentiate.

Then the slide was washed thoroughly with water. And smear was counterstained with 1% w/v methylene blue for 1minuites washed with water and the slide allowed to dry with air and view using oil immersion microscopy.

3.9.2 Typing of *Brucella* Isolates:

3.9.2.1 Growth anerobically in partial CO_2(10%):

cultured isolates in duplicate tryptose agar medium slops were incubated aerobically and in atmosphere of 10% CO_2 using CO_2 generating kites in an anaerobic jar (Gaspak) at 37^{o}c for 3-5 days and shown the result.

3.9.2.2 Productionof H_2S:

3.9.2.2.1 Preparation of strips:

Sterile filter paper prepared by sheets of filter paper were soaked in ten per cent w/v solution of lead acetate and hung up to dray at 37^{o}C in air free of H_2S when dry the sheets were cut into strips of 8*100mm warped in grease proof paper in bundle of and vacuum autoclaved at (121^{0}C) for 20 minute they should be stored away from culture or other sources of H_2S

3.9.2.2.2 Procedure:

The pure culture of two isolates were cultured on tryptose agar slops, filter papers impregnated with lead acetate was inserted in the tubes in such a way that it does not come in contact with the medium and is held in place between the plug and the side of the tube and fixed with the caps. The paper was examined daily for 5 days for blackening.

3.9.2.3 Growth in presence of dyes:

This method was prepared according to corbel (1979). Used three dyes, Safranin O .5% , Carbol fuchsine .2% and Thionine.2%

3.9.2.3.1 Procedure:

Dye sensitivity test media was prepared by the addition of the relevant dye to the basal medium serum dextrose agar is recommended for this purpose to prepare the media the dyes were dissolved in distilled water to produce stock solution these were sterilized by steaming for one hour. Concentration dyes was then added to the SDA basal media plates or slopes (table4).

Table (3): The volume added and the concentration of the stock solution of the dyes:

Inhibitor	stock solution	volume added	final concentration
Thionine	0.2%	10ml/litter	1:50,000
Basic fuchsine	0.2%	10ml/litter	1:50,000
Safranin o	0.5%	20ml/litter	1:10,000

3.9.2.3.2 Basic fuchsine (1/50 000):

A total of 0.2% (w/v) stock solution of basic fuchsin was prepared and sterilized by steaming in an autoclave by steaming for 15 minutes and added to a sterilized Brucella agar base at a rate of 10ml for 1 litter of medium (20µg/ml). A milky suspension was prepared from strain Rev1 and strain19 (as standard), *Brucella*. melitensis and *B*. *Abortus* isolates were cultured on the serum dextrose agar medium which was then incubated at an aerobic incubation at 37^{O}C and examined for growth after five days.

3.9.2.3.2 Thionine (1/50 000) :

A total of 0.2% (w/v) stock solution of thionin was prepared and sterilized by steaming in an autoclave for by steaming for 15 minutes and added to a sterilized *Brucella* agar base at the concentration of 10 ml for 1 litter (20µg/ml) A milky suspension was prepared from strain Rev1 and strain19 (as standard), *Brucella*. melitensis and *B*. *Abortus* isolates were cultured on the serum dextrose agar medium which was then incubated at an aerobic incubation and examined for growth after five days.

3.9.2.3.3 Safranin O:

A total of 0.5% (w/v) stock solution of safranin0 was prepared and sterilized by steaming in an autoclave for 15 minutes and added to a sterilized Brucella agar base at the concentration of 20 ml for 1 liter (20µg/ml) A milky suspension was prepared from strainRev1 and strain19

(as standard), *Brucella*. melitensis and *B. Abortus* isolates were cultured on the serum dextrose agar medium which was then incubated at anaerobic incubation and examined for growth after 5days.

3.9.2.4Phage lysis:

The phages (Weighbridge (wb), Tbilisi (Tb), Berkely (BK), Frinzy (fi)and rough canis R/c phage at the routine test dilution (RTD) were used for identification of the strains to the biovar levels. The culture on agar slops to be tested, harvested with normal saline solution. Serum dextrose agar medium were cultured by used 100µ from harvested culture *Brucella*. *melitensis* and *B. Abortus* isolates with Pasteur pipette let till absorbed, a drop of 20µ of the RTD dilution of the tested phage was placed on inoculated area of each culture. Then late the drop of the phage dried, incubated at 37^{o}C for 1-2 days and examined the complete, partial or no lysis.

3.9.2.5 Agglutination in Mono specific Anti sera:

The *Brucella* culture examined for agglutination with Abortus (A), Melitensis (M) mono specific anti-sera for *B.abortus* and *B. Melitensis* anti sera. Culture grew in slant of *Brucella*. *melitensis* and *B. Abortus* isolates were harvested with normal saline, the harvested heated in 65^{o}C in water path for one hour. The test employed on clean slide. A drop of each serum placed on the slide and a drop of the culture suspension added to each and mixed with a loop. The slides observed for agglutination .and shown the result. The appearance of agglutination was then read within 20 to 30 seconds.

3.9.2.6 Molecular biology studies:

Brucella DNA was extracted from the isolates using a commercial purification system (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) according to the manufacturer's instructions for DNA purification from cells. Final pellets were re-suspended in 50 µL of TE (10

Mm Tris, 1 m M EDTA, pH 7.2). Species-level identification was under taken by the AMOS PCR assay Bricker BJ, Halling SM (1994)

3.9.2.6.1 DNA preparation:

DNA preparation for *Brucella* genomic DNA extraction, *Brucella* sample strains were cultured for 24 h at 37°C on tryptic soy agar-yeast extract slopes and harvested, in 3 ml of sterile distilled water, by centrifugation at 2,000 × g for 10 min. The pellet was suspended in 567 µL of TE/sodium buffer (50 mM Tris, 50 mM EDTA, and 100 mM NaCl [pH 8.0]). Then, 30 µL of 10% (wt/vol) sodium dodecyl sulfate (SDS) solution and 3 µl of 2% (wt/vol) proteinase K solution were added, and the mixture was kept at 37°C for 1 h. The lysed cell suspension was extracted twice with phenol-chloroform, and nucleic acids were precipitated by gently mixing the aqueous phase with 2 volumes of cold ethanol. The precipitate was dissolved in 100 µL of TE (10 mMTris, 1 mM EDTA [pH 8.0]).

The amount of DNA was measured by electrophoresis of an aliquot of each sample through 1% agarose gels and was compared with standard DNA solutions (Salehi *et al*, 2006). The extracted DNA was then used for PCR amplification of part of omp2a gene.

3.9.2.6.2 PCR Amplification conditions: The reaction mixture contained 5 µL of 10x PCR buffer, 10 pMol of the primer mix, 0.2 µMol of dNTP mix, 1.5 mM MgCl2, 1 unit of Taq DNA polymerase, 100 pg of sample DNA in a volume of 50 µL. No template negative controls were routinely processed to monitor contamination with Brucella DNA and positive controls were included to monitor amplification success. Amplifications were initiated by denaturing the sample for 3 min at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. After the last cycle, samples were incubated for an additional 5 min at 72°C before storage at 4°C.

Each PCR run was done separately for each isolate.

CHAPTER FOUR

4RESULTS

4-1 Isolation of *Brucella*:

Brucella organisms were isolated from two samples each from human blood and animal knee joint), All were CO_2 dependent for growth. Brucella organisms were isolated according to morphology, colony appearance and growth characteristics according to typing procedures they were found to be *Brucella abortus* biovar 6. from animal, and *Brucella melitensis* from human. Preservation and maintenance of Brucella isolates: The growth of Pure Brucella isolate on the side of the agar slant were stored in a refrigerator at $4^{o}C$ for up to six months (Fig1).

(Fig1) shown the growth of Pure *Brucella abortus* and *B. melitensis* isolate on the side of the Tryptose agar medium

4.2 The Ecometric test:

Four quadrant streaking of each plate, each line was calculated as (5%), (1,2,3,4,5) and each quadrant as(25%), (A,B,C,D), the whole plate was 100%. Fig(2).

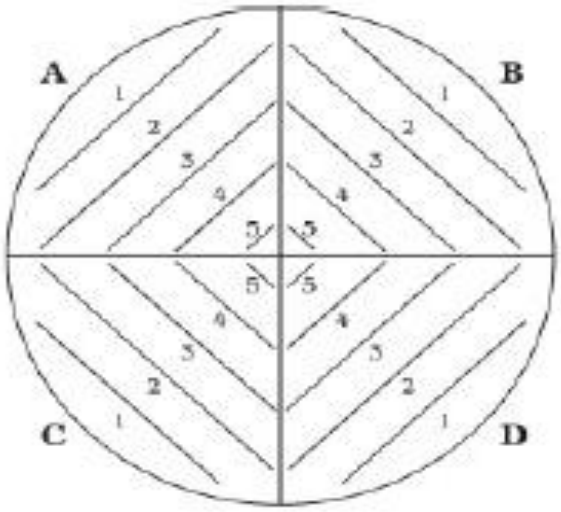
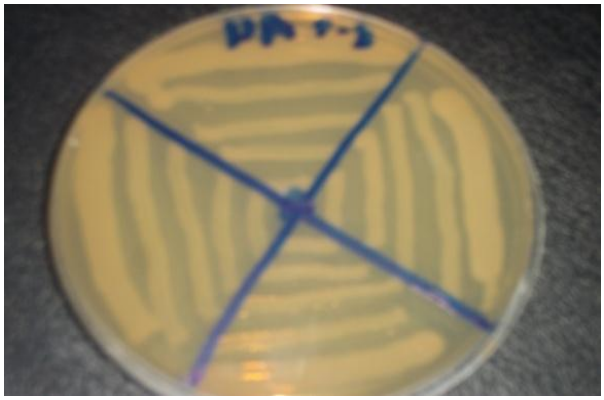


Fig (2) Schematic diagram of the ecometric testA, B, C and D= 25% of the culture plate. Line of loop 1,2, 3,4 and 5 =5% of the culture plate

4.3.1 Growth in Potatoes agar (PA):

4.3.1.1 *Brucella abortus:*

The growth of culture was 100% at an atmosphere of 10% CO_2 at anaerobic jar at 37^{o}C for 3-5 days. Fig (3) . The growth was 60% at aerobic incubation 37^{0}C for 3-5 days Table (4)



