بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Sero-prevalence of Brucellosis among women with history of abortion in Khartoum State

الانتشار المصلي لعدوى البروسيلا لدى النساء المجهضات سابقا في ولاية الخرطوم

A thesis submitted in partial fulfillment for the requirement of M.Sc degree in Medical Laboratory Sciences (Microbiology)

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B.Sc in Medical Laboratory Sciences (2011)

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2015

الآية

قال تعالي:

وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا

صدق الله العظيم

سورة الاسراء الآيه (85)

DEDICATION

To my beloved parents.

My brothers and sister.

My teachers specially:

Dr. Abbas Mohamed Ahmed Fadlelmula

My All friends.

ACKNOWLEDGEMENT

Thanks first to ALLAH for helping and blessing us in doing this work.

Then I would like to express my deepest thanks and gratitude to my supervisor Dr. Abbas for his great efforts and huge help and patience during my work, I ask ALLAH to bless him.

My thanks also extend to the colleagues in the department of Microbiology in Alrawda hospital for creating good environment to do the practical work.

To Dr.Mohamed Elsadig for help me.

To all those who supporting me help me and encouraged me.

I would like to thank every women participated in this work, asking god to cure and bless them.

ABSTRACT

This was a descriptive, cross-sectional study conducted to determine prevalence of human brucellosis and assess the detection of *Brucella* antibodies by using Rose Bengal Plate Test (RBPT), modified Rose Bengal Plate Test (mRBPT), and Slide Agglutination Test(SAT), among women with history of abortion in Khartoum State from May to July (2015) on a total of one hundred and twenty (n=120) samples.

All subject examined in this study were from different age groups and different times of abortion, 8/120(6.7%) of the screening women were positive for *Brucella abortus* antibodies by mRBPT, 5/120(4.2%) by RBPT and SAT. As for *B.meletensis,* only 2/120(1.7%) were positive by SAT.

The highest prevalence was found on women age between 20-37 years, while the low rate was found in pregnant women aging between 38-46 years.

According to frequency of abortion, 37/120(30.9%) of tested women aborted once, 64/120(53.3%) twice, and 19/120(15.8%) had abortion more than two times. When examined by mRBPT, positive cases were 3/37(8.1%) among those who had abortion once, 3/64(4.7%) among twice abortion and 2/19(10.5%) among women who had more than two time abortion. .

The Results in this study showed that the mRBPT was more sensitive than RBPT and SA

ملخص الدراسه

هذه دراسه وصفيه مقطعيه أجريت لتحديد مدى انتشار عدوى البروسيلا وتقيم فعالية اختبارات الروزبنغال والروزبنغال المعدل والتجلط بالشريحه في الكشف عن الاجسام المضاده للبروسيلا بين النساء اللاتي لهن سابق اجهاض في ولاية الخرطوم في الفترة من مايو وحتى يوليو 2015 وكان عددهم الكلي 120 .

كل المشتركين الذين فحصوا في هذه الدراسه من اعمار مختلفه وعدد مرات اجهاض مختلفه 120/80 (6.7%) من النساء المفحوصات اظهرن ايجابيه للاجسام المضاده للبروسيلا باستخدام الروزبنغال المعدل بينما نقصت النسبه الايجابيه الى 120/5(4.2%) باستخدام طريقه الروزبنغال والتجلط بالشريحه لنوع *B.abortus* و120/2 (1.7%) ايجابيات باستخدام التجلط بالشريحه لنوع *B.meletensis* والتي اجريت لنفس عينات فصل الدم.

وكان اعلى معدل انتشار في الائى تتراوح اعمارهن بين 20-37 سنه بينما كان المعدل اقل فى الحوامل بين 38-46 سنه.

تبعا لعدد مرات الاجهاض عند النساء المفحوصات , النساء اللاتي تعرضن للاجهاض مرتين اعلى تردد 120/64 (53.3%) من اللاتي تعرضن للاجهاض مره واحده 120/37 (30.8) واللاتي تعرضن للاجهاض اكثر من مرتين 120/19 (15.8%). الحالات الايجابيه كانت 37/3 (8.1%) للواتي تعرضن للاجهاض مره واحده و 64/3 (4.7%) للواتي تعرضن للاجهاض مرتين و 19/2 (10.5%) للواتي تعرضن للاجهاض اكثر من مرتين عندما فحصوا بالروزبنغال المعدل.

أوضحت النتائج في هذه الدراسه ان اختار الروزبنغال المعدل اكثر دقه وفعاليه من اختباري الروزبنغال و التجلط بالشريحه.

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List of Abbreviations

Abbreviation	Word
Ab	Antibody
AGID	Agar Gel ImmunoDiffusion
CFT	Complement Fixation Test
ELISA	Enzyme Linked Immune Sorbent Assay
FPA	Fluorescence Polarization Assay
HIG	Hemolysis In Gel
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHLT	Indirect Hemolysis Test
LPS	Lipopolysaccharide
ME	Mercapto Ethanol
mRBPT	Modified Rose Bengal Plate Test
MRT	Milk Ring Test
OMPs	Outer Membrane Proteins
PCFIA	Particle Concentration Fluorescence Immuno Assay
PCR	Polymerase Chain Reaction
RBPT	Rose Bengal Plate Test
RIA	Radio Immuno Assay
RNA	Ribonucleic Acid
SAT	Slide Agglutination Test
SRID	Single Radial Immuno Diffusion
STA	Standard Tube Agglutination

CHAPTER ONE

INTRODUCTION

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INTRODUCTION

1.1Back ground

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella*. It affects many mammalian species and is transmissible to humans, thus giving it an important socio-economic impact. Making a diagnosis of brucellosis may be difficult because of the unspecific symptoms and signs shared with other febrile illnesses (Kaltungo *et al.*, 2014).

Isolation and identification of the bacterium was first reported by Bruce and co-workers, when they isolated *Brucella melitensis* from military personnel in Malta (Bruce, 1887).

Brucellosis is a zoonotic disease, and virtually all infections derive directly or indirectly from exposure to animals and their products. Brucellosis generally presents as an acute or subacute febrile illness with protean clinical manifestations. To the unaware patient, the acute phase of the disease may be experienced as an innocent febrile illness that does not need consultation with a physician. However, brucellosis should be treated promptly because the infection may persist, and the patient may develop severe complications (Ali and Ameen, 2010).

Brucellosis in Sudan was first reported from human cases as early as1904. The organism was also isolated from camels in Butana area. *B.abortus* was first isolated from a dairy farm in Khartoum, while *B.melitensis* was isolated from goat's milk among british residents in the El Gezira area, Many investigators reported the disease from different parts of Sudan (Hassan, 2009).

Brucellosis can be manifested as three different clinical types according to the duration of symptoms: acute (initial 2 months),
subacute (2–12months), and chronic (12 months). The disease may also be asymptomatic, subclinical, and focal, or present with complications, relapses, and re-infections (Pabuccuoglu *et al*., 2011).

Human and animal infections caused by *Brucella* are distribute worldwide; the most important regions of endemicity are the Mediterranean countries, Asia, and Latin America. It has been estimated that the 500,000 new cases of human brucellosis that are reported annually only account for about 4% of the total number of cases , since many cases are either not communicated or are undetected. The disease is a health and economic problem in countries where programs to control cattle infection have been unsuccessful.(Baldi *et a1*., 1996).

Unequivocal diagnosis of brucellosis requires isolation of the causal agent. Blood culture is the method of choice, but specimens need to be obtained early, and cultures often need long periods of incubation. In addition, failure to detect the pathogen is a frequent occurrence. Although in the last few years PCR-based laboratory tests have been proposed, they cannot be considered a routine diagnostic method yet. These limitations make serology the most useful tool for laboratory diagnosis of *Brucella* infection. (Serra and Vinas,2004).

In endemic countries, brucellosis is typically acquired through the consumption of dairy products. It affects both genders equally, and special attention must be given to those with an impaired immunological status such as pregnant women, since entities such as typhoid fever, influenza, and hepatitis E have been shown to be more severe in pregnant women, presenting higher mortality rates (Vilchez *et al*., 2015).

Spontaneous abortion, which is the loss of a pregnancy without outside intervention before 20 weeks of gestation, is the most common complication of pregnancy. Up to 20% of the recognized pregnancies will end in spontaneous abortion causes of many abortions are unknown. One of the causes of the abortion is infection. Although brucellosis can also result in human abortion, it has been debated whether it is any more frequent than with the other bacterial infections. Reports from the areas where *Brucella melitensis* infection is endemic, suggest that there is an increased rate of abortion in asymptomatic pregnant women (Nassaj *et al.,* 2008)

Rationale

Brucellosis is one of the most important zoonoses and is common in countries and communities where a large proportion of the inhabitants are involved in livestock farming activities, people live in close contact with their animals or consume raw milk and other dairy products prepared from fresh milk.

