Comparative Study of Four Seroagglutiny Serum Tests for
Brucillosis in Dairy Cattle in Port Sudan City, Sudan

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October, 2005

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A dissertation submitted to the university Khartoum in
partial fulfilment of the requirements for the degree of
Master of Science in Microbiology (M.Sc. Micro)

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August, 2010
DEDICATION

To my teacher’s soul who gave me more than I was asked for the best man, teacher, father and colleague prof. Mohammed Musa M.A.

To my Parents: Zeinab & Ismail who always pray for me

To my Sisters, brother and my husband who always supported me

To my friends and colleagues
ACKNOWLEDGMENTS

First of all, thanks and praise are to ALLAH, the compassionate and the most merciful for giving me strength and health during the period of the study. Thanks to my supervisor Dr. Abdel Hafeez Hassan Nimir for guidance, advice and help during the study period, and all staff of Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum.

My thanks are to Dr. Al Waseela Mukhtar, Department of Agricultural Extension and Development, Faculty of Agriculture, University of Khartoum.

My deep thanks to Dr. Adil Ali Hassanin, Dr. Salah Omer and all staff of Port Sudan Research Laboratory for their invaluable assistance.

My thanks to Dr. Abdel Nassir Mohammed Taha and Dr. Maha Khojaly and all the staff of Department of Brucella, Central Veterinary Research Laboratory, Soba (CVRL).

My deepest thanks for prof. Mohammed Musa Mohammed-Ahmed, Department of Parasitology, College of Veterinary Medicine, Sudan University of Science and Technology for his encouragement and giving me his time and experience, to Dr. Majdi Badawi, Dr. Eiman and Dr. Nuseiba Department of Pathology, Central Veterinary Research Laboratory, Soba (CVRL).

I would like to express my thanks to the all members of Animal Resources Department, Red Sea State and to Mr. Musa Ahmed for his unlimited help and assistance in collection of samples. I would like to thank the staff of Port Sudan Research Laboratory for their help.
ABSTRACT

The objective of this work was to study the seroprevalence of brucellosis in dairy cattle in Port Sudan city, the Red Sea State, Sudan. Two hundred and fifteen blood samples were collected aseptically from three locations in the city bearing in mind the density of animals in each location. Sera were separated from clotted blood samples and tested by four screen tests: The modified Rose Bengal Plate Test, the Rose Bengal Plate Test, the Serum Agglutination Test and the competitive Enzyme-linked Immunosorbent Assay. The seroprevalence rate was 21% by modified Rose Bengal plate Test and 13.0% by Rose Bengal plate Test. The Serum Agglutination Test detected 93% of the 27 samples positive for the Rose Bengal Plate Test. The highest titre for SAT was 1488 IU/ml and the lowest was 20 IU/ml. Only 46% of 46 cattle found positive with the modified Rose Bengal plate Test were confirmed by the modified Rose Bengal plate Test prove positive by the competitive Enzyme-linked Immunosorbent Assay. The results showed that the modified Rose Bengal Plate Test was the most sensitive of the four followed by the Rose Bengal Plate Test but were less sensitive than the competitive Enzyme-linked Immunosorbent Assay. These findings are discussed with respect to improved diagnosis and control of brucellosis in animals and humans in Eastern Sudan with emphasis on urban areas.
الملخص

الهدف من هذه الدراسة هو استكشاف درجة الانتشار المصلي لمرض البروسيليا في الأبقار المنتجة للألبان في مدينة بورتسودان، ولاية البحر الأحمر، السودان وقد تم جمع مائتين وخمسة عشر عينة دم من ثلاثة مواقع في المدينة مع الوضع في الاعتبار كثافة الحيوانات في كل موقع. الأمصال تم فصلها من عيانات الدم واختبارها بواسطة أربعة اختبارات مصلية هي اختبار روز بنغال المعدل، اختبار روز بنغال الصحي واختبار التنصيب المصلي وفحص تنافسية الأنزيم المناعي المرتبط. وأظهرت هذه الاختبارات أن معدلات الانتشار المصلي في ترتيب تنازلي كما يلي: 21% باستخدام اختبار روز بنغال المعدل، 13.0% باستخدام اختبار روز بنغال الصحي، وأُكد اختبار التنصيب القياسي فقط 93% من 27 عينة موجبة لاختبار اختبار روز بنغال الصحي.