Fig(3)The 100% growth of *B.abortus* in potatoes agar at atmosphere of 10% CO_2 at 37^{o}c for 3-5 days

4-3-1-2*Brucella melitensis*:

The growth was 100% at an atmosphere of 10% CO_2 and was 25% at aerobic incubation 37^0C for 3-5 days Table (4)

4-3-2 The growth on Serum dextrose agar (SDA) (Farrell medium):

4-3-2-1*Brucella abortus*:

The growth was 100% at serum dextrose agar at an atmosphere of 10% CO_2 in anaerobic jar at 37^oC for 3-5 days Fig (4).The growth was 65%. at aerobic incubation 37^0C for 3-5 days . Table (4)

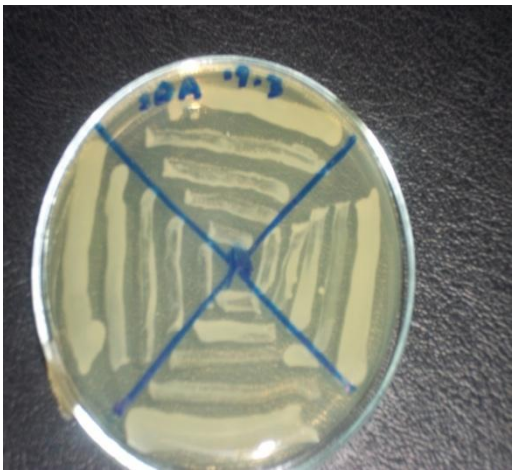


Fig (4) The 100% growth of *B.abortus* at serum dextrose agar at atmosphere of 10% CO_2 at 37^oC for 3-5 days

4-3-2-2 *Brucella. melitensis*:

The growth of culture at (SDA) was 100% at an atmosphere of 10% CO_2 in at $37^{O}C$. Fig (5) and was 25% at aerobic incubation.

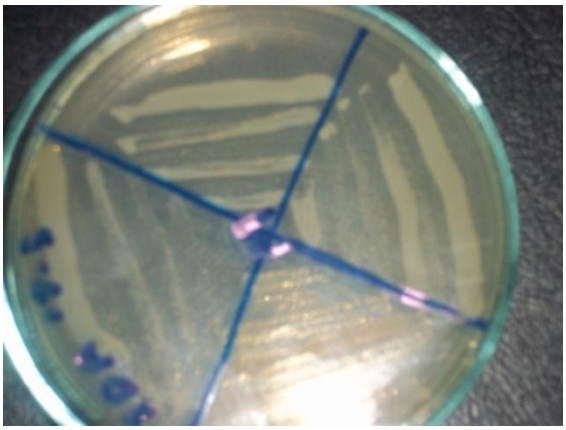


Fig (5) The 100% growth of *B. melitensis* at serum dextrose agar at atmosphere of 10% CO_2 at $37^{o}c$ for 3-5 days

4-3-3 Growth in Tryptose agar TA medium:

4-3-3-1*Brucella abortus*:

The growth was 100% at an atmosphere of 10% CO_2 at $37^{o}c$. Fig (6) and the growth was 75% at aerobic incubation at $37^{0}C$ for 3-5 days Table (4)

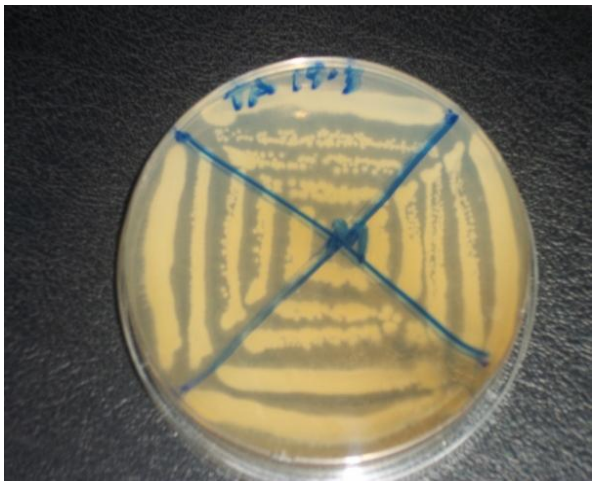


Fig (6) The100% growth of *Brucella. melitensis* in tryptose agar at atmosphere of 10% CO_2 at $37^{o}c$ for 3-5 days

4-3-3-2 *Brucella melitensis*:

The growth was 100.% at an aerobic incubation of 10% CO_2 Fig (7). And The growth was 30% at aerobic incubation 37^{0}C for 3-5 days .Table (4)

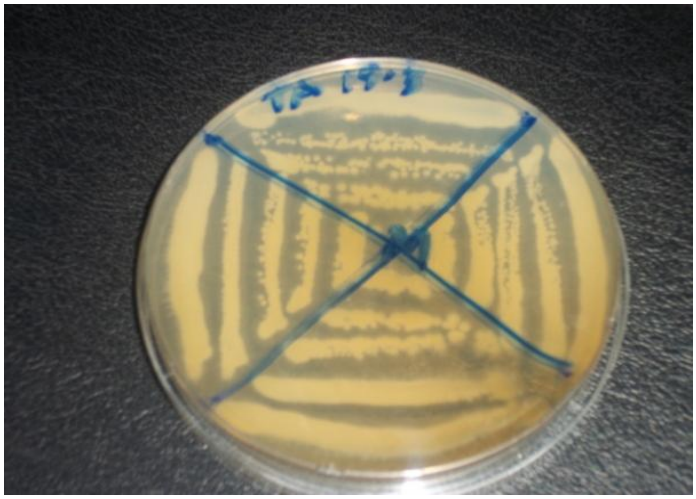


Fig (7) The 100% growth of *Brucella melitensis* in tryptose agar at atmosphere of 10% CO_2 at 37^{o}C for 3-5 days

4-3-4 Growth in Thyr.martin medium:

3-3-4-*1Brucella abortus*: The growth was 100.% at anaerobic incubation of 10% CO_2 at 37^{o}C for 3-5days. Fig (8).and it was 55% at aerobic incubation 37^{0}C for 3-5 days. Table (4)

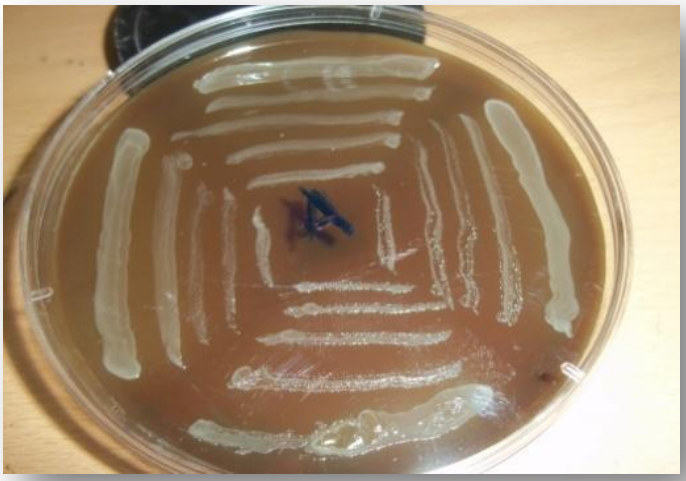


Fig (8) The 100% growth of *B.abortus* at anaerobic incubation of 10% CO_2 at 37^{o}C for 3-5 days in thyer –martin medium

4-3-4-2 *Brucella. melitensis*:

The growth was 100% At anaerobic incubation of 10% CO_2 at 37^{o}C for 3-5days and no growth at aerobic incubation 37^{0}C for 3-5. Table(4)

4-3-5 The growth in tryptose soya agar medium:

4-3-5-1 *Brucella abortus*:

The growth was 100% at anaerobic incubation of 10% CO_2 at 37^{o}C for 3-5days and at aerobic incubation 37^{0}C for 3-5 days was 85%. Table(4)

4-3-5-2 *Brucella melitensis*:

The growth at anaerobic incubation of 10% CO_2 at 37^{o}C was 100%. Fig (9) and at aerobic incubation 37^{0}C for 3-5 days was 100%. Table (4)

Fig (9) The 100% growth of *Brucella .militensis* in tryptose soya agar at atmosphere of 10% CO_2 at 37^{o}C

4-3-6 The growth in blood agar medium

4-3-6-1 *Brucella abortus*:

The growth was 100% at anaerobic incubation of 10% CO_2 at 37^{o}C and it was 100% at aerobic incubation 37^{0}C for 3-5 days Table(4)

4.3.6.2 *Brucella. melitensis*: The growth was 100%. at anaerobic incubation of 10% CO_2, the growth was 100% At aerobic incubation 37^{0}C for 3-5 days Table(4)

Table (4)The growth of *Brucella.abortus* and *Brucella. melitensis* in solid media (eco-metric test).

Media	Aerobic incubation at 37°c		Anaerobic incubation of 10% CO_2 at 37°C	
	Brucella. melitensis	*Brucella.abortus*	*Brucella. Melitensis*	*Brucella.abortus*
1.Potatoes agar(P.A)	25%	60%	100%	100%
2.Serum dextrose agar(SDA)	30%	65%	100%	100%
3-Tryptose agar	30%	75%	100%	100%
4-Thyr.m	No growth	55%	100%	100%
5-Tryptose soya agar	No growth	85%	100%	100%
6- Blood agar	100%	100%	100%	100%

4.4 The Viable Count of Broth media (Enriched Media):

The growth of *Brucella abortus* as measured by viable count was determined. Potatoes broth was the best medium for growth Fig (10) followed by Brain heart- infusion, then Tryptose broth and the least one was nutrient broth . The growth of B*rucella melitensis* as measured by detection of viable count and the best media was brain heart -infusion then Potatoes broth then Tryptose broth and the least one was nutrient broth. (Fig 11).