The disease prevalence is high among dairy cattle, sheep, goat and camel in Sudan .The treatment of the disease in human is difficult and cost a lot of money.

Brucellosis is a risk factor for women's general health and reproduction as well as for many obstetric complications during pregnancy, of which spontaneous abortion is the mostly known (Kurdoglu *et al.,* 2015).

Objectives

Genaral objective

Detection sero-prevalence of brucellosis in pregnant women with history of abortion in Khartoum state

Specific objective

1-To determine the prevalence of Brucellosis in women with history of abortion in Khartoum state using RBPT, mRBPT and SAT.

2-To determine the level of agreement between the three serological tests.

CHAPTER TWO

LITERATURE REVIEW

Chapter two

2. LITERATURE REVIEW

2.1 Taxonomy of Brucellae:

Brucellae are facultative intracellular coccobacilli belonging to the order Rhizobiales of the α-2 subgroup of Proteobacteria. The class Alphaproteobacteria includes organisms that are either mammalian or plant pathogens or symbionts . Within the family Brucellaceae, Ochrobactrum is the closest phylogenetic neighbour of *Brucella*. Historically, brucellae are differentiated by host tropism, pathogenicity and phenotypic traits. (Al Dahouk *et al.,* 2013)

On the basis of pathogenicity, host preference and phenotypic characteristics, six species of *Brucella* are commonly listed: *B. neotomae , B. canis , B. suis , B.ovis , B. melitensis* and *B. abortus* . Based on their cultural morphology, serotyping and biochemical characteristics, these species may be sub-divided into sub- types (also known as biovars, or biotypes) .(Sanogo *et al.,* 2013).

Currently ten species are recognized including the better known six classical species comprised of *B. abortus* (cattle, biovars 1-6, and 9) *B.melitensis* (goats, sheep, biovars 1-3), *B. suis* (pigs, reindeer and hares, biovars 1-5), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (desert wood rats). More recently, new members to the genus include *B. ceti* and *B. pinnipedialis* (dolphins/porpoises and seals respectively), *B. microti* (voles) and *B. inopinata*(reservoir undetermined) Of these species, *B. melitensis* has the greatest risk for human infection followed by *B.suis*and *B.abortus,* however several of the other species have been shown to be virulent for humans (Yohannes *et al.,* 2013).

2.2 Morphology and staining reaction:

It's generally accepted that, members of the genus *Brucella* is small, non motile, non- sporing, gram-negative, cocci, coccobacilli or short rods 0.5-0.7μm in diameter and 0.5-1.5 μm in length. They occur singly, in pairs (less frequently), short chains or small groups. (Hassan.2009)

2.3 Cultural characteristics:

Brucella is aerobic, many strains require supplementary carbon dioxide 10% for growth on primary isolation, the optimal temperature for growth is 37ºC and pH 6.6-7.4 (Garrity. 2005).

Growth improved by addition of equine or bovine serum to basal media. Blood agar, Tryptose-soy agar (TSA), columbia agar, serum dextrose agar (SDA) and potato agar are use for isolation. There are some selective media made by addition of different antibiotics supplement to suppress the growth of organisms other than *Brucella* e.g. Farrell's medium and Thayer-Martin's medium (OIE, 2009).

2.4 Biochemical characteristics

Brucella is catalase positive, oxidase positive but negative strains occurs, produce H_2S, hydrolyze urea, acid production does not occur from carbohydrates in conventional media except for *B.neotomae*, indole negative, methyl red negative, non-haemolytic and don't liquefy gelatine (Garrity. 2005).

2.5 Epidemiology of human brucellosis:

The disease is endemic especially in countries of the Mediterranean basin, the Middle East, the Indian subcontinent and parts of Mexico and Central and South America. Human brucellosis is found to have significant presence in rural/nomadic communities where people live in close association with animals. Worldwide, reported incidence of human

brucellosis in endemic disease areas varies widely, from <0.01 to >200 per 100 000 population (Nassaj *et al.,* 2008)

The Brucellosis 2003 International Research Conference estimated that 500,000 human infections occur per year worldwide, with incidences ranging from less than one case per 100,000 population in UK, USA and Australia, through 20 to 30 cases per 100,000 in southern European countries such as Greece and Spain, to more than 70 cases per 100,000 in Middle Eastern States such as Kuwait and Saudi Arabia (Yohannes *et al.*, 2013)

This organism causes debilitating illness in humans and is categorized as a potential bioterrorism agent. Since 1993, almost 2000 human brucellosis cases have been reported in the U.S. (http://dphhs.mt.gov/publichealth and http://liv.mt.gov. 2013)

2.5.1 Transmission and dose of infection to human:

The animal that are commonly known to serve as sources of human infection are goats, sheep, cattle, water buffalo and swine. Infection of reindeer, caribou, camels and yaks is of epidemiological importance in some parts of the world. Dogs have long been known as carriers of *Brucella* and the newly recognized species *B.canis* may be transmitted from dogs to man (FAO/WHO. 1971)

Brucella infects humans via aerosol inhalation, ingestion, or mucosal or skin abrasions. Suspicion of patient infection is raised when a detailed history reveals ingestion of contaminated dairy products or when contact with infected animals has occurred. Recent reports also suggest possible infection via blood transfusion and sexual transmission and from patients to medical personnel, especially in an obstetric setting (Baud and Greub 2011).

Consumption of contaminated milk with *Brucella abortus* or other species of the genus may lead to an infectious zoonotic disease. The risk of acquiring the disease from unpasteurized milk is the major cause of public health hazard. In Sudan, about 90% of milk sale is in the hands of illiterate farm who believe milk is hygienic and good nutritive source under the condition they are milking and purchasing. (Salman and El Nasri, 2012).

Brucella has a low infectious dose (10 organisms of *Brucella melitensis* are sufficient to cause infection in man), making infection a genuine risk to those occupationally exposed such as farmers, veterinarians and butchers and to the public through the consumption of contaminated unprocessed animal products which usually contain high numbers of *Brucella* and consequently pose significant infection risk factors if not properly handled and disposed. (Hassan. 2009)

2.5.2 Clinical manifestation:

The principle manifestation of brucellosis in animals is spontaneous abortion and the presence of erythritol in placenta of these animals which is believed to play an important role in the localization of *Brucella*. This is true only about *Brucella abortus.*

It is believed that brucellosis causes fewer spontaneous abortions in humans than it does in animals because of the absence of erythritol in the human placenta and fetus. An additional reason for the less significant role of *Brucella* infection in human abortion is the presence of anti-*Brucella* activity in the human amniotic fluid (Nassaj *et al.*, 2008)

In humans, brucellosis is a multisystem disease. Fever is the most common feature, followed by osteoarticular involvement (sacroilitis, spondylitis, peripheral arthritis, and osteomyelitis) . However, all systems might be affected, such as the nervous (neuropathies, chorea, and meningoencephalitis), gastrointestinal, hepatobiliary (hepatomegaly)

genitourinary (orchiepididymitis, glomerulonephritis, and renal abscesses), musculoskeletal, cardiovascular (endocarditis,mainly with involvement of the aortic valve) and pulmonary systems(pleural effusions and pneumonia). Approximately 10% of patients with brucellosis experiencerelapses, 90% of which occur within a year after discontinuation of antimicrobial drug therapy (Baud and Greub 2011).

2.5.2.1 Genitourinary complications:

Orchitis and epididymitis are the most frequent genitourinary complications of brucellosis in men. Usually unilateral, *Brucella orchitis* can mimic testicular cancer or tuberculosis. Although *Brucella* organisms have been recovered from banked human spermatozoa, there have been a few reports implicating sexual transmission. Renal involvement in brucellosis is rare, but it too can resemble renal tuberculosis. In women, rare cases of pelvic abscesses and salpingitis have been reported. (Corbel. 2006).

2.5.2.2 Human Brucellosis during Pregnancy:

During the early years of clinical description of Malta fever, Eyre recognised the occurrence of active brucellosis during pregnancy for the first time in 1908, with a statement of pregnancy frequently synchronizes with an attack of Malta fever and the course of pregnancy is unaffected, although lactation is frequently curtailed. Although it could not be proven microbiologically, De Forest *et al.* proposed a correlation between abortion and active brucellosis in humans firstly in 1917 . (Kurdoglu *et al.*, 2015).