على عيان كان 1488 وحدة دولية/مل، وأدنى 20 وحدة دولية/مل. وتُؤكد فقط 46% بواسطة اختبار تنافسية الأنزيم المناعي المرتبط من 46 عينة موجبة بواسطة اختبار روز بنغال المعدل. ومع ذلك، أثبتت عينة واحدة سلبية من قبل اختبار روز بنغال المعدل بأنها إيجابية بواسطة اختبار تنافسية الأنزيم المناعي المرتبط. أظهرت النتائج أن اختبار روز بنغال المعدل الأكثر حساسية من أربعة اختبارات يليه اختبار روز بنغال الصحي ولكن كانوا أقل حساسية من فحص تنافسية الأنزيم المناعي المرتبط.
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CHAPTER I

Introduction

Brucellosis is a worldwide public health hazard and has an economic importance. Brucella species have been identified: *Brucella abortus* (cattle), *B. canis* (dogs), *B. melitensis* (goats), *B. suis* (pigs, hares, reindeer, rodents and human), *B. ovis* (rams), *B. neotomae* (desert wood rats), *B. ceti* and *B. pinnipedialis* (marine mammals), *B. microti* (common vole) and *Brucella inopinata*, associated with a human infection (OIE, 2009). These strains are intracellular parasites, gram negative, short rods. *Brucella* species have a wide host range, but cattle and other bovidae is the preferred host of *B. abortus* which causes bovine brucellosis.

Bovine brucellosis is included in the OIE (World Organization for Animal Health) list of notifiable diseases as a multiple species disease. OIE-listed diseases are the diseases with the potential for international spread, significant mortality or morbidity within the susceptible species or potential for zoonotic spread to humans.

The World Health Organization (WHO) laboratory bio-safety manual classifies *Brucella* in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness (undulant fever) which may progress to a more chronic form and can also produce serious complications affecting the musculo–skeletal, cardiovascular, and central nervous systems. Therefore, strict precautions should be taken to prevent human infection. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes or through intact skin, but ingestion of dairy products constitutes the main risk to the general public where the disease is endemic (OIE, 2009).
Brucella species vary in their geographic distribution. B. abortus is found worldwide in cattle-raising regions except in Japan, Canada, some European countries, Australia, New Zealand and Israel, where it has been eradicated. Eradication from domesticated herds is nearly complete in the United State of America. Brucella abortus persists in wildlife hosts in some regions, including the Greater Yellowstone Area of North America.

Brucella species are readily killed by most commonly available disinfectants including hypochlorite solutions, 70% ethanol, isopropanol, iodophores, phenolic disinfectants, formaldehyde, glutaraldehyde and xylene; however, organic matter and low temperatures decrease the efficacy of disinfectants. Autoclaving (moist heat of 121°C for at least 15 minutes) can be used to destroy Brucella species on contaminated equipment. These organisms can also be inactivated by dry heat (160-170°C for at least 1 hour). Boiling for 10 minutes is usually effective for liquids. Xylene (1ml/liter) and calcium cyanamide (20 kg/m³) are reported to decontaminate liquid manure after 2 to 4 weeks. Brucella species can also be inactivated by gamma irradiation (e.g. in colostrum) and pasteurization. Their persistence in cheese which made from unpasteurized milk is influenced by the type of fermentation and ripening time. The fermentation time necessary to ensure safety in ripened, fermented cheeses is unknown, but is estimated to be approximately three months. Brucella is reported to persist for weeks in ice cream and months in butter. This organism survives for a very short periods in meat, unless it is frozen; in a frozen meat, survival times of years have been reported (OIE, 2009).

Brucellosis is usually an occupational disease: Most cases occur in abattoir workers, veterinarians, hunters, farmers, reindeer/caribou herders and livestock producers. Brucellosis is also one of the most easily acquired laboratory infections. People who do not work with animals, tissues or bacterial
cultures usually become infected by ingesting unpasteurized dairy products (OIE, 2009).