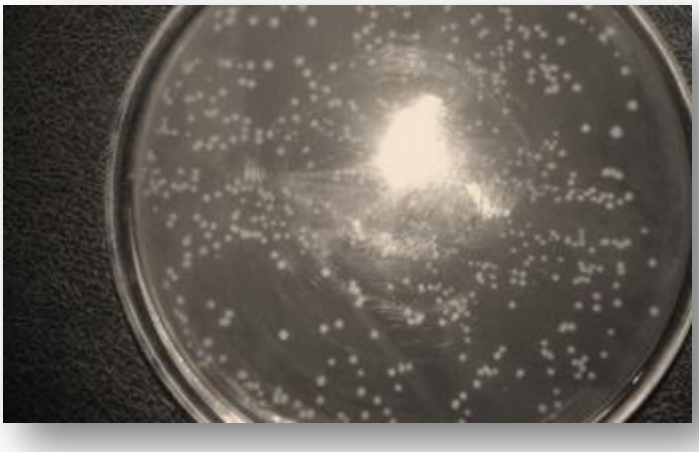


Fig (10)The Colony count of *Brucella abortus* in potatose infusion broth medium at 37^{o}C

Fig (11) The Colony count of *Brucella melitensis* in Brain heart infusion broth medium at 37^{o}C

4.5 The Identification of *Brucella* species:

4.5.1 Macroscopic appearance (Stereoscopic Microscope) :

4.5.1.1 The characterization of colony:

Individual colonies obtained over the surface of solid media were observed by obliquely transmitted light on low power stereoscopic microscope, the colony was round and honey pale color . When looked from above it was round and white color. Fig (12). Colony of *B*rucella. *Melitensis* was larger than *B*rucella.*abortus*. (Fig 13) *B*rucella.*abortus* and (Fig 14) *B*rucella. *melitensis*

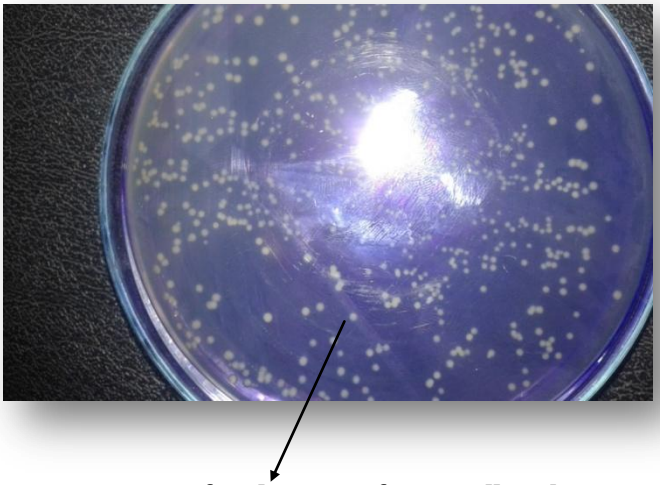


Fig (12): The appearance of colonies of *B*rucella.*abortus* in potatoes agar when viewed from above

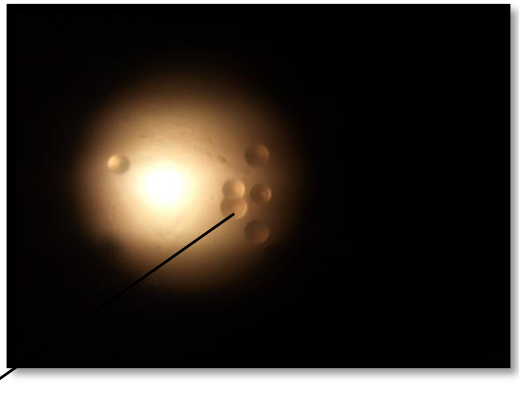


Fig (13) Colonies of *Brucella*.*abortus* in potatoes gar medium under stereoscopic microscope

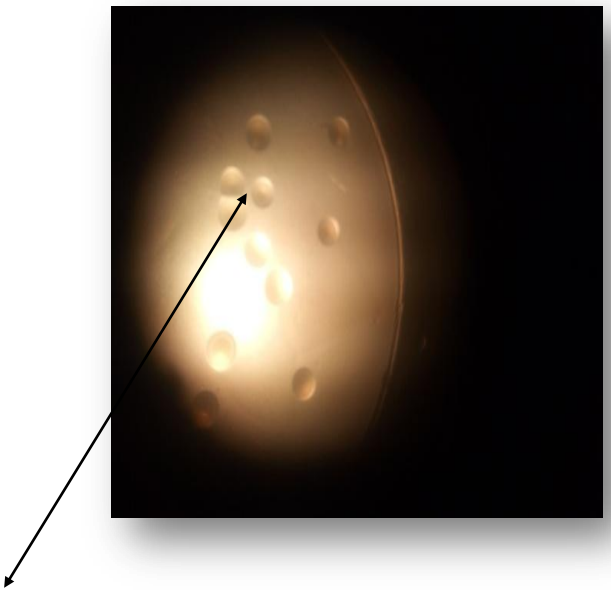


Fig (14) colonies of *B*rucella. *melitensis* on tryptose agar medium udder stereoscopic microscope

4.5.1-2 Staining of colonies by crystal violet:

Colonies did not take up the dye under the stereoscopic microscope; colonies were found pale yellow which indicated 100% smooth colonies. Fig (15).

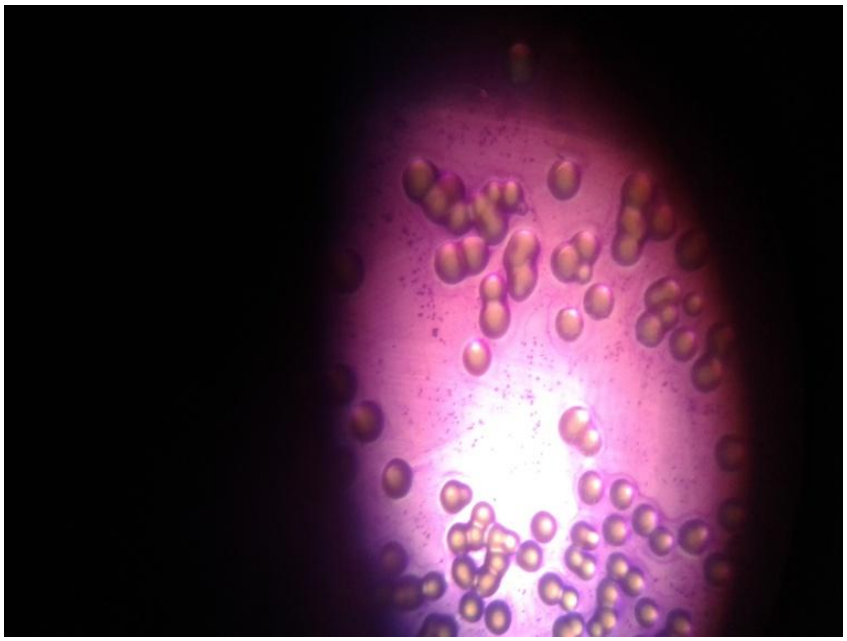


Fig (15) The uncolored smooth colonies of *B*rucella.*abortus* when staining by crystal violet and viewed by stereoscopic microscope

4.5.2 Microscopic appearance:

Smear from suspected samples or from pure culture isolates were fixed with heat and stained by the two methods: Gram's and Zeihl-Neelsen stain method. The organisms were Gram-negative, acid fast, coco bacilli, short and red color with gram stain Fig (16) and Brucella organisms appeared as red cocci or coco-bacilli against blue back ground and.

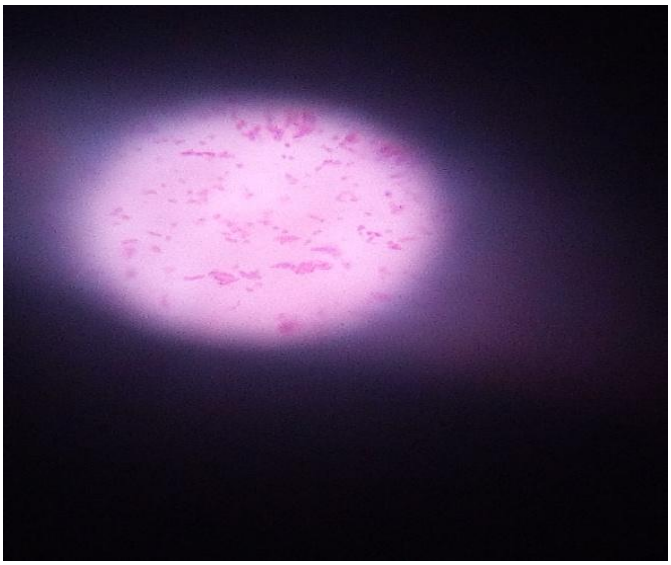


Fig (16) *Brucella melitensis* organism stained by gram stain under microscope

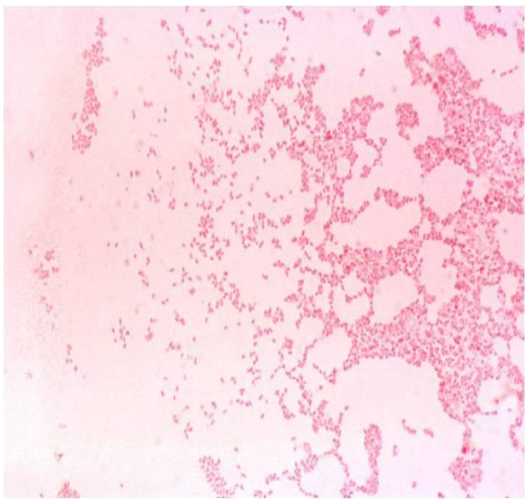


Fig (17) *Brucella melitensis* organism stained by Zeihl Nielsen under microscope

4.6 The typing of *Brucella* species:

4.6.1 The growth in a partial CO_2:

Brucella organisms isolated from two blood samples and rev-1,Strain19 (used as standard) all were CO_2 dependent for Growth. Table(5).

4.6.2 Production of H_2S:

4.6.2.1 *Brucella. melitensis* and Rev-1:

The strips were uncolored which indicated that H_2S was not produced by *B. melitensis* Fig(18) and Rev1 Fig (19)

Fig (18) *Brucella melitensis*

Flg19*Brucella melitensis*(M) *Rev-1*

4.6.2.2 *Brucella. abortus* and strain-19(as standard):

The strips in black colors that indicated H_2S was produced by *Brucella. abortus* Strain19 Fig (20) and *Brucella. abortus* (21) in potatoes agar medium and Table(5)

Fig (20) produced of H_2S by *b. abortus* Strain19

Fig (21) The black color of strips by H_2S produced by *Brucella.abortus* Fig (21)*B.* potatoes agar medium

4.6.3 The growth of *Brucella abortus* and *Brucella melitensis* in dyes:

4.6.3-*1Brucella.melitensis.*

grew on media containing the three dyes Basics fochsin.2%, Thionin (.2%) concentration 1:50000 and Safranin0 (.5%) concentration 1:100000 Table(5).