Brucellosis in pregnancy is associated with adverse outcomes such as spontaneous abortion, preterm delivery, chorioamnionitis, and fetal death. However, it is not clear whether these outcomes are more common than in other infectious diseases. The incidence of abortion is

higher and prompt therapy can be life-saving for the fetus (Vilchez *et al.*, 2015)

Various *Brucella* species are well-known causes of contagious abortion in cattle, sheep, goats, swine, and dogs. There is also evidence that brucellosis can produce spontaneous abortion in humans, which has been demonstrated by rare cases in which *Brucella* species were isolated from fetal or placental tissues, but it has not been demonstrated that brucellosis causes abortions more frequently than do other bacterial infections, It is believed that brucellosis causes fewer spontaneous abortions in humans than it does in animals because of the absence of erythritol in the human placenta and fetus Erythritol is a constituent of normal ungulate fetal and placental tissue and in cases of bovine abortion, promotes overwhelming infection of the placenta and fetus. An additional reason for the lesser role of *Brucella* infection in human abortion is the presence of anti-*Brucella* activity in human amniotic fluid.(Khan *et al.,* 2001).

2.6 Evidence in support of the diagnosis :

It is indicated by the history of recent exposure to a known or probable source of *Brucella* spp. This includes common host species, especially cattle, sheep, goats, pigs, camels, yaks, buffaloes or dogs; consumption of raw or inadequately cooked milk or milk products, and, to a lesser extent, meat and offal derived from these animals. In addition, the resistance of the organism and its high infectivity make environmental contamination a probable hazard, although this is always difficult to prove. Occupational exposure and/or residence in an area in which the infection is prevalent, also raise the probability of the diagnosis.

•Isolation of *Brucella* spp. from the patient.

•Demonstration by validated polymerase chain reaction (PCR) of the presence of *Brucella* genetic material in blood or other tissue sample.

•Demonstration by a validated serological method of *Brucella* antigen in blood or other tissue sample.

•Demonstration of a rising antibody titre in any serological test for brucellosis in the absence of exposure to any known source of cross-reacting antigens.

•Demonstration of a high sustained IgG antibody titre in the agglutination, complement fixation or ELISA tests with standardized antigens (Corbel. 2006).

2.6.1 Laboratory diagnosis:

The gold standard for the diagnosis of brucellosis is the isolation and identification of *Brucella* species from clinical specimens by culture. However, it is time-consuming and hazardous to the laboratory personnel. Therefore, most cases are diagnosed by serological testing. The most frequently used method is the standard tube agglutination test Enzyme-linked immune sorbent assay (ELISA) typically uses the cytoplasmic proteins as antigens and measures IgM, IgG, and IgA, which allow for better interpretation. It has been reported to be superior to others serology due to its higher sensitivity and specificity. (Nassaj *et al.,* 2008) The Rose Bengal (RB) test is commonly used to screen for brucellosis infections but it has been suggested that the results of RB should be verified by other tests . The standard agglutination test (SAT), which was developed by Wright and colleagues in 1897 in order to detect total antibodies, is the most frequently used test to diagnose brucellosis. If the SAT test yields negative results due to the presence of blocking antibodies, the Coombs' test may be used instead (Pabuccuoglu *et al.*, 2011)

Because symptoms of human brucellosis are usually nonspecific, laboratory tests are helpful for establishing the diagnosis. Though the blood culture is the only specific test, its sensitivity ranges from 17% to

85% depending on the culture conditions and the bacterial strain. This method has a good sensitivity for detecting *Brucella melitensis* but a low sensitivity for detecting *Brucella abortus* and *Brucella suis* . Because of this reason serological tests for diagnosing brucellosis are needed.

Serological tests are based on the use of either whole bacteria or antigenic extracts. Among the tests using whole *Brucella* are the plate agglutination test (Huddleson test), the standard tube agglutination (STA) test with or without 2-mercaptoethanol - ME), the Rose Bengal test, the Coombs' test, and CF. All of these tests mainly detect antibodies to lipopolysaccharide LPS . It has been clearly established that these antibodies are responsible for the cross-reactivity with other Gram-negative bacteria such as *Yersinia enterocolitica 0:9*, *Escherichia coli 0:157*, *Salmonella group N*, *Vibrio cholerae*, and *Francisella tularensis* . On the other hand, agglutination tests have low sensitivity because they are based on secondary interaction and interpretation of their results is largely subjective. The lack of standardization of commercially available *Brucella* suspensions is another drawback of these tests . ELISA, RIA, and gel precipitation have been developed as tests using antigenic extracts . These tests have improved the sensitivity of detection of antibodies, but the lack of specificity still remains, since the antigenic extracts used in these assays are rich in LPS. Levels of antibodies to LPS remain high in patients who have recovered from brucellosis , thus making it difficult to differentiate past from present bruceliar infection. Some studies of the changes in values of antibodies to LPS during the disease have been performed . However, to our knowledge, a similar follow-up of antibodies to cytoplasmic proteins of *Brucella* has not been reported. (Baldi *et al.,* 1996).

2.6.2 Overview of serological tests:

Common in-use tests:

Since 1897 aconsiderable number of serological tests have been developed. anumber of these tests were modified in various ways to increase performance. this tests include:

-Agglutination tests:

*standard tube (SAT)

*acidified antigen (RBT, BPAT)

*milk ring test (MRT)

-Complement fixation test:

*warm and cold (CFT)

*indirect hemolysis test (IHLT)

*hemolysis in gel (HIG)

-Precipitation tests:

*agar gel immunodiffusion test (AGID)

*single radial immunodiffusion test (SRID)

-Primary binding assays:

*radioimmunoassay (RIA)

*particle concentration fluorescence immunoassay (PCFIA)

*indirect and competitive enzyme immunoassay (IELISA, CELISA)

*fluorescence polarization assay (FPA) .(Nielsen. 2002)

2.6.2.1 Rose Bengal Plate Test (RBPT)

The RBPT is a spot agglutination technique which is also known as the card test or buffered *Brucella* antigen test. It uses a suspension of *B. abortus* smooth cells stained with the Rose Bengal dye, buffered to pH 3.65. At neutral pH, this test can measure the presence of IgM, IgG1 and IgG2. However, IgM appears to be the most active. At the buffered pH of 3.65, RBPT, prevents agglutination with IgM, and apparently, measures

only IgG1. Low pH of the antigen enhances the specificity of the test, while the temperature of the antigen and the ambient temperature at which the reaction takes place may influence the sensitivity and specificity of the test.(Kaltungo *et al.,* 2014)

2.6.2.2 Standard Agglutination Test (SAT):

This test is based on the reactivity of antibodies against the smooth lipopolysaccharide of *Brucella*. Excess of antibodies resulting in false negative reaction due to prozone effect can be overcome by applying a serial dilution of 1:2 through 1:64 of the serum samples thus increasing the test specificity. The test is performed at a near neutral pH, which makes it more efficient in detecting IgM antibody. Hence, it is best used to detect acute infections. It is less effective for IgG, resulting in low assay specificity (Kaltungo *et al.,* 2014).

2.6.2.3 Fluorescence polarization assay (FPA):

The FPA is based on the fact that, when polarised light excites fluorescent molecules, they will emit polarised light. In solution, the polarisation of the emitted light is inversely proportional to the molecule's rotational speed, which is influenced by the solution's viscosity, absolute temperature, molecular volume and the gas constant.

In brucellosis serology, small molecular weight subunit of OPS is labelled with fluorescein isothiocyanate and used as the antigen. When testing serum, blood or milk, if antibody to the OPS is present, the rate of rotation of the labelled antig3en will be reduced at a rate which is proportional to the amount of antibody present (Kaltungo*et al.*, 2014).

2.6.3 Molecular biology techniques:

The polymerase chain reaction (PCR) is a recent and promising technique that allows for rapid and accurate diagnosis of brucellosis without the limitations of conventional methodology. Several genus-specific PCR systems using primer pairs that target 16SRNA sequences and genes of different outer membrane proteins have been developed. The first brucellosis PCR-based test was introduced in 1990. The first species-specific multiplex PCR was called Abortus-Melitensis-Ovis-Suis (AMOS-PCR) assay, which is used to identify and differentiate *B. abortus* biovars 1, 2 and 4, *B. melitensis, B. ovis* and *B. suis* biovar 1. The PCR is based on the polymorphism arising from species-specific localisation of the insertion sequence IS711 in the *Brucella* chromosome.(Kaltungo *et al.,* 2014).

