COMPARATIVE STUDY ON CONVENTIONAL AND POLYMERASE CHAIN REACTION BASED DIAGNOSIS OF BOVINE BRUCELLOSIS

A Thesis Submitted in Partial Fulfillment of Master Degree of Veterinary Science (M.V.Sc.)

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PREFACE

The present study has been done at the Department of Preventive Medicine and Public Health, Faculty of Veterinary Medicine, University of Khartoum, under the supervision of Dr. Isam Mohammed Ali Elijalii.
DEDICATION

This work was dedicated to the souls of

My mother, father,

to

My sisters, brothers and relatives

With great love.
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ABSTRACT

Brucellosis is a widespread zoonotic disease which is caused by species of the genus Brucella. Diagnosis of brucellosis is the basic of any control program. Many methods are widely used for the diagnosis of the disease. Recently molecular techniques were introduced in the field of brucellosis.

This study was designed to isolate Brucella from milk samples and to evaluate the polymerase chain reaction as diagnostic method for brucellosis and to compare it with other conventional methods routinely used for the diagnosis of the disease. One hundred and sixty dairy cows from 12 farms from different localities in Khartoum State were examined for the presence of brucellosis. 160 milk samples and 160 serum samples were collected. All milk samples were examined bacteriologically for presence of the Brucella, 11 samples (6.9%) revealed colonial growth indicative to Brucella species. 149 (93.1%) showed no colonial growth. The 11 isolates were identified as B. abortus.

To compare polymerase chain reaction (PCR) with other tests, all milks samples were examined by PCR, Milk ring test and Modified Ziehl Nielsen Stain (MZN). Twenty (12.5%) milk samples showed Brucella species under microscope using MZN, although only 11 (6.9%) showed growth onto tryptose soy agar and Brucella media. 54 (33.8%) samples were showed positive reaction by MRT, while PCR detected brucella DNA in 33 (20.63%) milk
samples. On the other hand, 29 (18.13%) serum samples showed agglutinations using Rose Bengal plate test.

The sensitivity and specificity of these tests were evaluated. High sensitivity was reported for MRT and PCR (85% and 75%, respectively). While high specificity was obtained for PCR and RBPT (87.1% and 85.5%, respectively).

The prevalence of bovine brucellosis was found to be 12.5% for all farms examined. Most of the cows examined appeared healthy (97.5%), however application of vaccination program was found very limited. Furthermore, the level of hygiene was poor in most farms.

A positive correlation was found between mastitis and status of hygiene to the presence of bovine brucellosis (P< 0.05). In contrast, no relationship was observed between the disease and calving number (P> 0.05). It could be concluded that B. abortus could be isolated from milk samples and PCR showed high sensitivity and could be quick and useful method for diagnosis of bovine brucellosis.
ملخص الأطروحة

...
واختبار الحليقي النبّه

أعطى وقّد الاختبارات لهذه والخصوصية الحساسة.

تقييم تمت لوقّد الحساسية نسبتة أعلى المتسلسل البوليميري التفاعل في الاختبار.

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وين في الوالي العملي أعلاه أثناء الاختبار المتسلسل البوليميري التفاعل الاختبار كان خاصية نسبتة

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وسيلاّ البر مرض انتشار معدل كأحن الاختبارات تتمّ التي المزارات هذه أحن وام صحّة

ظاهرية كان الأبصار من إنا متدنيّ به ان البصّة المبكرة وان السحابة تفتقر كأحن.

كما تعلق وسيلة ووجد البر بروز المعالج، ووجود الصحة والحالة الاضطرابات بين جانبية العكّس أعلاه

والولداء وعدّ المرض بين علاقة توجد لا ﻧﺎن ﺖ.ر.ك

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INTRODUCTION

Brucellosis is a zoonotic bacterial disease which has a great effect on public and animal health in many countries of the world. It affects a variety of domestic and wild animals and man. It is caused by any one of the members of the genus *Brucella*.

*Brucella (B.)* is a group of bacteria which are morphologically and antigenically similar, it has six species according to the primary host, *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (swine), *B. ovis* (desert wood rat) (Bergy’s 1984); *B. neotomae* (Stonner and Lackman, 1957) and recently *B. maris*, a marine strain of *Brucella* (Ewalt et al., 1994; Ross et al., 1996). The first isolation of *Brucella* organisms from animals was made by Bang (1897), who was the first to report contagious abortion in cattle and other animal species and he named his isolate *Bacillus abortus*, Meyer and Shaw (1920) suggested the name *Brucella* for the genus.

Brucellosis has a major economic impact due to abortion, the consequent decrease in milk yield, death of infected animals and rejection of exported consignments containing infected animals. Also, the country incurs costs generated by prophylactic activities, control and eradication program, hospitalization of human patient, loss of work or income and failure in financial investment (Chukwa, 1987).
The disease in cattle is known by many names, such as infectious abortion, Bang’s disease, slinking of the calves and contagious abortion. In man the disease was also known by as Mediterranean fever, Malta fever, goat fever and undulant fever (Carpenter and Hubbert, 1963). *B. melitensis* was the first species reported as the case of human brucellosis due to consumption of raw infected goat milk (Bruce, 1887).

Bovine brucellosis is a highly contagious disease caused by *B. abortus* and at least nine biotypes have been recognized including a number of strain variants (Radostits *et al*., 2000).

In Sudan, animal brucellosis was suspected as early as 1904 and was first reported by Bennet (1943) in Khartoum. The milk supplying herd of cattle, sheep and goat were serologically tested and *B. melitensis* was isolated from the milk in Barakat (Daffalla and Khan, 1958). Subsequently many investigation of the disease were carried out (Habiballa *et al*., 1977, Suliman, 1987, Gameel *et al*., 1987).

Diagnosis of brucellosis is the cornerstone of any control program and is based on bacteriological and immunological findings. These methods are not wholly satisfactory that the bacteriological isolation is a time consuming procedure and handling of the microorganisms is hazardous and the use of serological test is recommended as a mean of indirectly diagnosing the disease. However, many current serological tests have proven to be either too sensitive,
giving false positive results, or too specific giving false negative results (Morgan and Mackinnon, 1979; Farina, 1985). The development of the new diagnostic tests for detection of *Brucella* species is of very important interest.

The polymerase chain reaction (PCR) has been shown to be available method for detecting DNA from different fastidious and non-cultivated agents. Recently it has been introduced in the field of brucellosis (Brikenmeyer and Mushahwar, 1991; Hamidy and Amin, 2001).

**Objectives**

The present study was carried out to investigate the following

1. To isolate *Brucella* from milk samples.
2. To evaluate the polymerase chain reaction for the diagnosis of bovine brucellosis from milk.
3. To compare PCR with MRT, MZN, culture for the diagnosis of bovine brucellosis.
4. To investigate the effects of some epidemiological factors on the disease.
CHAPTER ONE
LITERATURE REVIEW

1.1 Brucellosis

1.1.1 Definition

Brucellosis is a widespread bacterial disease of animals and man caused by any one of the members of the genus *Brucella* (Corbel and Hendry, 1983). It named brucellosis after David Bruce (1887), who was the first one to isolate the organism and recognized the disease. In animals the disease is characterized by bacterimia followed by localization of the organisms in the reproductive organs, reticuloendothelial tissues and sometimes joints (Gellespic and Timoney, 1981).

The disease in man is called Malta fever and is characterized by undulant fever, chills, headache, pains in legs, large joints and lumber regions, profuse nocturnal sweating, insomnia, sometimes laryngitis and bronchitis (Van Der Hoeden, 1964). Brucellosis is still a major problem, widely distributed throughout the world, mainly in developing countries due to traditional feeding habits and the failure to maintain standards of hygiene because of socioeconomic condition (Ozekicit *et al*., 2003).

1.1.2 Historical background

David Bruce (1887) was the first one who isolated the organism from spleen of a solider with Malta fever and named it “*Micrococcus melitensis*”.


Bang (1897) identified “Bacillus abortus” as the cause of abortion in cattle. Zammit (1905) identified goats as the reservoir of brucellosis. Malta fever, Mediterranean fever, Mediterranean gastric fever remittent and goat fever, which are often synonymously used for undulant fever (Carbenter and hubbert, 1963).

The first isolation of Brucella organisms from animals was by Bang (1897), who was the first to report contagious abortion in cattle and other animal species and he named his isolates “Bacillus abortus”, which was followed by other names “Corynebacterium abortus”, “Bacterium abortus” and “Alcaligenes abortus”, Meyer and Shaw (1920) suggested the name Brucella for the genus.

The disease in animals was known by many names before it named brucellosis; such as infectious abortion, Bang’s disease, slinking of the calve and contagious abortion.

In Sudan, the disease was suspected as early as 1904. Sympson (1908) reported 20 clinically diagnosed cases in the Blue Nile and Kassalla Province. In 1943 there was one sample of goat serum of high agglutination titer received. Bannet 1943 isolated Brucella abortus for the first time from a dairy herd in Khartoum. Haseeb (1950) was the first to confirm a case of human brucellosis. Haseeb (1950) and Dafalla (1962) stated that the disease was diagnosed in all provinces.
1.2 Bovine brucellosis

Bovine brucellosis is a highly contagious bacterial disease which has a worldwide distribution. It caused by *Brucella abortus*. Bang (1897) was the first to isolate the organisms “*Brucella abortus*” from a cow suffering from contagious abortion. *Brucella abortus* was isolated from various sources including milk, hygroma fluids, vaginal swabs and semen (Chatterjee *et al.*, 1995; Casolinaovo *et al.*, 1996), lymph nodes and aborted fetuses (Musa and Mitchell, 1985).