4.6.3.2 Strain -19 (as standard): did not grew on media containing Thionin (.2%) (10 ml/l medium) and Basic- fuchsine (**.**2%) (10ml/l medium) with final concentration 1:50000 but grew in media containing safranine0 .5% with final concentration1:100000. Table(5).

4.6.3.3 Rev1:

not grew on media containing Thionin (.2%) (10ml/lit medium) and Basic-fuchsine. 2% (10ml/l medium) last concentration 1:50000 and media containing Safranino0 .5% with final concentration1:100000 see Table(5).

4.6.3.4 *Brucella.abortus*:

not grew on media containing Thionin and basic fuchsine with final concentration 1:50000 and safranine0 (.5%) with final concentration 1:100000. Table(5).

4.6.4 phage lysis:

Culture of *brucella melitensis* isolate was not lysed by phage, Weighbridge (Wb), Tbilisi (Tb), Frinzy (Fi) and rough canis (Rc). Culture of *Brucella.abortus* isolate was lysed by phage Tb, Wb and FI. and did not lysed by R/C. Fig (22) Table 5..

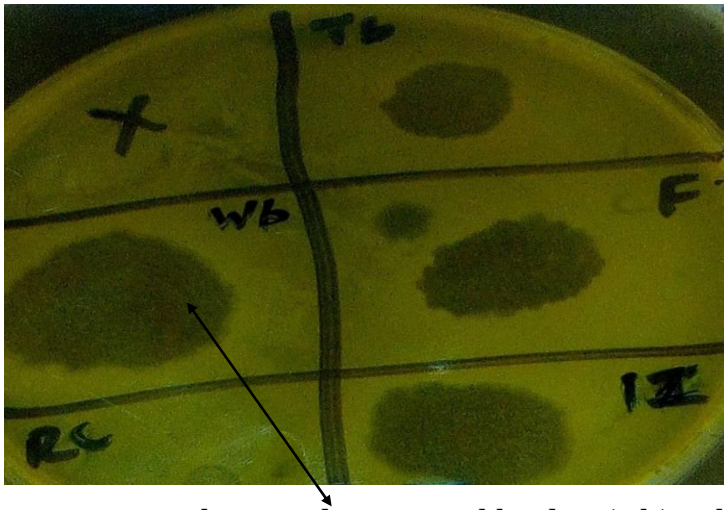


Fig (22) Phage Typing showing lysis Weighbridge (wb), Tbilisi (Tb), Frinzy (fi) and rough canis (RC)

4.6.5 Mono specific anti sera: There was agglutination by *Brucella. melitensis* isolate with Melitensis mono specific anti-sera Fig(23).

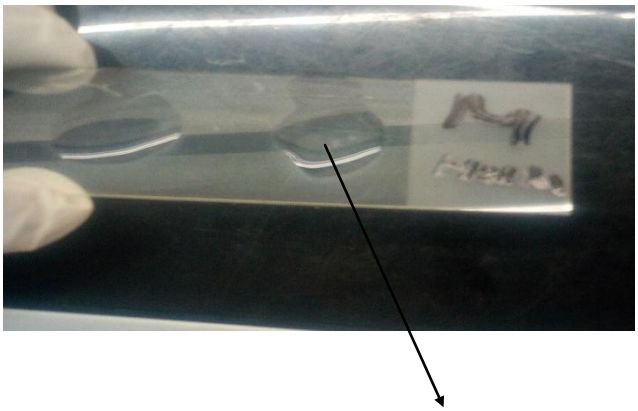


Fig (23) mono specific anti sera showing the agglutination of *Brucella. melitensis* with Mono specific Melitensis anti-sera(

In *Brucella.abortus* isolate there was agglutination with mono specific Abortus anti sera(A). (fig (24) and Table(5))

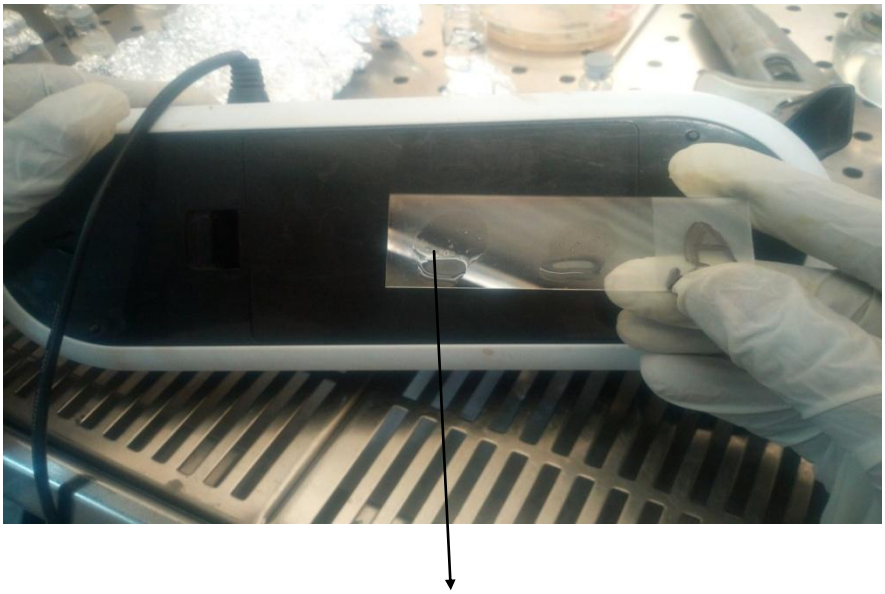


Fig (24) **mono specific anti sera agglutination of *Brucella.abortus* isolates with mono specific Abortus anti-sera(A).**

Table (5): The tests of *Brucella* Typing:

Species	CO_2	H_2S	Biochemical testes			Agglutination in sera		Phage lysis				
			Growth in dyesC 1:50000									
			Thionin	Basic fuchsine	Safranino	M	A	TB	IZ	WB	R/c	FI
B.abortus	±	+	_	_	_	-	+	L	L	L	NL	L
B.melitinsis	±	_	+	+	+	+	_	NL	NL	NL	NL	NL
Rev1	±	_	_	_	_	+	-	NL	NL	NL	NL	NL
Strain19	±	+	_	_	+	-	+	L	L	L	NL	L

M=mono specific *B.melitensis* serum, A=mono specific *B.abortus* antisera,TB=Tbilis=, IZWB= webridge, R/C rough strain, L=confluent lysis NL=no lysis and C=concentration of dyes

4.6.6 The amplification of the DNA by PCR:

Two isolates (*B.abortus* and *B. melitensis*) were tested by AMOS primers for Brucella species detection. The *Brucella abortus* measured 498 bp while the Brucella melitensis measured 720bp. DNAs fragments bp bands were visualized in electrophoresis.

Figure 24 indicated the *Brucella abortus* 498 bp. While figure 25 shows the characteristic 720 bp amplified DNA bands of *Brucella melitensis*

Figure(25).

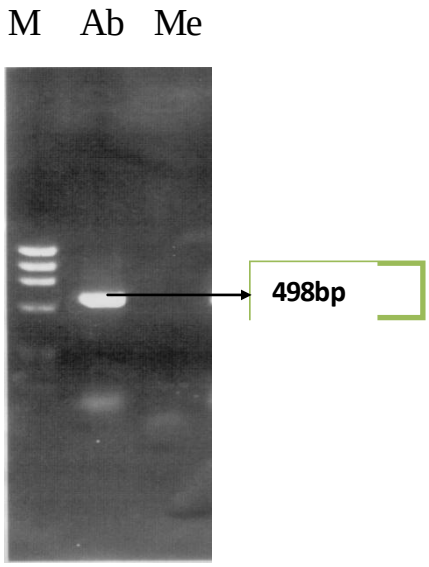


Fig. (25) The amplification of the field isolates DNAs by the *Brucella abortus* specific primers. Note the amplified 498bp DNAs fragments.

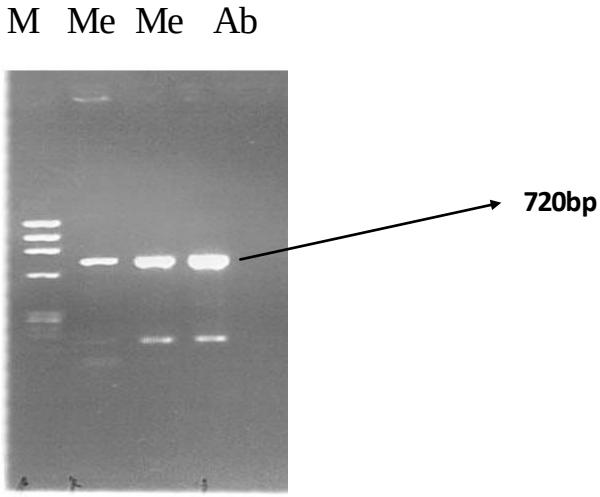


Fig. (26) The characteristic 720bp amplified DNA bands of *B*rucella. *melitensis* DNAs when amplified with its specific primes

CHAPTER FIVE

1.5 DISCUSSION

*Brucella*e are fastidious and slow growing organism when cultured from clinical material that is often grossly contaminated. It is necessary to use selective media for isolation trials, which must inhibit the growth of contaminants. There are many selective media available for the isolation of *Brucella* from contaminated material. Both serum Dextrose agar and Tryptose agar medium were currently recognized and used as an agreed international formulation recommended by the Reference laboratories. Most of the laboratories usually prepare their own media for routine diagnostics as well as research purposes. To ensure that the media is of good quality and capable of giving satisfactory results, proper quality management system is essential. For that purpose, certain parameters of media prepared were checked and then approved for laboratory use (Krisher *et al*, 2004).

This study aimed to develop a national Standard Operating Procedures (SOPs) for isolation, identification and characterization of *Brucella* field isolates presented to the laboratory.