2.7 Immune response :

Although humoral antibodies appear to play some role in resistance to infection, the principal mechanism of recovery from brucellosis is cell-mediated. Cellular immunity involves the development of specific cytotoxic T lymphocytes and activation of macrophages, enhancing their bactericidal activity, through the release of cytokines (e.g. gamma interferon and tumour necrosis factor) from specifically committed helper T lymphocytes. Coincident with the development of cell-mediated immunity, the host usually demonstrates dermal delayed type hypersensitivity to antigens of *Brucella* (Corbel, 2006) .

2.7.1 Antigenic structure of brucella spp.:

2.7.1.1 Lipopolysaccharide (LPS):

Lipopolysaccharide (LPS) is *Brucella* antigen that domain antibody response. in smooth strains the LPS comprises lipid A, core region and an O-chain, the structure of the LPS of non smooth strain (R-LPS) is basically similar to that of the S-LPS except that the O-chain is either absent or reduced to afew residues. The specificity of the R-LPS is therefore largely determined by the core polysaccharide (Corbel, 1997).

The presence of 4-amino, 4, 6 dideoxymannose in the LPS is also responsible for the antigenic cross-reactivity with *Escherichia hermanni* and *Escherichia coli O: 157, Salmonella O: 30, Stenotrophomonas maltophilia, Vibrio cholerae O: 1*, and *Yersinia enterocolitica O: 9* (Perry and Bundle, 1990).

2.7.1.2 Outer membrane proteins (OMPs):

The studies on the surface molecules composing the envelope of *Brucella* led to the characterization of two immunogenic fractions endowed with protective inducing activity: the sodium dodecyl sulphate (SDS-I) insoluble cell wall fraction and hot saline (HS) extract (Cloeckaert *etal.*, 2002).

The major OMPs have been shown to be surface-exposed by use of monoclonal antibodies (MAbs) and techniques such as enzyme-linked immunosorbent assay (ELISA), immunoelectron microscopy, and flow cytometry (Bowden et al., 1995). However, they appear to be much less accessible to antibodies on S than on R *Brucella* strains which are probably due to steric hindrance caused by the presence of the long LPS O-side chains at the surface of S *Brucella* strains (Jacques *et al.*, 1992).

2.7.1.3 Ribosomal proteins:

Ribosomal proteins have emerged as immunologically important components; crude ribosomal preparations were demonstrated to stimulate both antibody and cell-mediated responses and to confer protection against challenge with *Brucella* (Corbel, 1997). It has been established that the L7/L12 ribosomal proteins are important in stimulating cell-mediated responses. They elicit delayed hypersensitivity responses as components of brucellins (Bachrach *et al.,* 1994), and as fusion proteins, they have been shown to stimulate protective responses to *Brucella* (Oliveira and Splitter, 1996).

2.8 Pathogenicity and Virulence:

Brucella spp. are facultative intracellular organisms, surviving and multiplying within cells of reticuloendothelial system (RES) and their disease spectrum is partially explained by the ability of the organism to evade host defense mechanisms by virtue of intracellular existence. Survival and multiplication of *Brucella* organisms in phagocytic cells are features essential to establishment, development, and chronicity of the disease.(Till. 2014) Soon after entry into the body, the bacteria are ingested by polymorphonuclear and mononuclear phagocytes. After ingestion by phagocytes, the organisms proliferate in the local lymph nodes. The infection spreads hematogeneously to tissues rich in elements of RES, including the liver, bone marrow, lymph nodes and spleen. Organisms may also localize in other tissues, including joints, the central nervous system, the heart and the kidneys(Slack.2004) . Brucellae form granulomas made up of epitheloid cells, polymorphonuclear leukocytes, lymphocytes, and giant cells in tissues and organs. Granulomas are known to be more frequent in *B. abortus* infections. Although toxemia is

Commonly observed in *B. melitensis,* abscess formation in joints and spleen is more often related to *B. suis* (Gul and Erdem. 2015). Multiplication continues within macrophages and monocytes, and eventually the cells are killed, releasing the organisms. The “undulant” waxing-and-waning fever pattern seen in brucellosis is associated with the periodic release of bacteria and their components from phagocytic cells.Release of bacteria into the peripheral circulation results in hematogenous seeding of other organs and tissues, thereby leading to the protean clinical manifestations of human brucellosis. Relapses and recurrences of illness are kept in check to some degree by a balance between the virulence of the organism and the presence of an intact functional cellular immune response. As with other intracellular pathogens, humoral antibodies are produced, but cellular immune defense mechanisms are required to kill the bacteria (Winn *et al.*, 2006)

Cellular immunity has a fundamental role in controlling the disease. Although the presence of specific antibodies is of utmost importance in diagnosis, they play a limited role in the immune response. The IgM antibodies increase in the first week and the IgG antibodies in the second. After weeks of raising both antibodies, the levels decrease rapidly through a successful treatment. Furthermore, IgG levels decrease faster than IgM levels with treatment. Even after eradication of active infection, IgM antibodies can remain positive in low titers for months or even years. A high level of IgG and IgA antibodies for longer than 6 months is a sign of chronic infection or relapse (Gul and Erdem. 2015).

The clinical spectrum of brucellosis depends on many factors, including the immune status of the host, the presence of other underlying diseases or conditions and the species of infecting organisms. The greater virulence of *B. melitensis* and *B. suis* has been supported by in vivo studies with experimentally infected animals and by in vitro work

examining phagocytosis, intracellular survival, and lymphocyte responses to the different species. Disease caused by *B. abortus* and *B. canis* are insidious in their onset, but tend to cause milder constitutional symptoms and less severe complications (Winn *et al.*,2006).

2.9 Prevention and treatment:

Prevention is effectively achieved by testing and elimination of infected animals quarantine of imported animals, and pasteurization of milk .Acombination of tetracycline and rifampin or streptomycin taken for 3 to 6 weeks is usually effective in controlling infection. (Cowan and Talaro 2006).

Vaccination is the cornerstone of control programmes in livestock and although the S19, RB51 (both in cattle) and Rev 1 (in sheep and goats) vaccines have been successfully used worldwide, they have drawbacks and the ideal brucellosis vaccine is still awaited. There is no vaccine available for pigs and wildlife since there is no vaccine available for humans (Godfroid, 2004).

CHAPTER THREE

MATERIALS AND METHODS

Chapter three

3. Materials and methods

3.1 Study design

This was descriptive and cross sectional study.

3.2 Study area

This study was carried out at Khartoum State targeting women with history of abortion.

3.3 Study Period

Study was conducted during 1 May –30 July2015.

3.4 Study population and sample size

One hundred and twenty samples (n=120) were collected from hospitalized women having history of abortion.

3.5 Ethical consideration

Approval to conduct this study was obtained from the College of Graduate Studies, Sudan University of Science and Technology.

Permission was obtained from patients and verbal consent was taken.

3.6 Data collection

Personal and clinical data were collected from the subjects.

3.7 Specimens collection

Under aseptic condition after wearing the gloves, alcohol antiseptic (70% ethanol) was used to clean the skin. Four ml from venous blood were collected in plain container, used sterile disposable syringes and left to clot for 15 minutes , centrifuged at 3000 rpm for 5minutes and serum was separated and stored at -20ºC till used

3.8 Examination of serum samples

Slide agglutination was used as screening test and confirmed by Rose Bengal and Modified Rose Bengal Plate tests for all sample sera.

3.8.1 Slide agglutination test

3.8.1.1 Principle

This test is based on the reactivity of antibodies against the smooth lipopolysaccharide of *Brucella*. So that it's depending primarily on the reaction between the *Brucella* antigen and specific antibodies that were assumed to be present in sera of examined subjects.

3.8.1.2 Procedure

The serum samples and antigen were brought to room temperature.

Twenty microliters of each serum sample was placed on slide. After shaking the antigen bottle, expelled contents of dropper and refilled, one drop (50microliter) of each antigen (*B.abortus* and *B.melitensis*) was placed near each serum spot. They were mixed thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2cm in diameter. The mixtures were agitated gently for two minutes at an ambient temperature on rocker. Then the results observed under high intensity light.

Complete absence of agglutination and a clear suspension indicates negative result. Agglutination within one minute was reported as reactive or positive result.

3.8.2 Rose Bengal Plate test (RBPT)

3.8.2.1 Principle

The RBPT is a spot agglutination technique, It uses a suspension of *B. abortus* smooth cells stained with the Rose Bengal dye, buffered to pH 3.65. The acid pH diminishes agglutination by IgM but encourages agglutination by IgG1, generally reducing cross-reactions.