1.2.1 Etiology

Bovine brucellosis is caused by *B. abortus* which is a small Gram-negative, non-encapsulated coccus, coccobacillus or short rod, normally intracellular in host tissues. It is not acid-fast, but does resist decolourisation of weak acids, and stains red with Stamp's modification of the Ziehl-Neelsen stain (Bergy’s 1984).

1.2.2 World distribution

Until recently, bovine brucellosis has been present throughout the world. However, a number of countries have now succeeded in eradicating this disease. These include Australia, Canada, Israel, Japan, Austria, Switzerland, Denmark, Finland, Norway, Sweden and New Zealand.
1.2.3 Transmission

Bovine brucellosis is transmitted by ingestion, penetration of the intact skin and conjunctiva, and contamination of the udder during milking. Cows shed large numbers of organisms when they abort. Bacteria are also excreted intermittently in milk throughout the lactation. Animals become infected through ingestion of contaminated feed or water, or after licking an infected placenta, fetus or genitalia of another cow, after it has aborted. Most cows remain chronically infected. Uterine discharge, milk, urine, faeces and hygroma fluids are also sources of bacteria. Infected bulls may excrete the organism in their semen. Congenital transmission may occur through in utero infection. Importation of infected animals is the highest risk for introducing bovine brucellosis. Few infected cows do not recover from infection completely and should be considered as permanent carriers whether or not abortion occur. Bulls do not usually transmit infection from infected to non infected cows mechanically. Infected bulls may discharge semen containing organisms but are unlikely to transmit the infection. The risk of spread is much higher if the semen used for artificial insemination. (Radostits et al., 2000).

1.2.4 Clinical signs

The incubation period is variable and often cannot be accurately determined. The dominant feature of the disease in cattle (and usually the first observed clinical sign in a herd) is abortion, which typically occurs at about 5–
7 months of gestation. Sometimes, full-term calves are born but die soon after birth. The abortion rate in a herd depends on many factors, like the prior herd immunity. In fully susceptible herds, the abortion rate in the first year of infection may vary from 30% to 80%. However, even in fully susceptible herds, abortions may sometimes be more insidious.

Retained placenta, with secondary bacterial metritis, is a common sequel to abortion and may lead to permanent sterility. If infected cows remain fertile, subsequent pregnancies are usually carried to full term, although second and even third abortions may occur occasionally. In bulls, acute or chronic unilateral or bilateral orchitis, epididymitis and seminal vesiculitis occasionally occur. The scrotal circumference in such animals may be either normal or markedly increased. Unilateral or bilateral hygromas, particularly of the carpal joints, may occur in some animals in chronically infected herds (Geering et al., 1995).

1.2.5 Pathogenesis

The establishment and outcome of infection with *Brucella* depend on the number of infecting organisms and their virulence and also on host susceptibility (price *et al.*, 1990). Virulent *Brucella*, when engulfed by phagocytes on mucous membranes, are transported to regional lymph nodes. *Brucella* e persist within macrophages but not within neutrophiles. Inhibition of phagosome-lysosome function is a major mechanism for intracellular
survival and an important determinant of bacterial virulence. However, many of the mechanisms used by *Brucella* to survive within macrophages are not fully elucidated. Various stress proteins are thought to allow the organisms to adopt to harsh conditions encountered within macrophages (Rafie, *et al.*, 1996; Robertson and Roop, 1999). In addition, superoxide dismutase and catalase production may play a role in resistance to oxidase killing. Intermittent bacteremia results in spread and localization in the reproductive organs and associated glands in sexually mature animals. Erytheritol, a polyhydric alcohol which acts as a growth factor for *Brucella* e, is present in high concentration in the placenta of cattle, sheep, goat, and pigs. This growth factor is also found in other organs such as the mammary glands and epididymis, which are targets for *Brucella* e. In chronic brucellosis, organisms may localize in joints and intervertebral discs.

Abortion is a consequence of placentitis involving both cotyledons and intercotyledonary tissues. There is considerable variation in the severity of uterine lesions after abortion. There may be a mild to severe endometritis that becomes chronic. The chorion is not uniformly affected, and large parts may appear normal. Affected cotyledons are covered by a sticky, odorless brown exudates and are yellow-grey as a result of necrosis. Parts of the intercotelydonary placenta are thickened, oedematous, yellow-grey and may contain exudates on the surface.
Aborted fetuses may have varying degrees of subcutaneous edema and blood-stained fluid in their thoracic and abdominal cavities. Abomasal contents may be turbid, light yellow and flaky. The liver may be enlarged and discolored orange-brown. There may also be a fibrous pleuritis and grey-white foci of pneumonia. Some aborted fetuses appear normal. Mammary gland lesions and regional lymphadenitis are constant features but are not pronounced. In the bull, necrotizing orchitis occasionally results in localized fibrotic lesions (Quinn et al, 2002).

1.2.6 Diagnosis

Diagnosis of brucellosis is the basic of any control program and is depend on bacteriological and immunological finding. The use of serological tests is recommended as a mean of indirectly diagnosing the disease however, many current serological tests have proved to be either too sensitive giving false positive results or too specific giving false negative results (Morgan and Mackinnon, 1979; Farina, 1985). In addition the presence of antibodies does not always mean an active case of brucellosis, since vaccinated animals tend to yield persistent post vaccinated immune responses, and other Gram negative bacteria such as Yersinia enterocolotica may cross-react with smooth Brucella spp (Corbel, 1985; Diaz and Moriyon, 1989).

At present, diagnosis of brucellosis in live dairy cattle involve either the isolation of Brucella from milk samples or the detection of anti-Brucella
antibodies in serum or milk (Alton. et al., 1988). However these methods are not wholly satisfactory. Bacteriological isolation is a time consuming procedure, and handling the microorganisms is hazardous. Serological methods are not conclusive, because not all infect animals produce significant levels of antibodies and because cross-reaction with other bacteria can give false negative results (Alton et al., 1988).

The Milk ring test (MRT) is the most widely used for screening and monitoring brucellosis in dairy cattle (Alton et al., 1988). Although the sensitivity of the milk ring test is overemphasized (Huber and Nicoletti, 1986), its specificity has been questioned when prevalence is low (Rolfe, and sykes, 1987). In addition, false positive reactions may be given when milk is tested on the day of collection or taken from cows with mastitis (Morgan and Mackinnon, 1979).

The most specific diagnostic tests involves isolation of the causative organisms but this suffering along incubation period and low sensitivity, especially in the chronic stages of the disease moreover, the culture material must be handled carefully, as the Brucella organisms is a class III pathogen (Alton et al., 1988). According to these problems, the development of new diagnostic tests for the direct detection of Brucella species in milk is of very important interest.
Recently polymerase chain reaction (PCR) has been shown to be available method for detecting DNA from different fastidious and non-cultivated agents (Brikenmeyer and Mushahwar, 1991). Although there are several studies on *Brucella* DNA detection by PCR from pure culture (Fekete et al., 1990; Herman and Ridder, 1992), there are a few studies have been performed with clinical or field samples from cattle (Fekete et al., 1992; Amin et al., 1995; Leal-Klevenzas et al., 1995; Romero et al., 1995). No enough data are available to assess the performance of the PCR assay on milk samples from farm animals other than cattle.

There are many methods which are used for the diagnosis of brucellosis:

1.2.6.1 Culture

Culture of suitable material on one of the *Brucella* media and isolation of the causative agent was used for diagnosis.

1.2.6.2 Demonstration of *Brucella* organisms in suspected samples

by staining with Modified Ziel Nelseen Stain. This method has a drawback in not being specific for *Brucella* organisms, *Coxiella burnetti* was found to be stained similarly (Corbel, 1973a).

1.2.6.3 Microscopical identification by immunofluorescence

It was stated that the identification of *Brucella* organisms microscopically by immunoflourescence specific and dependable technique
could differentiate between *Brucella* infection and that of Q-fever. Moreover it was stated that counter staining of abortion material with Evans blue prevents autofluorescence and improves the results of direct fluorescent antibody tests for detection of *Brucella* (Meyer, 1966; Corbel, 1973b).

### 1.2.6.4 Guinea pig inoculation

This method is successful than direct culture especially from contaminated material. Injections are made intramuscularly inside the thigh and the guinea pigs are killed 4-5 weeks after inoculation and their sera be subjected to five tube agglutination test. Recovery of the organism from the spleen of guinea pigs or positive SAT at 1/10 or over are taken as evidence of infection (Brinely *et al.*, 1978).

### 1.2.6.5 Serological diagnosis

Recently, there are two types of serological tests available: very sensitive ones which are used for screening and definitive ones used for confirmation of infection. As a result, usually more than one type of tests are used for the diagnosis of brucellosis because there is no single test which is both sensitive and specific, has the ability to discriminate between vaccinated animals from non vaccinated ones and could distinguish between antibodies due to infection from those of cross reactions. Many serological tests were developed for diagnosis of brucellosis using body fluids such as sera, hygroma fluids, milk, vaginal mucus, semen, bursa and muscle juices. These tests are
Rose Bengal test (RBPT), serum and tube agglutination test (SAT or TAT), complement Fixation test (CFT), card test, plate agglutination test, buffered plate agglutination test (BPAT), modified serum agglutination test, antiglobulin test (AGT) or coombs test, indirect haemolysis test (IHLT), haemolysis in gel test (HIGT), indirect haemolysis test (IHAT), enzyme linked immunosorbent assay ELISA), milk ping test (MRT), whey agglutination test and allergic skin test (AST). According to the WHO working group on Brucellosis (1992) RBPT, MRT, ELISA and CFT are the conventional serological diagnostic methods and should continue in use for brucellosis surveillance until year 2000. The important serological tests which are used in diagnoses of brucellosis are:-

1.2.6.5.1 Rose Bengal plate test

This test is widely used as a screening test to detect the presence of *B. abortus* infection in cattle (Morgan *et al.*, 1969; Alton *et al.*, 1975). Also it can be used as a definitive test (Nicoletti, 1967).