Brucellosis is an emergency animal disease that have the potential to cause major national socioeconomic consequences through very serious international trade losses, national market disruptions and very severe production losses in the livestock industries that are involved (AUSVETPLAN, 2005). Bovine brucellosis impacts on public health because many Sudanese, regardless of location or region, have the habit of consumption of raw milk. Such unscrupulous consumers are under high risk of contracting serious diseases such as Tuberculosis, leptospirosis, brucellosis, salmonellosis ...etc. The present study was planned to investigate the seroprevalence of brucellosis in Port Sudan city. Residents in the city and its surrounding are avid consumers of raw milk and dairy products or otherwise.

1.1-Objectives:

1. To study seroprevalence rate in dairy farms in Port Sudan city and focus on the magnitude of the problem of dissemination of brucellosis as a zoonotic disease.

2. To increase public awareness of the dangers of contracting brucellosis through contaminated raw dairy products and raw meat (Marara, camel’s liver). Farmers are also encouraged to vaccinate their animals against brucellosis and produce safe products to public.

Activities:

Conduction of a seroprevalence survey of brucellosis using four diagnostic serological techniques: Modified Rose Bengal Test (mRBPT), Rose Bengal Plate Test (RBPT), Standard Tube Agglutination test (SAT) and competitive ELISA (cELISA).
2. - Literature review

2.1. - Higher order taxa:

Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Brucellaceae (The National Center for Biotechnology Information NCBI, 2007).

Genus *Brucella* (Meyer and Shaw 1920). *B. melitensis* (Hughes 1893; Meyer and Shaw 1920) is the nomenclatural type species of genus *Brucella*. Genus *Brucella* comprises the following ten described species:

*B. melitensis* (Hughes, 1893; Meyer and Shaw, 1920).

*B. abortus* (Schmidt, 1901; Meyer and Shaw, 1920).

*B. suis* (Huddleston, 1929).

*B. ovis* (Buddle, 1950).

*B. neotomae* (Stoenner and Lackman, 1957).

*B. canis* (Carmichael and Bruner, 1968).

*B. ceti* sp. (Cloeckaert et al., 2001; Foster, et al., 2007).

*B. pinnipedialis* sp. (Cloeckaert et al., 2001; Foster et. al., 2007).

*B. microti* sp. (Hubálek et al., 2007; Scholz, et. al., 2008).

*B. inopinata* (Scholz, et. al., 2010).
Table 1: Characters used in the differentiation of *Brucella* species and biovars (FAO/WHO, 2005)

<table>
<thead>
<tr>
<th>Species</th>
<th>Bio-type</th>
<th>CO₂ req’t</th>
<th>CO₂ prod’n</th>
<th>Growth on media containing</th>
<th>Agglutination with monospecific antisera</th>
<th>Lysis by phage† at RTD</th>
</tr>
</thead>
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<tr>
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<td></td>
<td></td>
<td>thionin*</td>
<td>fuchsin*</td>
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<td><em>B. abortus</em></td>
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<td>(-)‡</td>
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<td>+</td>
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<tr>
<td><em>B. suis</em></td>
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<tr>
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<tr>
<td><em>B. ovis</em></td>
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<td>(+)</td>
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<td><em>B. canis</em></td>
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<tr>
<td><em>B. neotomae</em></td>
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<td>+</td>
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</tr>
</tbody>
</table>

L = Confluent lysis        PL = Partial lysis        NL = No lysis

* Concentration = 1/50 000 w/v
† Phage R will lyse non-smooth *Brucella abortus* at RTD
Phage R/O will also lyse *B. ovis* at RTD
‡ (+) = Most strains positive  (-) = Most strains negative
** For more certain differentiation of *B. abortus* Type 3 and Type 6, thionin at 1/25 000 (w/v) is used in addition.  Type 3 = + ,  Type 6 = -.
*** Some strains of this biovar are inhibited by basic fuchsin
**** Some isolates may be resistant to basic fuchsin, pyronin and safranin O
2.2. - History and nomenclature:

The disease now called brucellosis and previous Malta fever first came to the attention of the British medical officers in Malta during the Crimean War in the 1850s. The causal relationship between an organism and the disease was first established by Dr. David Bruce (1887).