3.8.2.2 Procedure

The 120 serum samples were examined for brucellosis by RBPT using RBPT antigen prepared in Veterinary Research Institute, Soba. 30 µl of serum sample mixed thoroughly with 30 µl of antigen, and allowed to react for 4 minutes at room temperature under rotatory agitation.

Complete absence of agglutination and a clear suspention indicates negative result. Agglutination within 4minutes was reported as positive result.

3.8.3 Modified Rose Bengal Plate test (mRBPT)

The test was performed by increasing the amount of sera. 75µl of serum sample were added to 25µl of antigen, this method described by Ferreira *et al.* (2003)to increase the sensitivity of the test.

3.9 Data analysis

Statistical package of social science (SPSS version 16.0). Computer software was used for data analysis. Significant level were set at ($p<0.05$). The degree of agreement between the tests according to kappa analysis is determine by: less than < 0 chance agreement.

0.01- 0.20 = slight agreement.

0.21 - 0.40 = fair agreement.

0.41 - 0.60 = moderate agreement.

0.61 - 0.8 = substantial agreement.

0.81 – 0.99 = almost perfect agreement .

CHAPTER FOUR

RESULTS

Chapter Four

4. RESULTS

A total of one hundred and twenty (n=120) samples were taken from women with history of abortion. They were tested for *brucella* Abs. The pregnant women were categorized into three age groups, (20 – 28), (29 – 37) and (38 – 46) years. The abortion frequency was once, twice or more than two.

From the total serum samples, 5/120 (4.2%) were found positive to *B.abortus* when tested by RBPT, table (4.3), 8/120 (6.7%) positive when tested by mRBPT, table (4.4), 5/120 were found positive to *B.abortus* when tested by SAT, table (4.5). Only 2/120 (1.7%) was found positive for *B. melitensis* by SAT, table(4.6).

The degree of agreement between the three tests was found moderate between RBPT and mRBPT (0.56) and fair between RBPT and SAT (0.37) when analyzed by Kappa test.

The relationship was found significant between RBPT and mRBPT p.value (0.038) , no significant relationship between RBPT and SAT p.value (0.19) when analyzed by chi squire test (table 4.7 and table 4.8).

Table 4.1: shows that the percentage of women with twice number of abortion 64/120(53%) higher than women with once number of abortion 37/120(30.9%) and women with more than two number of abortion 19/120(15.8%).

Table 4.1: Frequency of abortion among screening women

Frequency of abortion	No. of aborted women	Percentage %
Once	37	30.9
Twice	64	53.3
More than two	19	15.8
Total	120	100

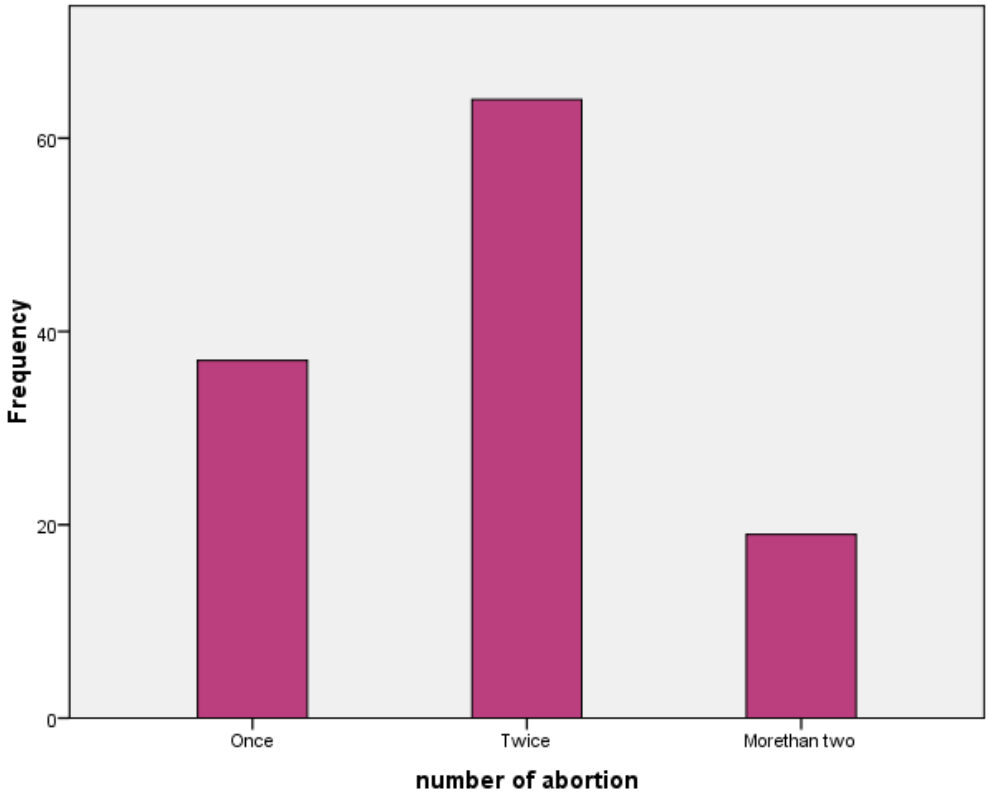

Fig 4.1: Frequency of abortion among screening women

Table 4.2: show that the percentage of age group (29-37) in screening women 60/120(50%) higher than age group (20-28) 42/120(35%) and age group (38-46) 18/120(15%).

Table 4.2: Age groups of the subjects and their percentage

Age group	Frequency	Percentage %
20-28	42	35
29-37	60	50
38-46	18	15
Total	120	100

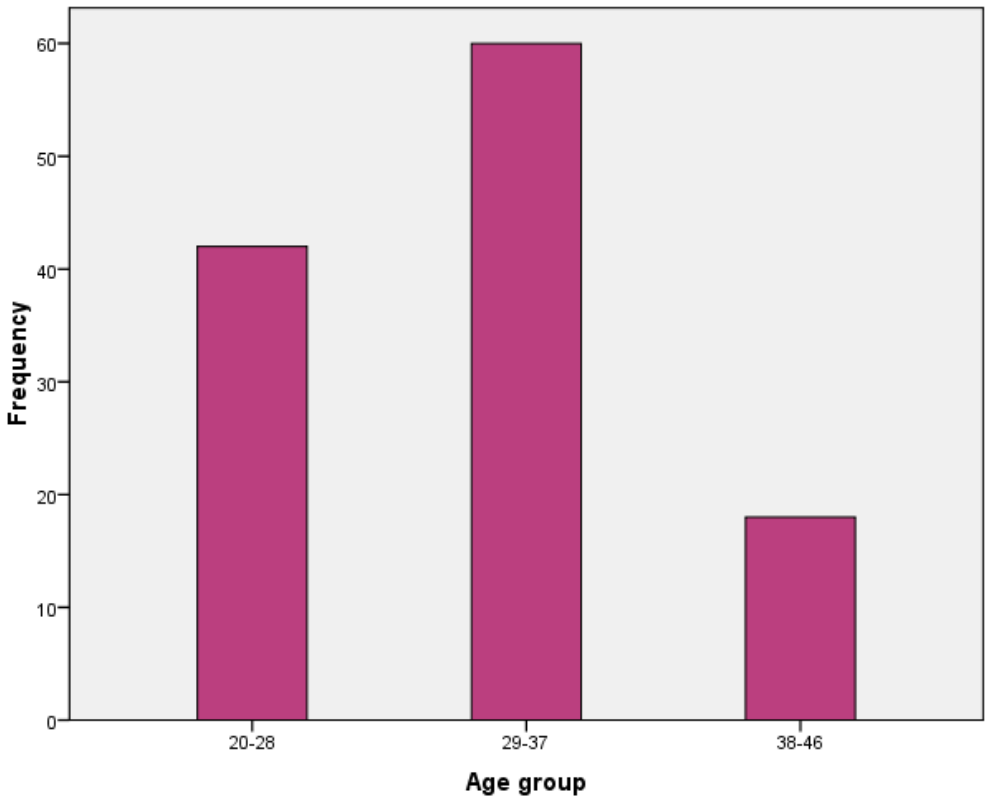

Fig 4.2: Age groups of the subjects and their percentage

Table 4.3: reveals that the screened women when tested by RBPT showed 5(4.2%) were positive for *Brucella* antibodies..