The rose Bengal stained antigen is buffered at 3.65 PH to inhibit non-specific agglutinins, but not those of *Brucella* (Rose and Roepke, 1957). However, recently it was found to detect IgG1 and IgM isotypes in bovine, sheep and goat sera and diagnosed the acute and chronic forms of the disease (WHO, 1993).
The RBPT is considered as a valuable screening test (Farina, 1985). It was recommended by Mikolon et al., (1998) because of its high sensitivity, ease of performance, it is cheap and rapid. The test is more sensitive, but less specific than SAT and CFT. Its efficiency affected by the cell concentration and the standardization procedure of the antigen (Hosie et al., 1985; Blasco et al., 1994).

1.2.6.5.2 Milk ring test

The milk ring test is widely used for screening and diagnosing of brucellosis. The test results are influenced by factors such as mastitis, mechanical agitation and vaccination with B. abortus S19 vaccine. The test used to detect brucellosis in dairy cattle but is not sensitive enough to detect brucellosis in goats (Shimi and Tabatabai, 1981). It can be used with milk from individual animals or bulk milk samples. The MRT was proved to be sensitive and specific for screening dairy herds and for identifying infected ones (Morgan, 1967). According to WHO Report, (1992) the MRT is not suitable for sheep and goats as ring formation does not readily occur.

1.2.6.5.3 Serum agglutination test

The test is widely used in some countries and its positive results are subjected to the definite CFT. Other than sera, the agglutination can be used for vaginal mucous and semen examination. The antigen used in the test is a
Brucella whole cell and the antibodies detected are those directed against the surface molecules.

SAT unlike the other tests, it detects antibodies of other isotypes (Macmillan, 1990). It can be performed in tubes or microtitre plates and the plate test was found to be more sensitive (Herr et al., 1982). SAT has international standardization; it is used for control program and in import and export policies (Macmillan and Cockrem, 1985). According to the Reports of FAO/WHO Expert Committee on Brucellosis (1964), the results of this tests in cattle with antibody level less than 50 I.U, should be considered negative in non-vaccinated animals or in those with unknown vaccination history, whereas in the vaccinated over 30 months of age, the level should be more than 50 I.U. In camels the level of a positive titer has not been established.

Morgan et al., (1969) mentioned that a proportion of sheep bacteriologically positive for brucellosis failed to react to the SAT. This proved the inferiority of SAT compared to the other conventional tests. Falade, (1978), compared RBPT, SAT and MRT for the diagnosis of brucellosis in caprine and concluded that SAT offered a better serological result.

1.2.6.5.4 The complement fixation test

This test is used for confirming the results of the RBPT and SAT. The test was found to be more accurate for bovine brucellosis (Morgan et al., 1973). Meyer (1979) stated that the test was superior to other tests in
sensitivity and specificity, and it was found to have the highest specificity in both non-vaccinated and vaccinated cattle when compared with SAT, haemolysis in gel, indirect enzyme immunoassay and buffered plate antigen tests. Although Corbel (1972) stated that RBPT and CFT reactions are probably due to the same antibody which is IgG\textsubscript{1}. Blasco \textit{et al.}, (1994) found that the CFT was less sensitive than RBPT.

Buxton and Fraser (1977) reported that the test was useful in detecting chronically infected animals in which the complement fixing antibodies disappear more slowly than agglutinins. But Sutherland \textit{et al.}, (1982) reported some limitation concerning the test, mainly its failure to differentiate between infected animals and those recently vaccinated in addition to the difficulty of performing the test.

1.2.6.6 Evaluation of sensitivity and specificity of some important serological tests

Huber and Nicoletti (1986) compared between Card test, Rivanol, CFT and MRT with the isolation rate of \textit{B. abortus} from cattle. the investigators concluded that CFT had the best balance of sensitivity and specificity in adult vaccinated cows. They also found that the false negative rates for Rivanol and CFT were higher in non adult vaccinated cows.

Dohoo \textit{et al.} (1986) compared between the BPAT, standard tube agglutination test, CFT, HIGT and Indirect EIA and concluded that CFT had
the highest specificity in both non vaccinated and vaccinated cattle. The Indirect EIA interpreted at a high threshold (high starting point) also exhibited a high specificity in both groups of cattle. The HIGT had very high specificity when used in non vaccinated cattle, but quite low one among vaccinated. The authors also realized that the exertion of CFT to all the tests had high sensitivities if interpreted at minimum threshold (minimum starting point).

Falade (1978) compared RBPT, SAT, MRT for the diagnosis of brucellosis in caprines and concluded that SAT gets a better serological diagnostic tool for goat brucellosis.

Boraker et al., (1981) described BrucELISA: an enzyme-antibody immunoassay for detection of B. abortus antibodies in milk: correlation with the Brucella test and with shedding of viable organisms. The BuceELISA results correlated highly with Brucella ring test reactions and culture positivity, eliminated false-positive. Brucella ring test reactions, detected antibody in some samples which were Brucella ring test negative, and distinguished between vaccinated and infected animals and concluded that the BrucELISA system is a sensitive, specific, and inexpensive method for screening large numbers of individual or bulk milk samples for the presence of antibody to B. abortus.

Three serological methods, the rose-Bengal test (RBT), the complement-fixation test (CFT) and an indirect enzyme-linked immunosorbent
assay (I-ELISA) were compared for the detection of *Brucella* -infected animals in unvaccinated cattle herds in Eritrea. The number of seropositive animals was higher by ELISA in herds that had positive animals. Serum samples which gave higher degrees of agglutination with the RBT need not be re-tested with CFT. Consideration of the seropositive status of a herd should be taken on defining the cut-off optical density readings for ELISA (Omer *et al.*, 2001).

Vanzini *et al.* (2001) evaluated an indirect enzyme-linked immunosorbent assay (ELISA) for detection of *Brucella abortus* antibodies in bovine bulk milk samples and they stated that the sensitivity of the ELISA was higher than the BRT but the specificity of the BPT was not statistically different from the ELISA.

Klaus (2002) stated that since serological diagnosis of brucellosis began more than 100 years ago with a simple agglutination test, it was realized that this type of test was susceptible to false positive reactions resulting from, for instance, exposure to cross reacting microorganisms. Also test format was inexpensive, simple and could be rapid, although results were subjectively scored. Therefore, a number of modifications were developed along with other types of tests but another problems occurs that interpretation was correct if all tests were performed and they did not agree, This led to the development of an assay that could distinguish vaccinal antibodies, starting with precipitin tests these tests did not perform well, giving rise to the development of primary
binding assays. These assays, including the competitive enzyme immunoassay (EIA) and the fluorescence polarization assay (FPA) and they are at the apex of current development, providing high sensitivity and specificity as well as speed and mobility in the case of fluorescence polarization assay.

The fluorescence polarization assay (FPA) is a simple, rapid, inexpensive method for the detection of \textit{Brucella abortus} antibodies in bulk tanks bovine milk samples at the farm level or at dairies with a sensitivity and specificity of 100 and 95.9\%, respectively, (Gall \textit{et al.}, 2002). The assay detect antibodies to \textit{B.abortus} in 15 min by testing undiluted whey produced by chemical and physical manipulation of milk from bulk tanks. this sampling is noninvasive and therefore costs less and is less stressful than blood-based tests.

\textbf{1.2.6.7 MOLECULAR METHODS}

\textbf{1.2.6.7.1 Polymerase chain reaction}

Polymerase chain reaction (PCR) is a powerful new technique that allows scientists to amplify a specific DNA sequence million of times in just a few hours. The technique was invented by Kary Mullis in 1983. PCR has already been cited in well over 5,000 scientific papers (as of 1992) and is revolutionizing many areas of genetic research including; genetic disease diagnosis, forensic medicine, and molecular evolution.
Polymerase chain reaction has been shown to be available method for detecting DNA from different fastidious and non-cultivated agents (Brikenmeyer and Mushahwar, 1991). Although there are several studies on *Brucella* DNA detection by PCR from pure culture (Fekete *et al.*, 1992; Leal-klevezes *et al.*, 1995; Romero *et al.*, 1995). In addition, not enough data are available to assess the performance of the PCR assay on milk samples from farm animals other than cattle, Although milk from other animal species such as a sheep, goats and camels is an important source of human brucellosis, particularly in those parts of the world were *B. melitensis* prevails (Alton, 1990).

PCR-based methods have the potential to be fast, accurate, and efficient in detecting *Brucella*. However, when PCR was applied to milk samples, its sensitivity was low with respect to bacterial culture and some false-negative PCR results have been reported (Romero *et al.*, 1995).