In this study, The absolute diagnosis of brucellosis requires isolation of the bacterium from blood or knee joint samples, culture is considered as the reference standard method for diagnosis of *Brucella spp*. in humans and in animals as it is the gold proof of the presence of the disease in specific areas. The organisms were used in this study sample of knee joint fluid collected from sero positive animal and blood sample from febrile human subjected for bacteriological examination, we recorded the isolation and identification of *Brucella abortus* biovar 6 from animal sample and *Brucella melitensis* biovar 1 from human sample.

Six selective media were used, versus serum dextrose agar supplemented with antibiotics it gave good result this agrees with Corbel and Hendry (1983) they said that where contaminating organisms are a possibility, selective media e.g. SDA supplemented with antibiotics was found to be the only useful tool. it has to be noted that present formulations of Brucella-selective media have only been tested extensively on *B. abortus, B. melitensis* and *B. ovis* and that, although unquestionably useful, they are inhibitory for some strains. Moreover, present formulations of selective media are different because of differences in antibiotic sensitivity, in particular, bacitracin and nalidixic acid, between *B.abortus* and *B. melitensis* (Marín *et al*., 1996). Although it is not known to what extent, these media are probably satisfactory for other Brucella species.

Alton *et al* (1975) found bacterial isolates were cultured on trypticase soy agar containing 5% sheep's blood. The Brucella AMOS PCR assay was developed to identify and differentiate specific Brucella species. In this study Brucella AMOS PCR test was evaluated to differentiate between *Brucella abortus* versus *Brucella melitensis*, Bricker and Halling (1995). The Brucella AMOS PCR correctly identified each isolate as defined by the conventional methods.PCR was shown to be a valuable tool for identifying field strains of *Brucella*. The conventional methods of identification require a minimum of 5 days to identify an isolate to Brucella species and biovar level. This can delay the results and allow for contamination of the Farm it was characterized for identity and differentiated from Brucella field strains

In this study all media in the trials passed the ecometric assessment, indicating that their formulation will provide an acceptable medium for isolation of *Brucella* even if the sample contains small numbers of viable organisms as 1bacterium in I ml of solution. The growth of culture at Potatoes, Serum dextrose, Tryptose, Thyr.martin, Tryptose soya and Blood

agar, were 100% at an atmosphere of 10% CO_2 at anaerobic conditions, the growth in Potatoes agar was 60%, Serum dextrose agar(SDA) 65%, Tryptose agar 75%, Thyr.martin55%, Tryptose soya agar 85% and blood agar 100% . This agrees with Huda Moneam (2021), at aerobic incubation at 37^0C for 3-5 days she found the growth was 100% in potatoes agar , Serum dextrose agar 85%, Tryptose agar 70%, tryptose soya agar 70% and blood agar 50%.

For the bacteriological examinations, *Brucella* were identified by morphological, cultural and biochemical characteristics such as CO_2 requirement, H_2S production, growth in the presence of Thionin and basic fuchsine (20 μg/ml). The strains were bio typed by agglutination with mono specific Abortus anti sera and Melitensis antiserum. After isolation, the Brucella are identified by cultural, oxidative metabolism tests, biochemical, phage lysis, and molecular biological criteria

In this study *Brucella.abortus* isolate was ± CO_2 dependent for growth this disagree with Nassir (2007) he found *Brucella* organisms was isolated (*B.abortus* biovar 6) was CO_2 dependent for growth, Alton (1988) found *B.abortus* biovar 6 was CO_2 dependent for growth, *Brucella*. *melitensis* isolate was ± CO_2 dependent for growth, this disagree with WHO (2006), Alton found *B. melitensis* biovar 1(-) CO_2 dependent for growth At H_2S production *B.abortus* isolate was positive, *Brucella melitensis* was negative results this agree with Nassir(2007) and Alton (1988).

Brucella abortus isolate failed to grow in media containing Thionin 2% , Basic 2% fuchsine and Safranin O 5% this disagrees with Nasser who found the growth at basic fuchsine was positive, *B. melitensis* grew in media contain basic fuchsine, Thionin 2%, and safranin O 5% .this agree with OIE (2016).

At Phage lysis no lysis on culture of *B. melitensis* and lysis with *B.abortus* this agrees with Nassir (2007) and Alton(1988).

The high specificity of the genus specific-PCR can be considered as a powerful alternate tool for rapid diagnosis of brucellosis,(Schuring, 2002). The high degrees of sensitivity and specificity of the PCR assays compared to the conventional methods, together with its speed, versatility in sample handling, and risk reduction for laboratory personnel, make this technique a very useful tool for the diagnosis of focal complications of brucellosis. This promising line of research will require further assessments of numbers of strains large enough to draw definite conclusions on the usefulness of particular protocols or methods used during this study. Identification and typing of *B.abortus* biovar 6 **.**The present document described detailed standard techniques that allows research and bacteriological identification of the genus Brucella in animal and human samples. These are isolation technique on both enriched liquid and solid culture agar media. The described identification techniques enable a presumptive identification of bacteria of the genus *Brucella* of the species: *B. abortus* and *B. melitensis*. These Procedures will be extensively employed to assist with working safely. This study successfully achieved advanced and direct molecular diagnostic procedures for the diagnosis of brucellosis. Using the genus-specific PCR assay. The high degrees of sensitivity and specificity of the PCR assay, together with its speed, easiness in sample handling, and risk reduction for laboratory personnel, make this technique a very useful tool for the diagnosis of brucellosis and this agrees with (Al Dahouk and Nockler (2011) and,(Schuring, 2002). The high specificity of the genus specific-PCR can be considered as a powerful alternate tool for rapid diagnosis of brucellosis,(Schuring, 2002).

During the course of this study, the conventional diagnosis of suspected samples performed using the cultural, morphological, biochemical and the Brucella phage typing revealed unequivocal results to the biovar levels.

In this study, two different methods of extraction of bacterial DNA from the Brucella isolates to improve its direct detection by PCR were evaluated. The inefficient bacterial DNA extraction could account for PCR-negative results.

The release of bacterial DNA by a single-step procedure based on freezing and thawing steps was evaluated. Simple sample-processing method did not enhance the efficiency of Brucella DNA; the PCR amplifications were always weak or even negative due to the degradation of the DNA.

Although the cell envelopes (CE) of most gram-negative bacteria are sensitive to Tris buffers and EDTA (Moriyón and Berman, 1982) and have shown that Brucella CE was more resistant to nonionic detergents, EDTA, and Tris than were those of Escherichia coli. Likewise, ionic detergents, such as SDS, have a limited action on *B abortus* CE under conditions used with CE of other gram-negative bacteria. These data proved that the Brucella CE is held by forces stronger than those acting in the CE of other bacteria.

One of the main targets of this study was to evaluate advanced and direct molecular diagnostic procedures for the diagnosis of brucellosis. Using the genus- specific PCR assay, when the identification of the clinical samples as *Brucella* is adequate, was successfully achieved. Specific primers for amplification of *B. abortus* and *B. melitensis* used. The results obtained yield a fragment size of 498bp for *B. abortus* and 720bp for *B. melitensis*. The results obtained came on line with Moulana *et al*., (2016).The Brucella AMOS PCR assay was developed to identify and differentiate specific Brucella species. In this study Brucella AMOS PCR test was evaluated to

differentiate between Brucella abortus versus *Brucella melitensis*, Bricker and Halling (1995). The Brucella AMOS PCR correctly identified each isolate as defined by the conventional methods.PCR was shown to be a valuable tool for identifying field strains of Brucella. The conventional methods of identification require a minimum of 5 days to identify an isolate to Brucella species and biovar level. This can delay the results and allow for contamination of the Farm.

CONCLUSION

- Well established Standard Operating Procedures (SOPs) were developed in the *Brucella* Laboratory.
- Brucella organisms were isolated according to morphology, colony appearance, growth characteristics and typing procedures. They were found to be *Brucella abortus* which was isolated from a sample of swelling in the knee joint of a cow and was found to be *B. abortus* biovar 6 and the other which was isolated from a sample of a febrile patient was proved to be *Brucella melitensis* biovar 1.
- The Ecometric technique was successfully used and was based on streaking inoculums of bacteria. It gave excellent numerical measurement of the performance of most of the media tested and was suitable and acceptable for isolation of *Brucella* even if the sample contains very small numbers of viable organisms.
- The sensitivity and specificity of the PCR make this technique a very useful tool for the diagnosis of brucellosis.

RECOMMENDATION

- For epidemiological sero-surveillances studies and bacteriological studies, isolations and characterization of organisms are most required to elucidate the exact situation of the disease in the country.
- Development of Molecular laboratory for brucellosis to help in advancement of diagnosis and reducing the bacteriological laborious and human risk.
- Knowing the exact causative agents of diseases will help in planning for applied operational control approaches in different parts of Sudan.

REFERENCES

Abbas, B. and Agab, H. (2002). *Areview of camel brucellosis. Prev Vet Med. 2002 Sep 10;55(1):47-56.*

Abbas, **B.**,**ElZubeir A. E. A. and Yassin, T. T. M., (1987).** *Survey for certain Zoonotic diseases in camels in Sudan*. Revue de ` Elevage ET Medicine Veterinaire des Pays Tropicaux 40(3), 231-233.

Abdalla, et al. (1966). Incidence of animal brucellosis in Wadi Halfa District. Sudan *J. Vet. Sci. Anim. Husb., 7: 28 – 31.*

Abou-Eisha, A. M. (2000): Brucellosis in camels and its relation to public health. *Asuit Veterinary Medical Journal 44 (87): 54-64.*

Abu Damir, H., Kenyon, S. J., khalfalla, E. A. and Idris, F. O., (1984).

Abubakar M, Mansoor M and Arshed M (2012). Bovine brucellosis :old and concepts. with Pakistan perspective. *Pakistan Veterinary Journal, 32:147-155.*

Acha, N.P. and Szyfres, B., (2003). *Zoonoses and Communicable Diseases Common to Man and Animals, third ed., vol. 1*. Pan American Health Organization (PAHO), Washington, DC.

Agab, H., (1997). *Clinical signs of animal brucellosis in eastern Sudan*. Revue de` Elevageet Medicine Veterinaire des Pays Tropicaux 50 (2), 97- 98.