Table 4.3: Positive and negative results by RBPT

RBPT	**Frequency**	**Percentage %**
Positive	5	4.2
Negative	115	95.8
Total	120	100

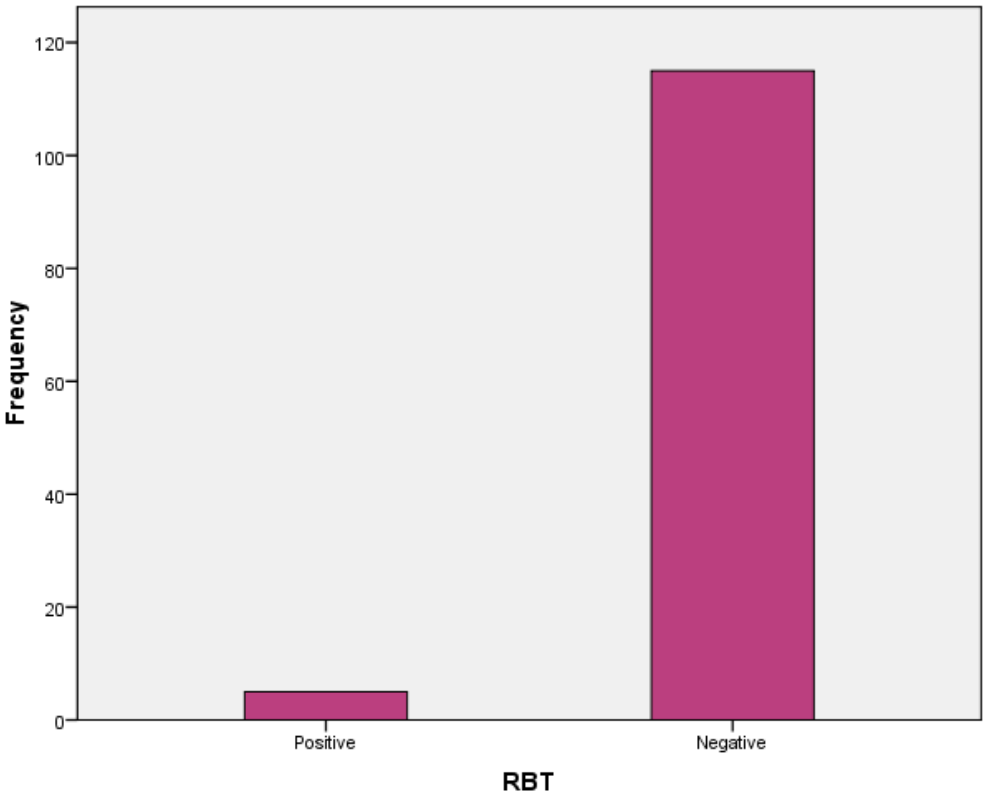

Fig 4.3: Positive and negative results by RBPT

Table 4.4: revealed that the screened women when tested by mRBPT8(6.7%) were positive for *Brucella* antibodies.

Table 4.4: Positive and negative results by mRBPT

mRBPT	**Frequency**	**Percentage %**
Positive	8	6.7
Negative	112	93.3
Total	120	100

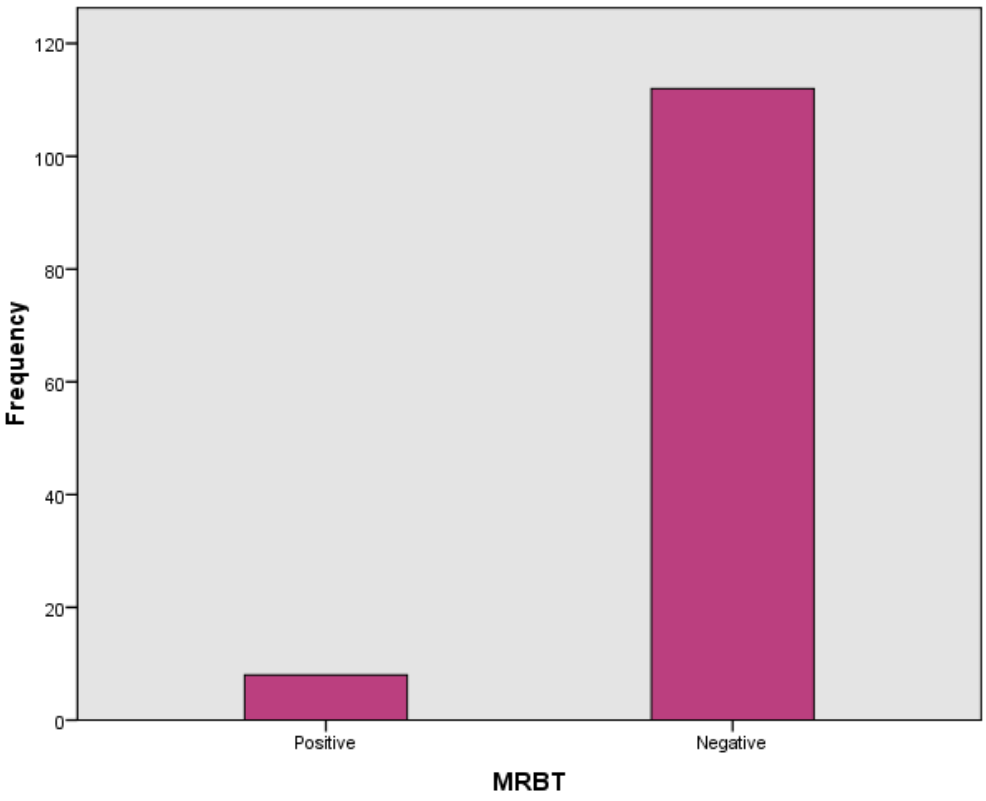

Fig 4.4: Positive and negative results by mRBPT

Table 4.5: showed that the screened women when tested by SAT were 5(4.2%) positive for *B.abortus* antibodies.

Table 4.5: Positive and negative results of *B.abortus* by Serum Agglutination Test (SAT)

Results	**Frequency**	**Percentage %**
Positive	5	4.2
Negative	115	95.8
Total	120	100

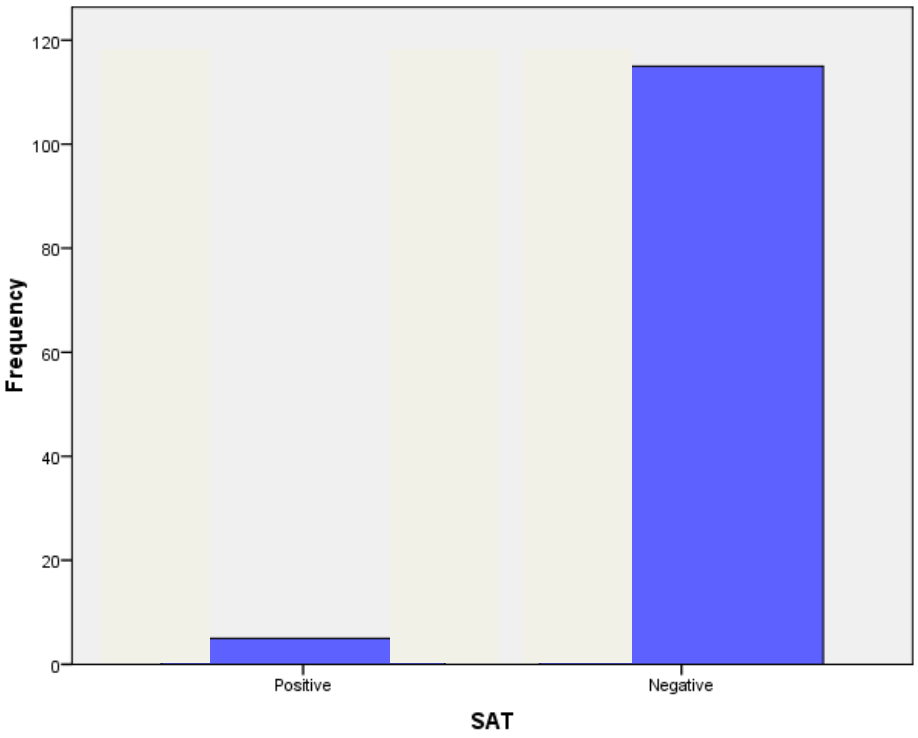

Fig 4.5: Positive and negative results of *B.abortus* by Serum Agglutination Test (SAT)

Table 4.6:The screened women when tested by SAT showed 2(1.7%) positive for *B.meletensis* antibodies.