Different methods of extraction of bacterial DNA from bovine milk to improve the direct detection of *Brucella* by PCR were evaluated. It was found that the use of lysis buffer with high concentration of tris, EDTA, and NACL, high concentration of sodium dodecyl sulphate and proteinase K, and high temperature of incubation was necessary for the efficient extraction of *Brucella* DNA. The limit of detection by PCR was 5 to 50 *Brucella* CFU/ml of milk (Romero and Lopez 1999).
Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by using SAT, RBPT, MRT, culture and PCR was performed. Milk samples were examined by culture and single step polymerase chain reaction techniques (PCR) for detection of *Brucella* species. It was evident that, PCR assay detected more positive samples from the milk of different animals except sheep than the culture method. This indicated that the sensitivity of the PCR was higher than the culture methods (Hamidy and Amin, 2001). The same result was reported by klevezas *et al.*, (1995) and Romero *et al.*, (1995) and this may be attributed to the fact that PCR detect living and dead organisms. PCR could detect fewer numbers of *Brucella* organisms per milliliter of milk than could be detected by direct culture. It is noteworthy to mention that all milk samples collected from *Brucella* free herds tested negative by PCR; This is finding indicated satisfactory the specificity of the assay. The speed and sensitivity of the PCR assay suggest that this technique could be useful for detection of *Brucella* organisms in bovine milk, as well as in sheep, goat, and camel milk.

**1.2.7 Epidemiology**

Epidemiology is the study of host parasite relationships in population. The interaction of host and *Brucella* species is complicated by many factors such as survival of the parasite within population are variable, absence of clinical signs, latency, incubation period and interacelluar inhabitation.
The changes in animal husbandry towards larger herds and greater cattle commerce result in great exposure of herds to brucellosis (Nicoletti, 1980) and nomadism cause major problems in delivery of veterinary services.

Brucellosis in food producing animals is caused by four species \( B.\text{abortus} \), \( B.\text{melitensis} \), \( B.\text{suis} \), \( B.\text{ovis} \), the organisms localized in the reproductive organs which they are all play role in transmission of the disease.

Bovine brucellosis is almost invariably transmitted from herd to herd through the movement of infected cattle. Cows shed large numbers of organisms into the environment when they abort. Cows that lactate following abortion excrete bacteria intermittently in milk throughout the lactation period. Urine, faeces and hygroma fluids are also a source of bacteria. There is a rapid decline of organisms soon after calving or abortion, and cows are then generally non-infective until the next pregnancy, when there is again a rapid build-up of \textit{Brucella} organisms in the reproductive tract even in the absence of abortion. Most cows remain chronically infected, with infection localization occurring in the udder and lymph nodes.

Cattle usually become infected after ingesting contaminated feed or water, or after licking an infected placenta, calf fetus or the genitalia of an infected cow after it has aborted. In pregnant cow uterus, there is a rapid multiplication of bacteria during the 2 \text{rd} and 3 \text{rd} trimester of pregnancy and
excretion of large numbers of the organisms at the time of abortion or normal parturition, also vaginal excretion may continue for up to 15 days. Excretion of the organisms in milk is intermittence and numbers of bacteria is varied (Morgan and McDiarmid, 1960). 52% of cattle with positive milk titers were culturally positive for Brucella (Nicoletti and Muraschi, 1978). The persistency of the organisms in the udder, appear to cause little udder damage (Nicoletti, 1984).

Susceptibility of cattle to B. abortus infection is influenced by the age, sex, and reproductive status of the individual animal. Sexually mature, pregnant cattle are more susceptible to infection with the organism than sexually immature cattle of either sex (Enright, 1990). Young cattle are less susceptible to B. abortus than older sexually mature cattle. Susceptibility appears to be more commonly associated with sexually maturity than age (Radostits et al., 2000)

Susceptibility to brucellosis increases with sexual development and pregnancy. Cunningham (1977) found weak and transient titers among young heifers exposed to virulent strains of B. abortus. Calves were least susceptible to infection while prevalence in lactating cows is the highest among different age groups.

Recent reports showed that number of heifer calves which were infected at early life, were negative to serologic tests and aborted or had an infected
calving during the first pregnancy, these were referred to as latent carriers (Cuninigham, 1977a) so it became a source of infection for the herd. Latent infection is difficult to diagnose early in the course of the disease and not easy to eradicate brucellosis in these cattle herds.

Bulls are more resistant to *Brucella* infection than sexually mature heifers and cows (Nicoletti, 1980). Bulls play a less important role in the spread of the disease but can be widely spread by infected semen used for artificial insemination (Blendixen and Blood, 1947).

The *Brucella* is an intracellular parasite, so they have protection from the innate host defenses and from the therapeutics agents. Natural or artificial infection usually persist indefinitely although about 10-15% recover spontaneously (Nicoletti, 1980).

The probability of infection increases by increases of the herd size (Christie, 1969). This permits contact with other cows in spite of hygienic or other control measures (Nicoletti, 1980).

Environmental survival of the organism depends on temperature and exposure to sunlight. It may survive for up to eight months in an aborted fetus in the shade, for 3–4 months in faeces, and for 2–3 months in wet soil. Human infection is acquired either occupationally through handling infected cows, their tissues, discharges and through drinking infected milk. The latter route of infection is prevented by pasteurization.
1.2.8 Zoonotic importance

Transmission of brucellosis to humans occurs through the consumption of infected, unpasteurized animal-milk products, through direct contact with infected animal parts such as the placenta by inoculation through ruptures of skin and mucous membranes, and through the inhalation of infected aerosolized particles. Brucellosis is an occupational disease in abattoir workers, veterinarians, dairy-industry professionals, and personnel in microbiologic laboratories. One important epidemiologic step in containing brucellosis in the community is the screening of household members of infected persons (Imuneef et al., 2004)

Airborne transmission of brucellosis has been studied in the context of using Brucella as a biologic weapon. In fact, B. suis was the first agent contemplated by the U.S. Army as a potential biologic weapon and is still considered in that category (Smart 1997). In a hypothetical attack scenario, it was estimated that release of an aerosolized form of Brucella under optimal circumstances for dispersion would cause 82,500 cases of brucellosis and 413 fatalities (Kaufmann et al., 1997). Cases of laboratory-acquired brucellosis are the perfect examples of airborne spreading of the disease (Ergonul et al., 2004).

After entering the human body and being taken up by local tissue lymphocytes, Brucella are transferred through regional lymph nodes into the
circulation and are subsequently seeded throughout the body, with tropism for
the reticuloendothelial system. The period of inoculation usually ranges from
two to four weeks.

The classic categorization of brucellosis as acute, sub acute, or chronic
is subjective and of limited clinical interest. Four species of *Brucella* can cause
human disease: *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis*. Disease from
marine species has also emerged (Sohn et al., 2003). The vast majority of cases
worldwide are attributed to *B. melitensis*. A recent study did not report any
clinical differences between cases caused by *B. melitensis* and those caused by
*B. abortus* (Dokuzoguz et al., 2005). Sufficient data on virulence and clinical
presentation of biotypes of *B. melitensis* are lacking, although separate
biotypes that predominate in various regions, for example type 2 in
Northwestern Greece, type 3 in Turkey, (Bodur et al., 2003), and type 1 in
Spain (Colmenero et al., 1997).

Human brucellosis is traditionally described as a disease of protean
manifestations. However, fever is invariable and can be spiking and
accompanied by rigors, if bacteremia is present, or may be relapsing, mild, or
protracted. Malodorous perspiration is almost pathognomonic. Constitutional
symptoms are generally present. Physical examination is generally nonspecific,
though lymphadenopathy, hepatomegaly, or splenomegaly is often present.
Osteoarticular disease is universally the most common complication of
brucellosis (Bosilkovski, 2004). The reproductive system is the second most common site of focal brucellosis. Brucellosis can present as epididymoorchitis in men and is often difficult to differentiate from other local disease (Navarro et al., 2001). Brucellosis in pregnancy poses a substantial risk of spontaneous abortion (Khan et al., 2001).

1.2.9 Economic importance

Brucellosis can resulted in decreased fertility, reduced milk production, abortion in susceptible replacement animals and testicular degeneration in bulls. Farmers suffer loss of income due to abortion, the consequences of decreased milk yield, killing of infected animals and prolonged fattening time.

The country incurs costs generated by prophylactic activities, control and eradication program, hospitalization of human patients, cost of research, loss of work or income and failure in financial investment (Chaukwa, 1987).

1.2.10 Treatment and control

All Brucella strains are sensitive in vitro to gentamycin, tetracycline and rifampin. Treatment is likely to be undertaken in animals. Streptomycin, doxycycline and rifampin have become the mainstay in antibiotic therapy for brucellosis (Solera et al., 1997). The combination of doxycycline plus streptomycin is found to be superior to that of doxycycline plus riflampcin. The combination with usually doxycycline is
necessity to prevent relapse on antibiotic withdrawal (Maurina and Raoult, 2001).

Several countries have made significant progress in the control of brucellosis in their animal population over the last few years: every country has its own vaccination program and control policy which depends on the pattern of animal husbandry practiced, species affected and the prevalence of the disease in the different livestock species (Shommein et al., 1987).

Prolonged treatment of infected domestic animals with a high dosage of antibiotics is not used due to the appearance of antibiotics in the human food chain, its interferes with the production of milk and the capacity of the organism to grow intracellularly.

The disease has negative impact on the economy and exportation so it must be controlled and eradicated. Plommet, (1986) recommended three ways for control and prevention of brucellosis:

- Vaccination of exposed herds or animals

- Protection of herds in disease free areas by restriction of animal movement and prohibition of importation of animals from infected areas.

- Segregation of infected animals or herds from free ones by testing and slaughter.
The maximum control and prevention is achieved when the three ways are combined (Nicoletti, 1980). Elimination by testing and slaughter is carried out only in small farms under closed systems.

1.3 The Genus Brucella

The genus *Brucella* (B.) is a group of Gram-negative bacteria, which are morphologically and antigenically similar (Evans, 1918). Six members of the genus are currently known. These are *B. melitensis* (Hughes, 1893); *B. abortus* (Schmit and Weis, 1901); *B. suis* (Huddleson, 1929); *B. neotomae* (Stonner and Lackman, 1957); *B. ovis* (Buddle, 1956) and recently *B. maris* a marine strain of *Brucella* (Ewalt *et al*., 1994; Ross *et al*., 1996). After molecular characterization the designation of *Brucella Maris* has been retained for practical reason (Betsy *et al*., 2002).