Agab, H., Abbas, B., El Jack Ahmed, H., Mamoun, I.E., (1994*).* *First report on the isolation of Brucella abortus biovar 3 from camel(Camelusdromedarius) in the Sudan*. Revue. delevage et de medicine veterinarie des pays tropicaux 47, 361–363.

Agab, H.; Abbas, B.; El Jack, H. and Mamon, I. E. (1995). First report on the isolation of *B. abortus* biovar 3 from camelusdromedarius in Sudan. Revere Elev. Med. Vet. Pays. Trop. 47, 361 – 363.

Al Dahouk S, Nockler K. (2011). Implications of laboratory diagnosis on brucellosis therapy. Expert Rev Anti Infect Ther. 2011; 9(7):833–45.

Alton *et al* (1975).Techniques for the brucellosis laboratory.2nd edition. Paris: institute National de la recherché eagronomique; 1975.

Ariza, J. (1999). Brucellosis: An update. The perspective from the mediterranean basin *Rev*. Med. Microbiol., vol. 10, 125–136.

Babiker *et al*. (2009). Spatial price transmission: *A study of Sheep marketing in Sudan.* in Alfajer. *Volume 3* (*1): pp 44*.

Baily, et al. (1992). Detection of Brucella melitensis and Brucella abortus by DNA amplification. *J. Tro.Med. Hyg , 95:271-5*.

Bang, B. (1897). The etiology of epizootic abortion. *J. Comp. Path. Therap., 10: 125-149.*

Bellis,M and roizes,G.(1988). DNA polymorphism in strains of the genus *Brucella*. *J. Bact., 170:4603*.Verger, J. M.,Gremont, F, Gremont,P. A .D. And Grayon M. (***1985***)*.Brucella*, a mono specific genus as shown by DNA hybridization, in *.J.Syst. Bact., 35:292***.**

Bennett, S. C.J. (1943). Annual report of the Sudan veterinary Service. pp29-30.Biochemical characteristics of *B. abortus and B. melitensis. J. Infect. Dis.Vol. 27, (1920), pp. (173– 184)*.

Blasco, JM. (1997). A review of the use of B. melitensis Rev 1 vaccine in adult sheep and goats. Prev. *Vet. Med. Vol. 31, (1997), pp. (275–283).*

Bosch J.; *et al* (1986). In vitro activity of ciprofloxacin, ceftriaxone and five other antimicrobial agents against 95 strains of *Brucella. melitensis J. Antimicrob.Chemother., vol. 17, 459–461*

Bricker, B. J., Halling, S. M. (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J ClinMicrobiol. 1995;33:1640–1642.*

Bruce, **D**. **(1887).** Note on the discovery of a microorganism in Malta fever. Practitioner, 39: 161-171.

Bruce, D. (1888). Note on the discovery of a microorganism in Malta fever. The Practitioner, 39: 160-170.

Buddle, M. B. (1956). *Studies on Brucella ovis (n. sp.), a cause of genital disease of sheep in New Zealand and Australia*. *J . Hygiene, vol. 54, 351–36.*

Capasso, L., (2002). Bacteria in two-millennia-old cheese, and related epizoonoses in *Roman Population, J. Infect. 45: 122–127*.

Carmichael, L.E. and Bruner, D.W. (1968). *Characteristics of anewly-recognized species of Brucella responsible for infectious canine abortions*. Cornell Vet., 48 (4) , 579–592.

Cetinkaya, B.; H. Ongor; A. Muz; H. B. Ertas; H. Kalender and H. M. Erdogan; (1999). *Detection of Brucella species DNA in the stomach content of aborted sheep fetuses by PCR* Vet. Rec., vol. 144, 239–240.

Claraveau, *et al*. (1998). *Phenotypic and molecular characterization of a Brucella strain isolated from a minke whale (Balaenopteraacutorostrata)*. Microbiology 144:3267-3273.

Corbel, M. *et al*. (1983). Method for the identification of *Brucella*. Ministry of Agriculture, Fisheries and Food, London.

Corbel, M. J. (2006). *Brucellosis in humans and animals*. Geneva, Switzerland, WHO pres UN.

Cutler *et al* (2005). Brucellosis –new asects of an old disease. *Journal of applied microbiology., 98:1270-1281*.

Dafaalla, .E.N., and Khan, A. (1958). *The occurrence, epidemiology and control of animal brucellosis in Sudan*. Bull.Epiz.Dis.Afr., 6:243-247.

Dafalla, E. N. (1962). Incidence of animal and human brucellosis in the Sudan. *Sudan J Vet. Sci. and Anim. Husb., 3 (2): 80 – 88*

Dahouk, *et al* (2007). Changing epidemiology of brucellosis, *Germany, 1962–2005*. Emerg. Infect. *Dis. 13, 1895–1900*.

Davies, G. and Casey, A. (1973). The survival of Brucella abortus in milk and milk products. *Brit. Vet. J.,129: 345 - 353*.

De Massis, *et al* (2005). *Correlation between animal and human brucellosis in Italy during the perio 1997–2002*. *Clin. Microbiol*. Infect. *11, 632–636*.

Detilleux, Philippe G., Billy L. Deyoe, and Norman F. Cheville. (1990). "Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro." Infection and Immunity, vol. 58, no. 7. American Society for Microbiology. (2320-2328).

Doern,G.V.,(2000). *Detection of selected fastidious bacteria*.Clin.Infect.Dis.30, 166.

Edgardo Moreno,dagger and Ignacio Moriyón Dagger(2002). "*Brucella melitensis*: A nasty bug with hidden credentials for virulence." PNAS. January; 99(1): 1-3.

El Sanousi, Enaam M. (1999). *The effects of various factors on the viability of Brucella abortus Strain 19 vaccines*. M. V. Sc. Thesis. University of Khartoum, Sudan.

El Sanousi, Enaam M. (2006). *Molecular Diagnosis of Brucella species*. Ph.D. Thesis, University of Khartoum, Sudan**.**

El Sanousi, Enaam M. and Judy Stack (2011).*OIE Twinning - A valuable concept for regional development of brucellosis .the Fifth Brucellosis* International Research Conference, Buenos Aires, Argentina, 21st to 23rd September 2011.

Elberg S. S.; (1973). *Immunity to Brucella infection*. Medicine, vol. 52, 339–356.

Elzer, P. H.(2007). *Brucellosis vaccines for 21st century.* NIAA Annual Meeting Proceedings Louisiana State University, L A, 70803**.**

Erwa, H. H. (1966). Isolation of Brucella abortus in the Sudan. *Sudan J. Trop. Med. HygSep; 69(9):201-2*

European commission (1999). The modification of technical annexes of council directive 64/432/EEC to take account of scientific Devolopments regarding tuberculosis, Brucellosis and Enzootic bovine leucosis. Report of the scientific committee on Animal health and Animal Welfare adopted 11 Octoper 1999. Sanco/B3/R10

Evans A. C. (1918). Further studies on Bacterium abortus and related bacteria. II. A comparison of Bacterium abortus and Bacterium bronchisepticus with the organism which causes Malta fever. *J. Infect. Dis., vol. 22, 580–593.*

FAO (1998). *A perspective of brucellosis surveillance in North Africa and the Middle East.* Studies in Algeria, Iran, Kuwait, Morocco, Oman, Sudan and Syria. FAO consultancy Mission Report**.**

FAO,(2009). *Brucella melitensis* in Eurasia and the Middle East,Technical meeting in collaboration with WHO and OIE Rome, May**.**

FAO. 2010. *Brucella melitensis* in Eurasia and the Middle East.FAO Animal Production and Health Proceedings. No.10. Rome.

Fayza, A. O.; *et al* .(1990). Survey of brucellosis among cattle, camels, goats and sheep in the Sudan, S. *J. Vet. Res. 9: 36-40.*

Fekete *et al*. (1990). Preliminary development of a diagnostic test for Brucella using polymerase chain reaction. *J. Appl. Bacteriol; vol. 69(2), 216-27.*

Fekete, *et al*. (1992). *Amplification of fragment length polymorphism in Brucella strains by use of PCR with arbitrary primers.* J. of Bact. 174, 7778 – 7783.

Félix J. *et al (*2000*).* "The genes for erythritol catabolism are organized as an "inducible operon in *Brucella abortus." Microbiology; 146, 487-495***.**

female camels for antibodies against *Brucella*, Chlamydia, Leptospira, BVD / MD, IBR / IPV and Enzootic bovine leucosis virus. *Deutche Tierärztliche Wochenschrift 97 (3), 134 –135.*

Flowers, A. I. (1977). *National programs, In: Bovine Brucellosis,* R. P. Crawford and R. J. Hidalgo, pp. (333–415), Texas A&M University Press College Station, TX.

Food and Agriculture Organization of the United Nations (FAO) (2003). Guidelines for coordinated human and animal brucellosis surveillance. FAO Animal Production and Health Paper No. 156. FAO, Rome

Foster G et al (2007). *Brucella ceti spp*. nov.and *B. pinnipedialis* spp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *International Journal of Systematic Evolution Microbiology,57: 2688-2693*.

Gameel, *et al* (1993). Prevalence of camel brucellosis in Libya. *Trop Anim Health Prod*. *25:91- 93*.

Geresu MA, *et al* (2016). Seropositivity and risk factors for Brucella in dairy cows in Asella and Bishoftu towns, Oromia Regional State, Ethiopia. *African Journal of Microbiology Research, 10(7): 203- 213*.

Godfroid J., Scholz H. C., Barbier T., Nicolas C., Wattiau P., Fretin D., et al. . (2010). Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. Prev. Vet. Med. 102, 118–131.

Gul, S.T and Khan, A., (2007*).* Epidemiology and epizootology of brucellosis. A review .*Pak. Vet.J. 27:145-151*.

Habiballa, N.; Dafalla, E. A. and Omer, E. E. (1977).*Studies on bovine and human in the Sudan*. The incidence of brucellosis and the species of *brucella*

Hadush A and Pal M (2013). Brucellosis: An infectious re-emerging bacterial Zoonosis of global importance. *International Journal of Livestock Health, 3:28-34*.

Hasseb, M. A. (1950). Undulant Fever in Sudan. *J. Trop. Med. 53: 241*

Higgins, R.; (2000). Bacteria and fungi of marine mammals: A review Can. *Vet. J., vol. 41, 105116*.