Table 4.6: Positive and negative results of *B.meletensis* by Serum Agglutination Test (SAT)

Resuts	**Frequency**	**Percentage %**
Positive	2	1.7
Negative	118	98.3
Total	120	100

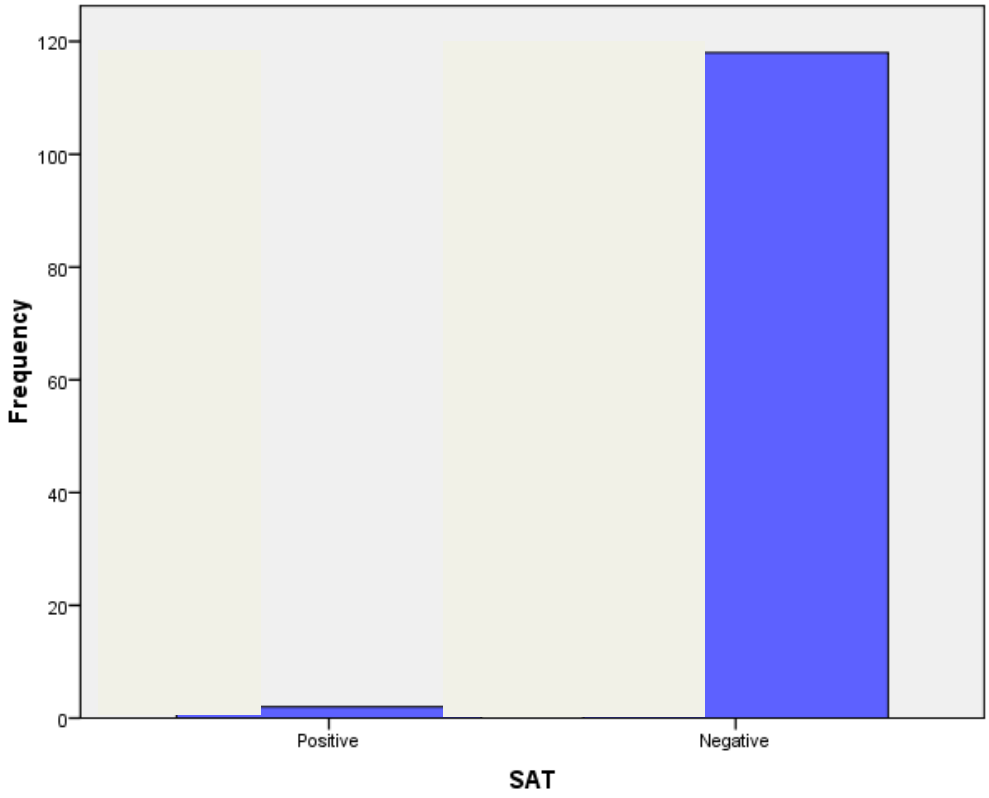

Fig 4.6: Positive and negative results of *B.meletensis* by Serum Agglutination Test (SAT)

Table 4.7: Degree of agreement and relationship between RBPT and mRBPT:

mRBP			RBPT		Total
			0	1	
	0	Count	4	0	4
		% within mRBPT	100.0%	.0%	100.0%
		% within RBPT	57.1%	.0%	33.3%
		% of Total	33.3%	.0%	33.3%
	1	Count	3	5	8
		% within mRBPT	37.5%	62.5%	100.0%
		% within RBPT	42.9%	100.0%	66.7%
		% of Total	25.0%	41.7%	66.7%
Total		Count	7	5	12
		% within mRBPT	58.3%	41.7%	100.0%
		% within RBPT	100.0%	100.0%	100.0%
		% of Total	58.3%	41.7%	100.0%

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement	Kappa	.526	.209	2.070	.038
N of Valid Cases		12			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Table 4.8: Degree of agreement and relationship between RBPT and SAT

SAT			RBPT 0	RBPT 1	Total
	0	Count	3	4	7
		% within SAT	42.9%	57.1%	100.0%
		% within RBPT	42.9%	80.0%	58.3%
		% of Total	25.0%	33.3%	58.3%
	1	Count	4	1	5
		% within SAT	80.0%	20.0%	100.0%
		% within RBPT	57.1%	20.0%	41.7%
		% of Total	33.3%	8.3%	41.7%
Total		Count	7	5	12
		% within SAT	58.3%	41.7%	100.0%
		% within RBPT	100.0%	100.0%	100.0%
		% of Total	58.3%	41.7%	100.0%

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement	Kappa	-.371	.257	-1.287	.198
N of Valid Cases		12			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Table 4.9 summarized the result which demonstrated that the percentage of positive results in women of age group (38-46) was (11.1%) 2/18, in women of age group (20-28) was (7.1%)3/42, and (5%)3/60 in women of age group (29-37) when tested by mRBPT.

Table 4.9: Distribution of mRBPT results according to age group

mRBPT	Age group			Total
	20-28	29-37	38-46	
Positive	3	3	2	8
Negative	39	57	16	112
Total	42	60	18	120

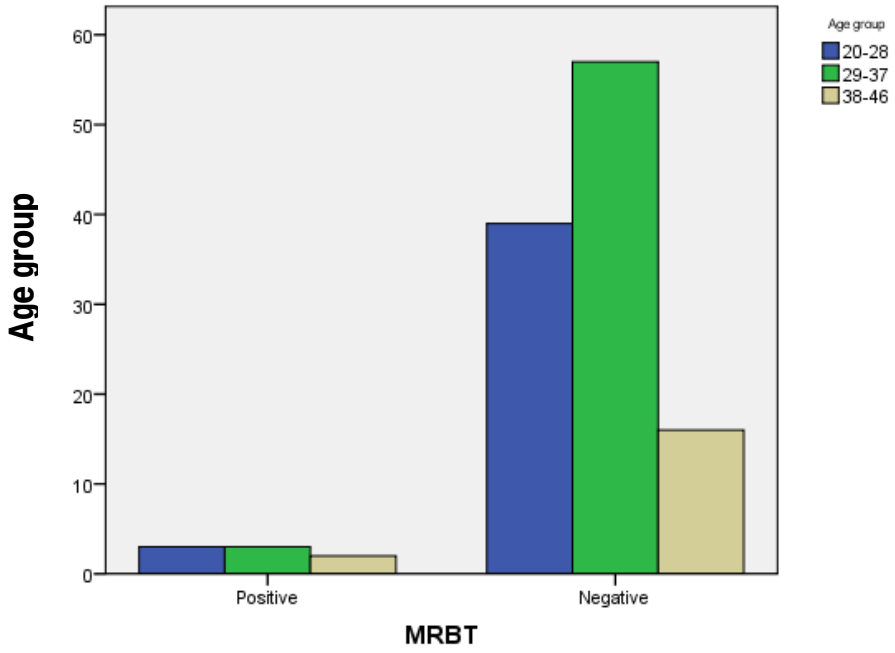

Fig 4.7: Distribution of mRBPT results according to age group

Table 4.10: summarized the result which demonstrated that the percentage of positive results in women of more than two number of abortion was (10.5%)2/19, women with one abortin (8.1%)3/37, and (4.7%)3/64 in women of twice abortion when tested by mRBPT.

Table 4.10: Distribution of mRBPT results according to number of abortion

mRBPT		**Number of a bortion**			**Total**
		Once	**Twice**	**More than two**	
	Positive	3	3	2	8
	Negative	34	61	17	112
Total		37	64	19	120