1.3.1 Taxonomy

Improvement of the knowledge on the taxonomy of *Brucella* plays a significant role in the solution of the questions of effective identification and differentiation study of museum cultures and their control as well as in scientifically grounded development of novel diagnostic and prophylactic preparations.

The classification of the species and identification of the Genus *Brucella* which based on recommendations made by the subcommittee on taxonomy of the International Committee on Bacteriological Nomenclature in
1963 and subsequently extended in later reports (1975, 1982 and 1984), depend on two sets of properties: lysis by phages and oxidative metabolic profiles on selected amino acids and carbohydrates substrate. Corbel (1990) mentioned that the oxidative metabolism patterns showed fairly close relationship with phage lysis patterns and that both procedures were useful for identification of the species.

The species and biovars of the genus *Brucella* were listed in Bergy’s Manual of Systemic Bacteriology (1984). Of the six species currently classified, *B. abortus* was further divided into 9 biovars, *B. melitensis* into 3, *B. suis* into 5, but *B. neotomae, B. ovis* and *B. canis* have no biovars identified, however, *B. abortus* biovar 8 no longer exists (Meyer and Morgan, 1973) and *B. abortus* biovar 7 was reported to be examined culture of *B. abortus* biovars 3 and 5 (International Committee on Systematic Bacteriology, Subcommittee on Taxonomy of *Brucella*, 1986). As a result both biovars were not included in recent classification (Alton *et al.*, 1988; Corbel, 1990). *B. melitensis, B. abortus, B. suis, B. neotomae* occur in smooth phases especially on primary isolation, while *B. ovis* and *B. canis* occur in rough forms.

Genetically determined variants in the properties of *Brucella* cultures occur. These include changes from the smooth to rough colonial phase, loss of CO₂ requirement or loss of H₂S production, changes in sensitivity to lysis by phages or resistance to dyes and antibiotics (Meyer, 1976; WHO, 1986).
The extending potencies of using modern methods of biochemistry, molecular biology, and genetics in the *Brucella taxonomy* will enable rapid and reliable identification of brucellosis agents (Dranovskaya, 1995).

For taxonomic purposes, all *Brucella Spp.* should be classified as *Brucella Melitensis* as DNA-DNA hybridization studies have shown that the genus contains only one species (Veger, *et al.*, 1985).

### 1.3.2 Morphology

Members of the genus *Brucella* are cocci, coccobacillia or short rods, measuring 0.5-0.7µm in diameter and 0.6-1.5µm in length. The organisms arranged singly and less frequently in pairs, short chains or small groups. They are Gram negative, non motile and do not form spores and capsule (Bergey’s, 1984).

### 1.3.3 Cultural and biochemical characteristics

The organisms are aerobic but many strains require supplementary CO₂ for growth especially on primary isolation. Growth is slow and is usually visible after 48 hours of incubation at 37°C and growth occurs between 20-40°C optimum PH is 6.6 -7.4°C. Colonies are usually 0.5-1.0 mm in diameter, transparent, raised, and convex, with an entire edges and smooth glistening surface. *B. canis* and *B. ovis* characteristically produce non smooth colonies. Non smooth variants of the other species also occur.
According to Bergey’s Manual of Systemic Bacteriology (1984), most strains require complex media containing several amino acids, thiamin, nicotinamide ions. Some strains may be induced to grow on minimal media containing an ammonium salt as the sole nitrogen source. Growth is improved by serum or blood. Enriched media such as serum agar, liver infusion, dextrose potato and glycerol potato are recommended for primary isolation and optimum growth (Buxton and Fraser, 1977). Some strains of *Brucella* require the presence of serum in the medium for their growth especially on primary isolation; Serum dextrose agar, serum-tryptose agar and serum-tryptocase soy agar are recommended as the best basal non selective media (Alton, 1988).

The organism does not produce acid from carbohydrates in conventional media except *B. neotomae*. The catalase, oxidase, H₂S and urease tests are positive but the indole and vogus-proskaur tests are negative.

1.3.4 Resistance to physical and chemical agent

Members of the genus *Brucella* are sensitive to the heat and are killed by pasteurization or exposure to 60°C for 30 minutes. It’s ready killed by UV or Gamma ray’s under complete exposure. The organisms are susceptible to an acid PH, disinfectant and direct sunlight (Buxton and Fraser, 1977).

1.3.5 Survival of the organisms

*Brucella* has substantial capacity to survive and persist in the environment under suitable condition compared with non pathogenic bacteria.
At low temperature *Brucella* can survive in soil for up to ten weeks and in liquid manure for up to 2.5 years and in frozen carcasses for many years. If dried in the presence of excess protein and protect from sunlight may retain infectivity for years (Bergey’s, 1984).

### 1.4 Bovine Brucellosis in Sudan

The first incidence of bovine brucellosis in Sudan was reported in a dairy herd in Khartoum when *B. abortus* was isolated from an aborted cow (Bennett, 1943), then after that bovine brucellosis was reported in all parts of the country and the prevalence rate was found to be higher in cattle compared to other animal species (Ragaa, 2000). Many investigators isolated the organisms from various sources in different localities in the country (Khan, 1956; Daffalla, 1962; Shigidi and Razig, 1971-1973; Ibrahim, 1974; Musa and Mitchell, 1985; Khalafalla *et al.*, 1987; Musa *et al.*, 1990a). *Brucella* was isolated from knee hygromas of cattle by Shigide and Razig (1971–1973) and Khalafalla *et al.*, 1995. *B. melitensis* was isolated from cows milk in Elgazira, Central Sudan (Daffalla and Khan, 1958). Brucellosis appears to be widely distributed in Darfur states (Western Sudan). Musa *et al.*, (1990b) reported the prevalence of the disease in different animal species including cattle and concluded that the highest prevalence rates were encountered in intensive farming system and under nomadic conditions.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Samples

2.1.1 Sources of samples

A total of 320 samples consisting of 160 milk and 160 serum were collected from dairy farms with known history of brucellosis. Animals were of different breeds and ages. The farms were in Khartoum State from different localities in Omdurman and Hilat Kuku.

2.1.2 Collection of samples

2.1.2.1 Milk samples

Milk samples were collected from dairy cows after examination of udder and teat abnormalities. The whole udder was washed, dried and the tip of the teat was disinfected with 70% alcohol. The first stream of milk was discarded and then 20 ml of foremilk from each half of the udder were taken directly into a labeled sterile universal bottle and placed on ice in a thermo flask.

2.1.2.2 Serum samples

Five ml of blood were collected in sterile tubes from the jugular vein using a disposable syringe after disinfecting the area with methyl alcohol. The tubes were placed in slanting position and left to clot, then taken to the laboratory on ice and placed in the refrigerator overnight. Then the serum was
collected into Bijou bottles. The sera were tested immediately after collection or kept at -20 °C until used within 48 hours.

2.1.3 Transportation of the samples

All samples were placed on ice in a thermos flask then taken to the laboratory and tested immediately.

2.2 Milk samples

All milk samples were examined serologically for presence of antibodies to *Brucella*. Also all milk samples were examined by modified Ziehl-Nielsen stain, culture and single-step polymerase chain reaction (PCR).

2.2.1 Bacteriological examination

2.2.1.1 Culture media

2.2.1.1.1 Solid media

2.2.1.1.1.1 Tryptic soy agar (Difco)

This is a dehydrated medium, it is a general purpose medium used with or without blood or other enrichment for the isolation and cultivation of wide variety of fastidious microorganisms. This medium contains tryptone, soya peptone, sodium chloride and agar. It was prepared according to the manufacturer’s instructions by suspend 40 grams in 1000/ml distilled or deionized water and bring gently to the boil to dissolve completely. Sterilized in the autoclave for 15 minutes at 15Ibs pressure (121°C), the PH was 7.3± 0.2.
2.2.1.1.1.2 Brucella medium (MAST DIAGNOSTICS, UK)

This is a dehydrated culture medium used for the isolation and cultivation of Brucella. It contains peptone mixture, dextrose, yeast, sodium chloride and agar. It was prepared according to the manufacturers’ instructions as follow: 45.5g of powder were suspended in 1 liter of distilled water, autoclaved at 121°C (15 p.s.i.) for 15 minutes, Cooled to 50°C and add 5% inactivated horse serum was added (to inactivate hold at 56°C for 30 minutes. Antibiotics may also be added if required, then mixed well before pouring. The PH was adjusted to approx. 7.4.

2.2.1.1.1.3 Serum dextrose agar (SDA)

2.2.1.1.1.3.1 Preparation of serum

Sera for media were separated from blood collected from horses through the jugular vein, then tested for Brucella antibodies by RBPT. Negative sera were filtered through 0.2-µ membrane filter, and then distributed in sterile McCartney bottles or sterile flasks (50 ml.). Sera were tested for sterility by incubation at 37°C for 24 hours, and then inactivated in a water bath at 56°C for 30 minutes. This sera were obtained from the Central of the Veterinary Laboratory (CVL).

2.2.1.1.1.3.2 Preparation of medium

The medium was prepared by reconstitution of Brucella medium base or tryptose soy agar (Difco), which had been sterilized as mentioned above. The
basal medium was left to cool to 56°C, then sterile horse serum (5-7%) was added for enrichment. The mixture was distributed into sterile Petri dishes (20 ml) and left to solidify. Slants were made by placing 5 ml of the medium into sterile McCartney bottles and left to solidify in a sloping position.