Huda M. A (2020) *Compliance of National Standard Operating Procedures for Brucella Biological Products*. Department of *Brucella*, central veterinary research laboratory. P. O. Box 8067,Amarat, Khartoum, Sudan.

Huddleson I. F.; (1929). The differentiation of the species in the genus *Brucella*. Michigan State College Agricultural Experiment Station Technical Bulletin No. 100. East Lansing, MI Agricultural Experiment Station, Michigan State University E. Lansing, MI.

Ibrahim, A. E. and Habiballa, N. (1975). Brucellosis in Messeriya cows of the Sudan. *J. Trop. Vet. Med.And Prod., 11: 245-246*.

International Air Transport Association (2003). Dangerous Goods Regulations, 44th Edition. International Air Transport Association, 800 Place Victoria, P.O. Box 113; Montreal, Quebec H4Z 1M1, Canada, pp 815

Ismail, Adil Abdel Rahman Ali (2007). *Prevalence of brucellosis in kuku dairy,Khartoum state and the susceptibility ofIsolates to some chemotherapeutic agents.MScthesis in Microbiology*. Department of Pharmaceutics, Faculty of Pharmacy. University of Khartoum.

Jahans, K. L.; G. Foster and E. S. Broughton (1997). The characterization of Brucella strains isolated from marine mammals *Vet. Microbiol., vol. 57, 373–382*

Jiménez de Bagüés, *et al* (1995).The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. *Vaccine, vol. 13, 301–304***.**

Kaoud,2010 H. A., Zaki, M.M., EL-Dahshan, A.R.and Nasr, S.A.(2010). Epidemiology of Brucellosis among farm animals Nature and science, *8(5): 190-197*.

Khalafalla, M. A.; Dafalla, E. A. and Bakhiet, M. R. (1987). Isolation and characterization of brucella organisms in the Sudan. Symposium on animal brucellosis in the Sudan.15-16 September 1987, Sudan.p.25-34

Krisher K, Callihan DR, Jones RN, Luper DC, Miller JM, Sharp SE, (2004). Quality Control for Commercially prepared Microbiological Culture Media; Approved Standards-3rd edn. NCCLS M22-A3:24.

M. and Zinsstag, J., (2003). Brucellosis and Q-fever seroprevalences of nomadic pastoralists and their livestock in Chad. Preventive Veterinary Medicine, 61, 279–293.-RFLP analysis. *Iran J Pub Health. 2006; 35:22–27*.

MacMillan, A.P. and Cockrem, D.S. (1985). Reduction non specific reactions to the *Brucella abortus* serum agglutination test by the addition of EDTA. Res. Vet. Sci., 38: 238-291.

Majid, A.A., Goraish, L.A and El Mansoury, Y.H.A. (1999). Sero-epidemiological observation of camel brucellosis in Eastern and Western Sudan. *Sud., J. Vet. Sc.Anim., Husb 38 (1-2) pp 178-184*.

Mantur, B. G., Mangalgi, S. S. and Mulimani, B. (1996). *Brucella melitensis*: A sexually transmissible agent? *Lancet 347: 1763*.

Mantur, *et al*. (1996). *Brucella melitensis***:** A sexually transmissible agent? Lancet 347: 1763.field, T.L. and Hoover, D.L., (2001). Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*. *Am. J. Vet. Res. 62, 398405*.

Marin, C, M: Alabart, J. L. and Blasco, J.M. (1996). Effect of antibiotics containd in tow Brucella selective media on growth of *B. abortus, B. melitensis* and *B. Ovis. J .Clin. microbial., 34: 426-428.*

Marín, C. M. et al. (1989). Efficacy of long-acting oxytetracycline alone or in combination with streptomycin for treatment of Brucella ovis infection of rams. *Amer. J. Vet. Res., Vol. 50, (1989), pp. (560–563)*.

Masoumi, J. P., Sheikh, M. A., Ahmad, R., Naeem, M., Ahmad, M. and Hussain, I., (1992). Seroprevalence of brucellosis in sheep, goat and man in Lahore, India. Indian Journal of Dairy Science 45 (6), 298 – 299.Romero, C. et al. (1995a). Specific detection of *Brucella* DNA by PCR. *J. Clin. Microbiol., Vol. 33, (1995), pp. (615–617)*.

Matar, G. M., Khneisser, I. A., Abdelnoor, A. M. (1996). Rapid laboratory confirmation of human brucellosis by PCR analysis of a target sequence on the 31-kilodalton *Brucella* antigen DNA. *J ClinMicrobiol.;34(2):477-8. doi: 10.1128/JCM.34.2.477-478.1996. PMID: 8789045; PMCID: PMC228827*.

Mayfield, J. E., Bantle, J. A., Ewalt, D. R., Meador, VB., Tabatabai LB. (1990) Detection of Brucella cells and cell components In: Animal Brucellosis, *K. Nielsen and J. R. Duncan, pp. (98–120), CRC Press Boca Raton, FL.*

McDonald, W. I., Compston, A., Edan, G., (2001). Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann

Mense, M.G., Van De Verg, L.L., Bhattacharjee, A.K., Garrett, J.L., Hart, J.A., Lindler, L.E., Hadfield, T.L. and Hoover, D.L., (2001). Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*. *Am. J. Vet. Res. 62, 398–405.*

Meyer KF. & Shaw EB.(1920).A comparison of the morphologic, cultural and

Miles,A.A.and misra,s.s.(1938) G,Hyg. (lond.),38,732.

Montasser, A.M., (1999). Some epidemiological and diagnostic studies on ovine and caprine brucellosis in Egypt. In: Proceedings of the 5th Science Congress on Egyptian Society for Cattle Disease, Assiut, November 1999.

Morata, P. *et al*. (1999). *Author's reply. J. Clin. Microbiol., Vol. 37, pp. (1654–1655).*

Morata, P. et al. (2003). Development and evaluation of a PCR-enzyme-linked immunosorbent assay for diagnosis of human brucellosis. *J. Clin. Microbiol., Vol. 41, pp. (144-148).*

Moreno,E., Moriyon,1.,(2002). *Brucella melitensis*: an sty bug with hidden credentials for virulence.proc.natl.Acad.Sci.U.S.A.99,1-3.

Mossel *et al* (1983).Quality assurance of selective culture media for bacteria moulds and yeast: An attempt to standardization at international level. *J Appl Bact; 54:313- 27.*

Moulana Z, Roushan MR, Marashi SM (2016). Evaluation of Different Primers for Detection of Brucella by Using PCR Method. Electron Physician. *2016; 8(11):3222– 3227.*

Mousa, RM. *et al*. (1986). Brucella meningitis: presentation, diagnosis and treatment—a prospective study of ten cases. *Quart. J. Med., vol. 233, (1986), pp. (873–885).*

Musa *et al.* (1990). *Brucella* biovars isolated from nomadic cattle in the Southern Darfur province, *Western Sudan. J. Comp. Path., 102: 49 – 54.Musa,* **M.T; Enaam M. Elsanousi; Angra, Tamadur E.E. and Ali, A.A. (2008).** Brucellosis, a challenge to veterinarians in Africa: The situation of the disease in the Sudan. The proceeding of the first scientific conference 17-21/8/2008. Friendship Hall, Sudan pp:51.

Musa, M. T. and Shigidi, M.T.A.(2001). Brucellosis in camels in intensive animal breeding areas of Sudan: implications in abortions and early life infection. Revued' Elevageet Me´dicineVe´te´rinaire desPaysTropicaux,54,11-15.

Musa, M. T., Jahans, K. L. and Fadlalla, M. E. (1990). *Brucella* biovars isolated from nomadic cattle in theSouthern Darfur Province of western Sudan. *Journal of Comparative Pathology, 102, 49-54.*

Musa, M.T. (1995). *Brucellosis in Darfur States: the magnitude of the problem and methods of diagnosisand control. Ph.D*. Thesis, University of Khartoum, *pp. 73-98.*

Nasri, M. E. (1960). Brucellosis in southern Sudan. Vet. Rec., 72: 1200-1201.

Nassir (2007) *Serological,* *Bacteriological and Molecular study of Camel Brucellosis (Eastern Sudan)*. Department of *Brucella*, central veterinary research laboratory. P. O. Box 8067,Amarat, Khartoum, Sudan.

Navarro, E. *et al*. (2002).Comparison of three different PCR methods for detection of *Brucell* spp in human blood samples. FEMS Immunol Med Microbiol, *Vol. , pp. (147-151).Neurol.;50:121–127. [PubMed]*

Navarro, E.; Casao, M.A.; Solera, J. (2004). Diagnosis of human brucellosis using PCR. Expert Rev Mol Diagn. 4: 115-123.

Nicoletti, P. (1980). The epidemiology of bovine brucellosis. *Adv. Vet. Sci. Comp., 24, 69- 98.*

Nicoletti, P. *et al*. (1985). Efficacy of long-acting oxytetracycline alone or combined with streptomycin in the treatment of bovine brucellosis. *J. Am. Vet. Med. Assoc., Vol. 187, pp. (493–495).*

Nicoletti, P. et al. (1989). Efficacy of various treatment regimens, using liposomal streptomycin in cows with brucellosis. Amer. *J. Vet. Res., Vol. 50, pp. 1004–1007.*

Nielsen, K and Wu, W.L., (2010). Serological Diagnosis of Brucellosis, Contributions, Sec. Biol. Med. Sci., MASA, XXXI, 1, p. 65–89.

Nimri LF. (2003). Diagnosis of recent and relapsed cases of human brucellosis by PCR assay.BMC Infect Dis; 3:5.Nonphagocytic Cells In Vitro. Infection and Immunity, *Vol. 58, No. 7, (2003), pp. (2320-2328).*

OIE [Office international des epizooties] terrestrial manual (2009a), caprine and ovine brucellosis. (excluding Brucella ovis)Ch (2.7.2.). pp: 10.