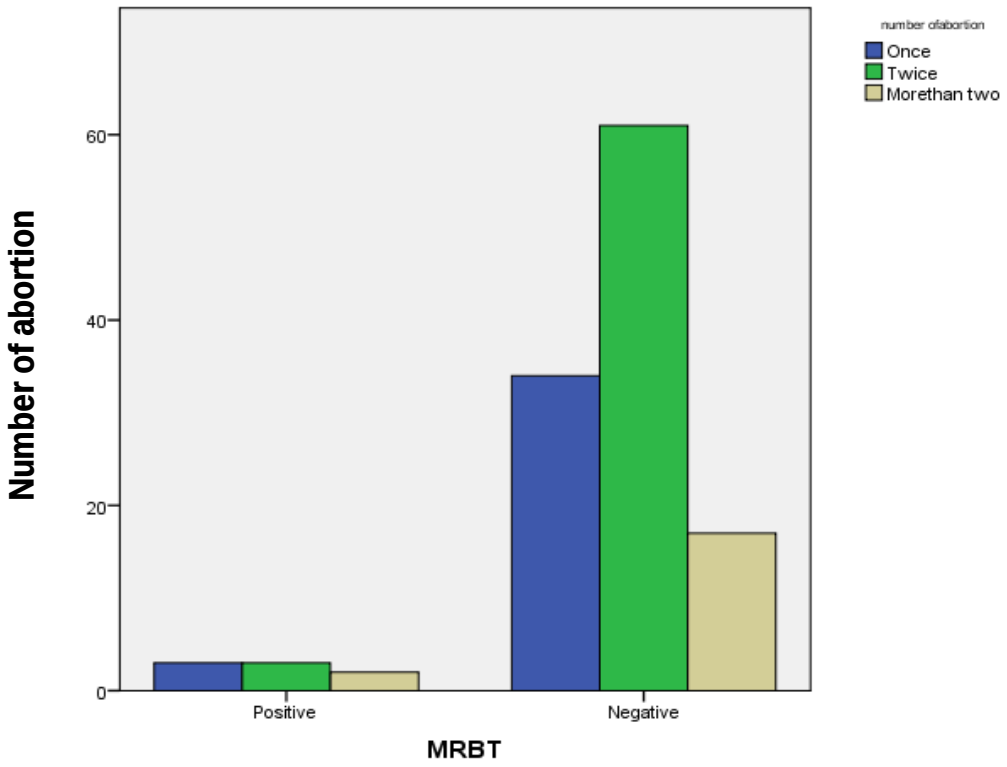

Fig 4.8: Distribution of mRBPT results according to number of abortion

Table4.11: Distribution of age groups and number of abortions

Age group	Number of abortion			Total
	Once	Twice	More than two	
20 – 28	25	17	0	42
29 – 37	12	45	3	60
38 – 46	0	2	16	18
Total	37	64	19	120

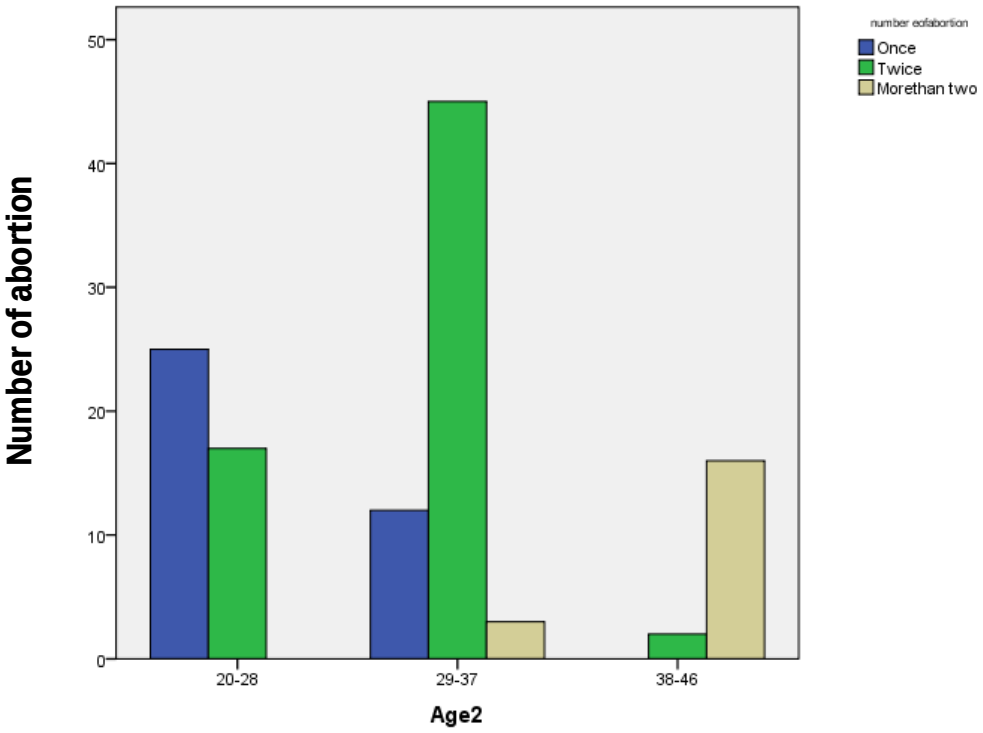

Fig4.9: Distribution of Age group and number of abortion

Table (4.12): Results of RBPT, mRBPT, and SAT together.

No. of sample	RBPT	mRBPT	SAT (*B.abortus*)	SAT (*B.melitensis*)
1	Positive	Positive	Negative	Negative
2	Positive	Positive	Negative	Negative
3	Positive	Positive	Negative	Negative
4	Positive	Positive	Positive	Positive
5	Negative	Positive	Positive	Negative
6	Negative	Negative	Negative	Negative
7	Negative	Negative	Positive	Positive
8	Negative	Negative	Positive	Negative
9	Negative	Positive	Negative	Negative
10	Negative	Negative	Negative	Negative
11	Negative	Positive	Positive	Negative
12	Positive	Positive	Negative	Negative

CHAPTER FIVE

DISCUSSION

Chapter five

DISCUSSION

According to the present results, it was found that 5/120 (4.2%) of the samples from screened women were positive for *Brucella abortus* when tested by RBPT and SAT whereas 8/120 (6.7%) were found positive by mRBPT. However, 2(1.7%) were positive for *Brucella meletensis* by SAT. The positive samples were not similar in the entire test except one which was positive by all the three tests for *Brucella abortus*. There was moderate agreement between RBPT and mRBPT(0.526) and the association was significant (*P*= 0.038) but the agreement was fair between RBPT and SAT and the association was not significant (*P*=0.198). This result agrees with previous study done by Karaji *et al*. (2011) in Iran where the number of positive serum samples with modified rose Bengal was twofold higher than conventional rose Bengal test, which included all positive samples with rose Bengal, the number of positive diagnosed serum samples with modified rose Bengal were nearly two times more than SAT, but it did not include all positive samples detected by SAT. On the other hand, this result was not agreed with those of Sadeghian *et al*. (2015) where there was highest level of agreement between RBPT and SAT.

In the present study there was significant relationship between RBPT and mRBPT, and there was statistically insignificant relationship between RBPT and SAT but in another study carried out by Karaji *et al*., (2011) the relationship between RBPT and SAT was significant. However, Ana *et al*. (2003) suggesting that the mRBPT and Indirect ELISA could replace RBPT when they tested infected sheep with *Brucella meletensis*.

In this study we had no gold standard test to determine the sensitivity and the specificity of the tests because we did not try to isolate the organism.

The study showed that the number of positive samples was higher in mRBPT than in RBPT and SAT, this result was in agreement with Karaji *et al*. (2011) and Ferreira *et al*. (2003), in which the mRBPT has specificity and sensitivity same as indirect ELISA and high level of agreement between them, this makes the mRBPT a good screening test for detection of *Brucella* antibodies from serum sample.

The study revealed that the number of women having twice number of abortion were (53.3%), higher than women having once abortion (30.8%) and women having more than twice abortion (15.8%). The percentage of positive cases of *Brucella* infection was 3/64(4.7%) among women of twice number of abortion, 3/37(8.1%) among women of once abortion, and 2/19(10.5%) among women of more than twice abortion. However, Kurdoglu, *et al.* (2015) who showed high rate of spontaneous abortion was a more consistent finding rather than high rates of preterm delivery and intrauterine fetal death in pregnant women with brucellosis. The occurrence of abortion was not associated with the magnitude of `serum agglutination titre or the clinical type of disease. In another study, done by Khan, *et al.* (2001) they demonstrated that the incidence of spontaneous abortion among pregnant women with brucellosis is high, and that the pregnant women should receive prompt therapy with antimicrobial agents when they present for medical care, which found that the incidence of spontaneous abortion and intrauterine death among a retrospective cohort of 92 pregnant women with acute brucellosis due primarily to *B. melitensis* was (46%) which is more higher than the result we have obtained (1.7%).

Conclusion

Results of this study confirmed that modified rose Bengal able to detect more positive serum sample than conventional rose Bengal and it would be substituted rose Bengal. However, SAT should be used aside with modified rose Bengal, because it could not cover all positive samples detected by SAT.

Recommendations

1-More studies are required, using large sample size from different hospitals to acquire more accurate result.

2-Employing more confirmatory tests eg. ELISA, PCR, to compare the sensitivity and specificity.

3-Good and early diagnosis should be processed to give the right treatment specially for pregnant women to avoid possibility of abortion or any problem outcome.

4- Cases with unexplained spontaneous abortion should be investigated for brucellosis.

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APPENDIX

APPENDIX

Appendex(1) : image of degree of agglutination in RBPT result.