2.2.1.1.4 Farrell’s medium

It is SDA with antibiotics and antimicrobial agents to the basal medium. These antibiotics are bacitracin, cyclohexamide, naladixic acid, nystatin, polymyxin B and vancomycin. These antibiotics obtained from the Central Veterinary Laboratory (CVL).

2.2.1.1.5 Nutrient agar (Oxoid)

This dehydrated medium is composed of beef extract, peptone, Nacl and agar. The medium was prepared according to the manufacturer instructions by dissolving 28 g of the powder in one liter of distilled water. It was distributed into 100 ml amounts in bottles and sterilized. Then distributed into sterile Petri dish (20 ml.) or McCartney bottles in slant position and left to solidify. It has a pH of 7.4. This medium was used for catalase test.

2.2.1.1.6 Christensen’s medium (Urea Medium)

The basal medium contains peptone, sodium phosphate, dextrose, potassium dihydrogen phosphate, phenol red and agar. It was prepared according to the manufacturer’s instructions by dissolving 2.4 grams of urea agar base (Oxiod) in 95ml distilled water by boiling. Sterilized and the PH was
adjusted to 7.1 then cooled to 50°C -55°C. Five ml of sterile 40% urea solution were added aseptically. The mixture was distributed in 10 ml amount into McCartney bottles and allowed to solidify in slope position. This medium was used for detection of urea splitting organisms.

2.2.1.1.7 Motility medium

The motility medium is composed of tryptose, sodium chloride and agar. It was prepared according to the manufacture’s instructions by dissolving 20/grams of the dehydrated powder in one liter distilled water, distributed in 5 ml amounts into test tubes, covered with cotton wool and sterilized.

2.2.1.2 Sterilization

2.2.1.1.2.1 Sterilization of glassware

Glassware such as test tubes, pipettes, flasks and Petri dishes were sterilized in the hot air oven at 160ºC for an hour. Others like McCartney, Bijou and universal bottles were sterilized in the autoclave at 15 Ib. /in² for 15 minutes (121 ºC). Instruments such as forceps, spatulas, scissors and scalpels were sterilized in the hot air oven at 160 ºC for an hour or by flaming after dipping in 70% alcohol while used (Merchant ant Packer, 1971).

2.2.1.1.1.2.2 Sterilization of culture media

Culture media such as tryptose soy agar and Brucella agar base, peptone water, blood agar base, urea agar base, nutrient gelatin, Hugh and liefson medium, citrate medium, methyl red and Vegoes-Proskauer media (M-R and
V-P media), nitrate broth, motility media and nutrient agar were sterilized in
the autoclave at 15 lb. /in² for 15 minutes.

2.2.1.1.2.3 Sterilization of solutions

Normal saline, phenol saline and distilled water were sterilized by
autoclaving at 115°C for 10 minutes.

2.2.1.1.2.4 Sterilization of serum

Serum was sterilized by filtration using 0.45-micrometer Millipore filter
(Oxoid).

2.2.1.3 Culture methods

2.2.1.3.1 Primary culturing

Milk is centrifuged at 3000 g for 15 minutes then discarded the layer
between the cream and sediment to obtain the mixture of sediment and cream
which was used for culture (Alton, 1988). A loopful of the mixture was
streaked onto tryptose soy agar supplied with antibiotics in duplicate using a
sterile loop.

2.2.1.3.2 Incubation of cultures

All inoculated plates were incubated at 37°C aerobically and in the
presence of 5-10% CO₂ (microaerophilically) using a candle jar.
2.2.1.3.3 Examination of culture

Plates were examined with the naked eye on the third day of incubation and re-examined daily for growth and colonial morphology. Plates showing no growth or heavy contamination were discarded after ten days.

2.2.1.3.4 Subculturing

Typical and well isolated *Brucella*-like colonies from the primary culture were picked with a wire loop and streaked on the surface of fresh plates of the corresponding medium. Pure cultures were obtained by replating the subcultures on T.S.A. These were identified macroscopically by the presence of small in diameter, transparent, raised, and convex, with an entire edges and smooth glistening surface colonies along the streak lines. Microscopically, modified Ziehl-Neelsen’s stained smears gave positive reaction, partial acid fast often branching filament which fragments into rods and cocci.

2.2.1.4 Identification of isolates

Purified isolates from the primary or from subcultruing plates were identified to the species level according to the criteria outlined by Barrow and feltham (1993).

2.2.1.4.2.1 Appearance of the colony

All well purified growth colonies were examined for shape, colour and consistency. Growth is slow and is usually visible after 48 hours of incubation at 37°C and growth occurs between 20-40°C at optimum pH (6.6 -7.4)°C.
Colonies are usually 0.5-1.0 mm in diameter, transparent, raised, convex, with an entire edges and smooth glistening surface.

2.2.1.4.2 Staining

2.2.1.4.2.1 Preparation of Smears from Culture

Smears were prepared by emulsifying part of a typical and well isolated colony in a drop of sterile saline and spread in a clean slide. The smears were then allowed to dry by air then fixed by gentle flaming.

All smears were examined by Modified Ziehl Neelsen’s stain and Gram Stain.

2.2.1.4.2.1.1 Gram Stain

The prepared smears were fixed by gentle flaming and put on a glass holder. The slides then flooded with crystal violet stain for one minute, washed with tap water. Covered with iodine and then washed, the slides were decolorized with acetone for 15 seconds and slides were washed with tap water. The slides were stained with diluted carbol fuchin for one minute and washed with water again and allowed to dry.

A drop of immersion oil was added to each slide and examined under microscope.

2.2.1.4.2.1.2 Modified Ziehl Nielsen’s stain

After fixation of the slide, it was flood with dilute carbolfuchsin for 15 minutes, and then the slides were washed thoroughly under running water. Decoloried in acetic acid 5% for 15 seconds, it was washed well in water.
When decolorized was complete, counter stain with methylene blue for one minute, washed and dried.

2.2.1.4.2 Motility

Motility was determined by the hanging-drop technique (Barrow and feltham, 1993). A drop of bacterial suspension was placed in the center of a cover slip. The cover slip was inverted over the concaved area of the slides and examined under the microscope. It was also done by inoculation of the organism into semi-solid motility media by means of a wire loop in a straight line then incubated at 37ºC and examined daily for five consecutive days. A positive reaction was indicated by the bacterial growth towards the surface.

2.2.1.4.4 Biochemical tests

All biochemical tests were performed according to Barrow and feltham (1993) and they included:

2.2.1.4.4.1 CO₂ Requirement

The isolates were inoculated onto two SDA plates and incubated at 37ºC for 2-3 days, on air + 10% CO₂ using a candle jar. Then the plates were observed for growth for 3-15 days.

2.2.1.4.4.2 Oxidase test

The oxidase test was performed by removing a portion of freshly grown colonies with a sterile glass rod and rubbing it on a strip of filter paper, which had been impregnated, with 1% solution of oxidase reagent. The immediate
development of a dark purple colour within 10 seconds indicated a positive reaction.

2.2.1.4.4.3 Catalase test

Organisms were grown on nutrient agar or *Brucella* agar slope. A drop of 3% hydrogen peroxide solution was added over the culture. Immediate production of gas bubbles was considered a positive reaction.

2.2.1.4.4.4 Urease production

A heavy inoculum of the organisms was seeded onto a slope of Christensen’s urea agar medium, incubated at 37°C (in 10% CO₂) and examined at intervals of 15 minutes, hourly for 24 hours and daily for five consecutive days. A positive reaction was indicated by the development of red or purple pink colour.

2.2.1.4.4.5 H₂S Production

The organisms were grown on SDA slopes and lead acetate paper strips were inserted in the MacCarteny bottle without touching the medium then fixed in position by the bottle lid. The strips were examined daily for three days for blackening due to H₂S production.

2.2.2 Microscopic examination

2.2.2.1 Preparation of Smears from milk samples

Milk was centrifuged at 7000 rpm for 15 minutes then discards the layer between the cream and the deposit was discarded clean slides were flamed
then a loopful of the deposit and cream of the centrifuged milk was spread on the slide.

All prepared smears were examined by microscopic following Modified Ziehl Nelsen stain.

2.2.3 Milk ring Test

2.2.3.1 The antigen

The test was done according to Morgan et al., (1978). The antigen used for MRT was supplied by the Central Veterinary Laboratory (CVL). It is a suspension of the organism stained with Haematoxylin (blue).

2.2.3.2 Test procedure

The test was done by adding 0.03ml of stained milk ring test antigen to one ml of milk. Both were mixed well and incubated at 37ºC for three hour and then the test was observed for ring formation.

2.2.4 Polymerase chain reaction (PCR)

2.2.4.1 DNA Extraction

2.2.4.1.1 DNA Extraction from the milk samples

2.2.4.1.1.1 DNA Extraction by boiling

The extraction method was adapted locally. Mixture of the cream and sediment obtained from the centrifugation of 20 ml of the milk sample at 3000 g for 15 minutes was taken and then diluted to 1:80 by using deionized distilled water in screw capped micro centrifuged tube. The dilute mixture was
vortexed then placed on water bath at 100 °C for 5 minute then the supernatant were gently aspirate and centrifuged for 2 minutes, then gently aspirated the supernatant which used directly for PCR amplification without any further processing.

2.2.4.1.1.2 DNA Extraction by Kit (GenElute™ bacterial genomic DNA Kit), (SIGMA).

The extraction was done by using commercial kit as follows:

20 ml of the milk sample was centrifuged at 3000 g for 15 minutes then a mixture of the cream and sediment was used.