Omer M.M., Abdelaziz A.A., Abusalab M.A. & Ahmed A.M.(2007). Survey of brucellosis among sheep, goats, camelsand cattle in Kassala area, eastern Sudan. *J. Anim. Vet. Adv.,6 (5), 635–637.*

Omran, Ehssan O. M. (2011). Prevalence of Brucellosis in different animal species and man in Sinnar state, Sudan. M.V.Sc. Thesis. Sudan Academy of Science. organisms isolated from cattle in three provinces. *Sudan Med. J., 15: 9-16*

Pappas, G., Akritidis, N., Bosilkovski, M., Tsianos, E(2005). Brucellosis. *The New England journal of medicine; 352(22); 2325-36.*

Pappas, G., *et al* (2006). The new global map of human brucellosis. Lancet Infectious Diseases 6, 91–99. *PCR. J Clin Microbiol 32:2660-2666*

Paris and *B. suis*), in Handbook Series in Zoonoses. FL, Vol. 2, 99

Pérez-Sancho M, García-Seco T, Arrogante L, García N, Martinez I, Diez-Guerrier A, (2013). Development and evaluation of an IS711-based loop mediated isothermal amplification method (LAMP) for detection of *Brucella* spp. on clinical samples. *Res vet sci. 2013;95(2):489–94.*

pp. (3198–3200).

Queipo-Ortuno, M. I. *et al*. (2005).Comparison between Light Cycler Real-Time Polymerase Chain Reaction (PCR) assay with serum and PCR enzyme - linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. *Clin Infect. Dis. Vol. 40, (2005), pp. (260-264).*

Quinn, P. J., Markey, B. K., Carte, M. E., Donnelly, W. J., Leonard, F. C. (2002): Brucella species. In: Veterinary Microbiology and Microbial Diseases. London: *Blackwell Science Ltd., pp. 168 – 172.*

Quinn. *et al* (1994). Clinical veterinary Microbiology. Elsevier limited London, UK. 24: 26-267, Radositis,O. M., Blood, D. C., Gay, C. C. (1994): Veterinary medicine. VII edition, Bailliere Tindall London, *pp.1379-1394; 1495-1499*

Radostits, O. M., Blood, D. C. and Gay, C. C., (1994). Brucellosis causedby B. abotus and B. melitensis. In: Veterinary Medicine: Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 8th ed. London: Bailliere Tindall, pp. 787-792.

Radwan, A. I. et al. (1993). Successful therapeutic regimens for treating Brucella melitensis and *Brucella abortus* infections in cows. Rev. Sci. Tech., *Vol. 12, (1993), pp. (909–922).*

Radwan, *et al* (1992). Serological and bacteriological study of brucellosis in camels in central Saudi Arabia. Rev. Sci. tech. Off. *Int. Epiz. 11(3): 837-844*.

Ray, W.C. and Steele, J.H., (1979). Brucellosis (due to *Brucella abortus*

Raytheon Polar Services Company (2006). McMurdo Medical Standard Operating Procedures and Protocols. Medical Department. ME – M 311. No. OPP 0000373.

Robichaud, S., Libman, M., Behr, M. and Rubin, E., (2004). Prevention of laboratory-acquired brucellosis. Clin. Infect. Dis. 38, 119–122.

Romero C. & López-Goñi I. (1999). Improved method for purification of bacterial DNA from bovine milk for detection of Brucella spp. by PCR. Appl. Environ. Microbiol., *Vol. 65, (1999), pp. (3735–3737).*

Romero, C. *et al*. (1995b). Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *J. Clin. Microbiol., Vol. 33, (1995),*

Roop, R. Martin; Jennifer M, Gaines;Eric, S. Anderson and Eric S Anderson (2009). Survival of the fittest: How *Brucella* strains adapt to their intracellular niche in the host. Medical Microbiology and Immunology 198(4):221-38

Salehi *et al* (2006). Isolation of *Brucella abortus* using PCR Schelling, E., Diguimbaye, C., Daoud, S., Nicolet, *J., Boerlin, P., Tanner*,

Schelling, E., *et al* (2003). Brucellosis and Q-fever seroprevalences of nomadic pastoralists and their livestock in Chad. Preventive Veterinary Medicine, *61, 279–293.cademic Publishers, pp. 358-362*.

Schloter, *et al* l. (2008). Genetic diversity and phylogenetic relationships of bacteria belonging to the Ochrobactrum-*Brucella* group by recA and 16S rRNA gene-based comparative sequence analysis. Syst. Appl. Microbiol. 311-16.

Scholz C, *et al* (2009). Isolation of *B.microti* from man dibular lymph nodes of Red foxes, vulpes, in Vector Borne Zoonotic Disease, *9: 153-156. Scholz, H. C., S. Al Dahouk, H. Tomaso, H. Neubauer, A. Witte, M.*

Scholz, H. C. *et al.* (2008). *Brucella microti sp*. nov., isolated from the common vole Microtusarvalis. Int. *J. Syst. Evol. Microbiol, Vol. 58, (2008), pp. (375–382)*.

Schurig, G.G *et al*, 2002. Brucellosis vaccines: Past, present and future. Vet. Microbiol., *90: 479-496. PMID: 12414166*

Seifert, S. H. (1996). Tropical Animal Health, 2nd Edition. Dordrecht: Dordrecht Kluwer: A

Selma Elsadig Ahmed Gabir (2006).*Brucellosis in Dairy Cattle in Khartoum State. MSc* Thesis in Microbiology, Department of Microbiology, Faculty of Veterinary Science, University of Khartoum.

Shallali, ***et al*** (1982). preliminary survey of mastitis and brucellosis in some dairy farm in the Blue Nile Province, Sudan. *J. Vet. Rs. 4: 34-41*.

Shigidi M.T.A (2010) Brucellosis in the Sudan, its epidemiology and control. (Unpublished)

Shigidi, M. A. and Razig, S. A. (1971-1973). Isolation of *Brucella abortus* from knee hygroma in a bull. Sudan *J. Vet. Sci. Anim. Husb., Vol. 14, (19971-1973), pp. (33-35)*.

Stamp, *et al* (1950). Enzootic abortion in ewes. I. Transmission of the disease. *Vet. Rec., 62, 251–254.*

Stoenner, H. G. and Lackman, D. B. (1957). A new species of Brucella isolated from the desert wood rat, Neotoma lepida.Thomas. Am. *J. Vet. Res. Vol. 18, (1957), pp. (942-951)*.

Sulieman, I .A (2011). *Epidemiology and Bacteriology of brucellosis in Sheep and Goats in south Kurdofan state Sudan*. *M.V.Sc*. Thesis. Sudan Academy of Science.

Tabak, *et al* (2008). family screening necessary in brucellosis Infection? *36, 575–577*.

Terzolo *et al* (1991). Skirrow agar for simultaneous isolation of *Brucella* and Campylobacter species. Veterinary \ Records *129 (24), 531 – 532*.

The Center of Food Security and Public Health (2009). Brucellosis. United States Department of Agriculture (USDA). CFSPH, Iowa State University.

Traum, J. (1914). Report of the chief of the Bureau of animal industry, U.S. Department of Agriculture, p.30.U.S.Department of Agriculture, Washington, D.C.

Unger F., *et al* (2003). Risk associated with bovine brucellosis in selected study herds and market places in four countries of West Africa. Animal Health Working Paper 2, International Trypanotolerance Centre, Banjul.

Verger, J.M. (1985). *B. melitensis* infection in cattle. In: *Brucella melitensis*, Plommet and Verger, eds. MartinusNijhoff Publ., Dordrecht-Boston-Lancaster. 197–203.

Vitas, A. I., et al., (1997). Protective effect of liposomal gentamicin against systemic acute murine brucellosis. *Chemotherapy, Vol. 43, (1997), pp. (204–210)*.

Vizcaíno, N., et al., (2000). DNA polymorphism in the genus *Brucella*. Microb. Infect. *Vol. 2, (2000), pp. (1089–1100)*.

Walker, R. L. (1999): *Brucella*. In: Hirsh, D. C. and Zee, Y. C. (eds). Veterinary Microbiology. London: *Blackwell Science Inc., pp. 196-203*

Wallach, J.C., Samartino, L.E., Efron, A. and Baldi, P.C., (1997). Human infection by *Brucella melitensis*: an outbreak attributed to contact with infected goats. *FEMS Immunol. Med. Microbiol. 19, 315–321.*

Wernery, U. and Kaaden, O. R. (2002). Infectious Diseases of Camelids. Blackwell Science, *Berlin, pages 23, 33, 87, 137, 181, 276, 285, 373.*

Wernwry, U. and Wernery, R., (1990). Seroe pidemiological investigation in

Whatmore A. M., *et al.* . (2014). *Brucella papionis sp*. nov., isolated from baboons (Papio spp.). *Int. J. Syst. Evol.Microbial. 64, 4120–4128.*

White, P. G. and Wilson. J. B.(1951). Differentiation of smooth and non-smooth colonies of Brucellae . *journal of bacteriology 61 239-240.*

WHO/EMC/ZDI/98.14 (1997). The Development of New/Improved Brucellosis Vaccines: Report of WHO Meeting with the participation of the Food and Agriculture Organization of the United Nations (FAO) and the Office International des Epizooties (OIE). Emerging and other Communicable Diseases, Surveillance and Control, Geneva, *Switzerland 11-12*.

World Health Organization, (2006). Brucellosis in humans and animals WHO/CDS/EPR/2006.7.

World Organization for Animal Health (OIE) (2016). Brucellosis (*Brucella abortus, B. melitensis and B. suis*). Chapter 2.1.4. OIE Terrestrial Manual 2016.

Wright, A.E. and Smith, F., (1897). On the application of the serum test to the differential diagnosis of typhoid and Malta fever. Lancet; 1: 65 9.Yongqun He, Sherry Reichow,Sheela Ramamoorthy, Xicheng Ding, Raju Lathigra, Johanna C. Craig, Bruno W. S. Sobral, Gerhardt G. Schurig, Nammalwar Sriranganathan, and *Brucella melitensis* Triggers Time-Dependent Modulation of Apoptosis and Down-Regulation Stephen M. Boyle (????). "Mitochondrion-Associated Gene Expression in Mouse Macrophages." Infection and Immunity, Sept. (2006), p. 5035–5046

Young, E.J. and Corbel, M.J., (1989). Clinical manifestations of human brucellosis, in Brucellosis: clinical and Laboratory Aspects. In: CRC.

Zammit T., (1905). A preliminary note on the examination of the blood of goats suffering from Mediterranean fever, reports of the Commisssion on Mediterranean Fever, Part III, Harrison and Sons, London, p. 83.