Cells were harvested by suspending one millimeter of the mixture at 12,000- 16,000 x g for 2 minutes then cells were resuspended in 180 µl lysis solution T. then cells were lysed by two ways first 20 µl proteinase K was added to cells suspension, vortex or pipet to mix. Incubate at 55 C for 30 minutes second 200 µl lysis solutions C was added vortex or pipet to mix and was incubated at 55 C for 10 minutes.

500 µl of column preparation solution was added to each binding column and spin at 12,000 x g, for one min. 200 µl ethanol was added to the lysed cells, vortex or invert to mix. The mixture was transferred to binding column. Spin at 6500 x g for one min. the column was transferred to new collection tube then 500 µl wash solution 1 was added to column. Spinnet at 6500xg for one minute then the column was transferred to new collection tube.
500 µl wash solution concentrate was added to column and spinet at 12,000xg for three minutes to dry column. Finally the column was transferred to new collection tube. 200 µl of Elution solution was added and spinet at 6500xg for one minutes.

2.2.4.1.2 DNA Extraction from the Standard Bacteria (strain 19) by Boiling.

One to three colonies from the growing cultures were picked up into 100 µl deionized distilled H₂O in 1.5 ml screw capped micro centrifuged tube then placed on boiling water bath for 10 min. The supernatant was took and used directly for amplification.

2.2.4.2 Oligonucleotide Primers

The primers used were previously described by Bricker and Hallng, (1994). They were obtained from MWG- Biotech (AG 32-1074-1/6). The sequences of the primer were as follows:

AB

5´ GAC, GAA, CGG, AAT, TTT, TCC, AAT, CCC  3´

IS711

5´-TGC, CGA, TCA, CTT, AAG, GGC, CTT, CAT-3
2.2.4.3 PCR Amplification

2.2.4.3.1 PCR Reaction mixture

PCR assay was done by DNA thermocycler following the instructions of the manufacturer (Bioline) with some modifications. PCR amplification was carried out in 50 µl reaction mixture consisted of 5 µl of a sample (or genomic DNA) containing template DNA, 1.5 U of Tag DNA polymerase, 5 µl of 10 x PCR amplification buffer, 20 pmol/µl each primer, 200mM deoxynucleoside triphosphate (dNTPs), 25mM MgCl and double-distilled water to a final volume of 50 µl. To minimize evaporation, 25 µl of mineral oil was added to the reaction mixture on top of each PCR tube.

2.2.4.3.2 Thermal cycler program

The amplification was done by the program which described by Bricker and Halling (1994). So the thermal cycler device (Techne) was programmed to provide the following thermal profiles:

First denaturation at 95 °C for 5 minutes, cycling condition consists of 35 cycles. DNA denaturation at 95 °C for 1.15 minutes, primers annealing at 55.5 for 2 minutes and extension of the two strands at 72 °C for 2 minutes. After the final cycle, the reaction was terminated by an extra run at 72°C for 10 minutes for final extension.
2.2.4.4 Electrophoresis

Amplified products from the samples were confirmed by electrophoresis on 1.5% agarose gel, which was prepared as follows:

0.6 gram agarose were dissolved in 40 ml 1X TBE solution (Tris Boric acid EDTA) under heat and then 0.5µg/ml ethidium bromide (BDH, U.S.A) was added. The liquid agarose gel was poured into gel electrophoresis apparatus and 10 slots were made using a comb and then left to coal. 20µl of PCR product was mixed with 5µl of loading buffer and dye was transferred into each slots of the gel. 2µl of DNA ladder (100 bp DNA ladder, MBI Fermentas) were put into one slot of each run. Positive and negative controls were applied in each run. The gel electrophoresis apparatus was conducted into a power pack (Volts = 75 for 45 minutes). The amplicons were visualized under ultraviolet illumination (BDH) and photographed afterwards using gel documentations system. The product fragments were identified by comparing the products bands with the band of the positive control and with the DNA size marker,.

2.3 Serum Samples

All serum samples were examined by RBPT.
2.3.1 Rose Bengal test (RBPT)

2.3.1.1 The antigen

The antigen used in the RBPT was obtained from CVL, It was prepared and standardized as described by Alton et al., (1988).

2.3.1.2 The test procedure

The serum samples and the antigen were removed from the refrigerator and placed at room temperature for an hour. The test was done by dispensing 0.03ml of each serum to be tested to an enamel plate. The same amount of rose Bengal antigen was added to each serum and both were mixed together, rocked by hand for four minutes after which the test was immediately read. Agglutination appeared as weak positive, positive, strong positive or very strong positive.

2.4 Epidemiological data

Epidemiological data of farms examined was collected. The collected data included, breed, hygiene, general condition, routine check of Brucella, vaccination program, history of abortion, culling practice and type of insemination.

2.4 Statistical analysis

The statistical analysis of the data was done using Chi square test.
CHAPTER THREE

RESULTS

3.1 The Results of milk samples and blood samples with different tests

Out of the 160 milk samples, cultured for the presence of *Brucella*, only 11 samples (6.9%) revealed colonial growth indicative to *Brucella*. 149 (93.1%) showed no colonial growth indicative to *Brucella* organism. According to colonial morphology, motility, microscopical appearance with Gram and modified Zeil Nelson’s stains and biochemical tests the isolated were identified as *B. abortus*.

The colonial morphology of isolated *B. abortus* were small in diameter, transparent, raised, convex, with an entire edges and smooth glistening surface. All isolates were non motile, Gram –ve cocci, coccobacillia or short rods. With modified Ziel-Nelson’s stain, they appeared acid fast bacilli. All the grown organisms proved to be non *Brucella* according to colonial morphology and stains reactions, were not further identified.

When examined by modified MZN, 20 (12.5%) out of 160 milk samples demonstrated *Brucella* organism. 140 milk samples showed no organisms microscopically. The seen organisms showed acid fast Bacilli, pink cell with blue background.
With Milk Ring Test, the 160 milk samples gave 54 (33%) positive reactions. 106 (67%) gave negative reactions. Positive samples showed formation of clear blue ring at the top column of the milk in the test tube.

Examination of the milk samples with PCR reveal amplification of *Brucella* DNA from 33 (20.6%) samples. The size of band produced from all samples was 498 bp. However 127 (70.4%) samples showed no PCR product.

With Rose Bengal Test, 29 (18.1%) serum samples gave agglutinations to *Brucella* antigens. While 131 (81.9%) were negative. The 29 positive samples showed varied degrees of agglutination varied from + to ++++. 

The variation of reactions of milk samples and serum samples to different tests is shown in Table3.
Table 3. Reactions of Milk and Serum Samples to Different Tests

<table>
<thead>
<tr>
<th>Samples</th>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>Milk</td>
<td>MZN</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.5%</td>
</tr>
<tr>
<td>160</td>
<td>Milk</td>
<td>Culture</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.9%</td>
</tr>
<tr>
<td>160</td>
<td>Milk</td>
<td>MRT</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33.8%</td>
</tr>
<tr>
<td>160</td>
<td>Milk</td>
<td>PCR</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20.6%</td>
</tr>
<tr>
<td>160</td>
<td>Serum</td>
<td>RPBT</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18.1%</td>
</tr>
</tbody>
</table>
3.2 The Prevalence of bovine Brucellosis in examined farms

Epidemiological data was obtained during the study from the 12 farms examined, the farm data revealed that the total population of the farms examined was 437 animals. The prevalence of bovine brucellosis in all farms examined based on MZN was found to be 12.5%.

The results revealed that most of the animals examined from all farms were cross bread (88.1%) and only (11.9%) were native cattle. There were 7 farms had one bull in each farm while 5 farms had two. All farms had a natural insemination except one farm. All bulls in the farms examined found to be negative for brucellosis except in one farms it was positive. Most of the cows examined were appear healthy (97.5%), however application of vaccination program was very limited (19.38%). One farm performing the culling practice. Furthermore the level of hygiene was found to be poor in most farms (Table 4).
Table 4: Summary of epidemiological herd data of bovine brucellosis in Khartoum state

<table>
<thead>
<tr>
<th>Farm number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed or % of Friesian blood</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>62%</td>
<td>67%</td>
<td>67%</td>
<td>52-62%</td>
<td>62%</td>
<td>62%</td>
<td>62%</td>
<td>62%</td>
<td>67%</td>
</tr>
<tr>
<td>Number of bulls</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Way of insemination</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N+A</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>General condition</td>
<td>H</td>
<td>H</td>
<td>P</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Routine check of brucellosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Last check of brucellosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Every 6 month</td>
<td>-</td>
<td>Before 2 years</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Annually</td>
</tr>
<tr>
<td>Vaccination and last vaccination</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>History of abortion</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Culling practice</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Milking practice hygiene</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>G</td>
<td>P</td>
<td>G</td>
<td>G</td>
<td>F</td>
<td>P</td>
<td>P</td>
<td>G</td>
</tr>
<tr>
<td>Feeding and drinking hygiene</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>G</td>
<td>P</td>
<td>G</td>
<td>G</td>
<td>F</td>
<td>P</td>
<td>P</td>
<td>G</td>
</tr>
</tbody>
</table>

N, natural; N+A, natural and artificial; A, artificial
H, healthy; G, good; P, poor; F, fair.
3.3 The Sensitivity and Specificity of the MRT, RBPT and PCR Based on MZN

Based on MZN, the sensitivity of the MRT was found to be 85% while the specificity was 73.4%. The sensitivity of the RBPT was low 45% while its specificity was 85.5%. The sensitivity of the PCR was found to be 75% however its specificity was very high 87.1% (Table 5).
Table 5: The Sensitivity and Specificity of Milk Ring Test, Rose Bengal Test and Polymerase Chain Reaction Based on Modified Ziehl Neelson's Stain

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk Ring Test</td>
<td>85%</td>
<td>73.4%</td>
</tr>
<tr>
<td>Rose Bengal Test</td>
<td>45%</td>
<td>85.5%</td>
</tr>
<tr>
<td>Polymerase Chain Reaction</td>
<td>75%</td>
<td>87.1%</td>
</tr>
</tbody>
</table>
3.4 The Relationship Between Some Factors and Occurrence of the Brucellosis

Out of the 160 animals examined, 23 cows were found to be mastatic. The number of calving of examined animals varied from 1-6. A positive correlation was found between mastitis and the occurrence of brucellosis (P<0.05). In contrast, no relationship was observed between the disease and calving number (P>0.05) Table 6.
Table 6: chi square and P. value

<table>
<thead>
<tr>
<th>Factor</th>
<th>$X^2$</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastitis</td>
<td>7.7967</td>
<td>0.005*</td>
</tr>
<tr>
<td>Calving number</td>
<td>4.7072</td>
<td>0.788</td>
</tr>
<tr>
<td>Hygiene</td>
<td>9.5154</td>
<td>0.009*</td>
</tr>
</tbody>
</table>

* The difference was statistically significant (P < 0.05).
Figure 1: Number of positive reaction of serum and milk samples with different tests.
Figure 2: The Sensitivity and specificity of different tests
Figure 3. Gel Electrophoresis of Genomic DNA obtained from milk samples. Lane: 1, Molecular size marker (base pairs, bp); Lanes 2,3 milk samples.
Figure 4(a). Gel Electrophoresis of PCR products obtained from milk samples. Lane 1, Molecular size marker (base pairs, bp); Lanes 2, 3, 4, 5, 6, 7, 8 milk samples.
Figure 4(b). Gel Electrophoresis of PCR products obtained from milk samples. Lane 1, Molecular size marker (base pairs, bp); Lanes 2, 3, 4, 5, 6, 7, milk samples.
CHAPTER FOUR
DISCUSSION

Brucellosis is an important disease of animal and man. It is distributed all over the world. The disease transmitted by many routes mainly by ingestion. The disease in cattle is characterized by abortion, hygroma, orchitis, placentitis and infertility. The prevalence of infection varies considerably between herds, areas, and countries (Radstitis et al., 2000). Cattle are the most important source of infection with \textit{B. abortus} which was isolated from various sources including milk, hygroma fluids, vaginal swabs and semen (Chatterjee \textit{et al.}, 1995; Casolinaovo \textit{et al.}, 1996), lymph nodes and aborted fetuses (Musa and Mitchell, 1985).

Bovine brucellosis is of major economic importance in developing countries which have not had a national brucellosis eradication program. Diagnosis of brucellosis is one of the most important way for eradication program which it based on bacteriological and immunological finding, recently brucellosis was diagnosed by polymerase chain reaction in many country of the world (Hamidy and Amin, 2001).

The present study on bovine brucellosis was carried out in Khartoum State mainly in Hilat Kuku Area where the bulk of cattle population in Khartoum is kept, approximately about 5039 head of cattle are found in this
area (Mahlab 2). On the other hand, Kuku area was one of the early dairy units established.

This study was aimed to evaluate the polymerase chain reaction (PCR) in diagnosis of brucellosis and to compare between different tests namely MRT, RBPT, MZN, PCR and culture. Also, attempts were made to isolate *Brucella* species from milk samples.

In the present study, from 160 milk samples 11 isolates of *Brucella abortus* were recorded. In Sudan, many investigators had isolated *Brucella* species from various sources rather than milk in different localities in the country (Khan, 1956; Dafalla, 1962; Shigidi and Razig, 1971-1973; Ibrahim, 1974; Khalafalla *et al.*, 1987; Musa *et al.*, 1990a and Musa and Mitchell, 1985). Although our isolates were few but they could open the way for further isolation of *B. abortus* from bovine milk. On the other hand, isolation of *Brucella*, in Sudan from milk was directed to *B. melitensis* specially from goats. Usually the isolation of *Brucella* species is very difficult because of the long incubation period needed moreover the organisms is fastidious. Also, milk contains other types of bacteria which may contaminated the culture. Hence specific antibiotics should be added to the media when culturing species of the genus *Brucella* (Alton *et al.*, 1988).

Milk Ring Test showed high sensitivity for detection of the infection (85%). Although the high sensitivity, MRT may give false positive reactions
because that the test results are influenced by many factors such as mastitis, mechanical agitation and vaccination (Morgan and Machinnon 1979). And MRT is most widely used for screening and monitoring brucellosis in dairy cattle (Alton et al., 1988).

Although the rose Bengal plate test is considered as a valuable screening test (Farina, 1985) and recommended by Mikolon et al., (1998) due to its sensitivity, it showed low sensitivity (45%) compared to other tests used in this study. This may be attributed to the fact that efficiency of the test is affected by the cell concentration and the standardization procedure of the antigen (Hosie et al., 1985; Blasco et al., 1994)

Polymerase chain reaction has been shown to be a reliable method for detecting DNA from different clinical samples (Brikenmeyer and Mushahwar, 1991). The main objective of the present study was to evaluate the PCR as diagnostic method for bovine brucellosis from milk samples which for the first time in Sudan was performed. in the diagnosis of brucellosis directly from milk. PCR showed high sensitivity (75%) and high specificity (85%) compared to other tests used.

Extraction of Brucella DNA from milk was done for samples by boiling and by using GeneElute™ Bacterial Genomic DNA Kit (SIGMA) to compare between them and the two methods showed quite similar extraction results so the boiling methods could be use for extraction with the advantage of low cost.

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The PCR detected more positive samples (n=54) from the milk than the culture method. This indicated that the sensitivity of the PCR was higher than that of the culture method. The same conclusion was reached by Leal-Klevenzas et al., (1995), Romero et al., (1995), Adel et al, 2001 and Hamidy et al., (2001). This may be attributed to the fact that PCR detects living and dead organisms, while culture detects only living organisms. Also, PCR could detect fewer numbers of Brucella strains per ml of milk than could be detected by direct culture.

Lower numbers of Brucella strains were detected in milk using different extraction protocols. This indicated that the sensitivity of PCR is primarily affected by the effectiveness of the DNA-extraction protocol and the amount of sample processed by the assay. Since low numbers of Brucella strain are able to transmit the disease, the sensitivity of the PCR should be increased to detect the lower numbers of Brucella organisms in field samples.

It was evident from our results that Brucella was not detected by culture or PCR from some seropositive animals and this was expected, as the excretion of the organisms in milk is intermittent (Morgan and Mackinnon, 1979; Alton et al., 1988) and is more common during late lactation and can persist for several years. Although the sensitivity of the serological tests is better than the culture, the specificities are low and false positive reactions may occur (Whicher, 1981).
The prevalence of bovine brucellosis was found to be 12.5% among examined farms which it considered high. This may be attributed to the fact that there was no routine check of brucellosis to detect the infected ones and most of these farms (88.12%) were lacking the culling practice, however application of vaccination program was very limited. Furthermore, the level of hygiene was poor in most farms. The prevalence obtained in this study is relatively low when compared with Suliman (1987), who has investigated brucellosis in Khartoum and Elgazira provinces and reported a prevalence of 15.2%. This difference may be due to the large numbers of samples they examined (2085 milk and 710 serum samples) at the same time El Gezira is considered as an endemic area. Gameel et al., (1987) examined 20 dairy herds for brucellosis in Khartoum province, 9 farms revealed positive cases. No much work was carried out on bovine brucellosis in Khartoum State. This study is considered as a new update of bovine brucellosis in this State. However, bovine brucellosis was reported in all parts of the country as the prevalence was found to be higher in cattle compared to other animal species (Raga, 2000).

In the present study it was observed that no relation was found between age of examined animals and occurrence of brucellosis. The disease was found in cows with different ages without significant differences between calving numbers. Enright (1990) reported that brucellosis occurred in all ages of cattle.
on the other hand, Radostits et al., (2000) stated that young cattle are more susceptible to infection than older ones.

All bulls in the 12 farms examined were found to be negative for brucellosis except one farm in which the bull used for insemination was positive. The prevalence in this farm was found to be relatively high (16.1%). Although bulls play less important role in the spread of the infection but can spread infection by semen used for artificial insemination (Blendixen and Blood, 1947). The high prevalence in this farm may be disagreed with above idea.

The results revealed that most of animals examined from all farms were cross bread (88.1%) and 11.9% were local. The incidence of brucellosis is high in cross bread than in local. Bakhiet (1981) studied the incidence of brucellosis in cross breed cattle and local bread in El Gezira using serum agglutination test and found that the rates of reactors was 22.5%, 1.2% among the cross bread and local cattle respectively.
Conclusions

The present study concluded that:

1. Eleven isolates of *Brucella abortus* were isolated from milk samples.
2. PCR showed high sensitivity and specificity compared to other tests.
3. PCR has the potential to be useful method for diagnosis of brucellosis from milk samples.
4. The prevalence of bovine brucellosis in examined dairy farms was found relatively high (12.5%).
Recommendations

1. Introduction of PCR in the field of diagnosis of brucellosis in Sudan would be useful among references laboratories.

2. Good hygiene, routine check of brucellosis, culling practice and vaccination program must be conducted for all farms to reduce the disease.

3. National program for control and eradication of brucellosis should be done by proper diagnosis.
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