In 1897, a Danish veterinarian Bernhard Bang isolated \textit{B. abortus} as the agent, and the additional name (Bang's disease) was assigned. In modern usage, (Bang's disease) was often shortened to just (Bangs) when ranchers discuss the disease or its vaccine. A Maltese doctor and an archaeologist Sir Themistocles Zammit earned a knighthood for identifying unpasteurized milk as the major source of the pathogen in 1905, and it has since become known as Malta fever. The popular name undulant fever originates from the characteristic undulance (or wave-like nature) of the fever which rises and falls over weeks in untreated patients. In the 20th century, this name, along with brucellosis (after Dr Bruce), gradually replaced the 19th century names Mediterranean fever and Malta fever. In 1989 a Saudi neurologists discovered neurobrucellosis, a neurological involvement in brucellosis.

The following synonyms have previously been applied to brucellosis:

Brucelliasis, Bruce's septicaemia, Continued fever, Crimean fever, Cyprus fever, Febris melitensis, Febris undulans, Goat fever, Melitensis septicaemia, Melitococcosis, Milk sickness, Mountain fever, Neapolitan fever and Slow fever (en.academic.ru/dic.nsf/enwiki/240290).

2:3. - \textit{Brucella abortus}:

2:3:1. - Description and significance:

\textit{Brucella abortus} is a gram-negative bacterium that is found in cattle populations. This intracellular parasite is a blood borne pathogen that causes
premature abortion of a cattle fetus. What makes this bacterium so dangerous is that it is zoonotic, meaning it can be transferred from an animal to a human host and still remains pathogenic. In humans this disease causes both acute and chronic symptoms, but can be treated with antibiotics. Because of its economic effect on the cattle business and the disease potential in humans, the United States of America (USA) has spent close to $3.5 billion trying to vaccinate cattle herds in the country. It is possible for *B. abortus* to be spread from wild populations of elk and bison into domestic cattle herds and this is why the USA government continues to be vigilant in tracking potential cases within herds. (AUSVETPLAN, 2005)

2.3.2. - Genome structure:

*B. abortus* genome contains 2 circular DNA chromosomes. The first chromosome is 2,124,241 nucleotides long and codes for 2200 genes. The second chromosome is 1,162,204 nucleotides long and codes for 1156 genes. The genome has a guanine and cytosine (GC) content about 57% and 81% of the genome is a coding region. This pathogen is different from many in that it does not contain any plasmids or genomic islands that relate to pathogenicity within its genome. In addition to lacking these two features, the genome also lacks many other genes that code for common virulence factors including (capsules, fimbriae, exotoxins, cytolyisins, resistance forms, antigenic variation, plasmids, or lysogenic phages). The genes that do encode for virulence in *B. abortus* are being examined but they are not well enough understood to say for sure what the mode of virulence is for this intracellular pathogen.

2.3.3. - Cell structure and metabolism:

*Brucella abortus* are Gram-negative rod-shaped bacteria that do not have flagella or pili, nor do they create capsule slime. They also do not produce
spores. These heterotrophic bacteria carry out either aerobic or anaerobic respiration because they are facultative anaerobes. This means that the bacterium can grow with or without oxygen presence. In order to grow *B. abortus*, a very complex medium is required, because it is a fastidious bacterium that requires most essential nutrients to be imported into the cell from the host. Although it is a fastidious bacterium, *B. abortus* does has (all major biosynthetic pathways) available to it. In its primary host, cattle, the metabolic pathway for the breakdown of erythritol is one that is most desirable, it is even used (preferentially to glucose). This is a possible factor in the bacteria’s virulence because erythritol is found in bovine placenta. (From MicrobeWiki, the student-edited microbiology resource, 2007)

2.4. - Brucellosis in cattle:

The *B. abortus* is the principal cause of brucellosis in cattle. It is shed from an infected animal at or around the time of calving or abortion. Once exposed, the likelihood of an animal becoming infected is variable, depending on age, pregnancy status, and other intrinsic factors of the animal, as well as the amount of bacteria to which the animal was exposed. The most common clinical signs of cattle infected with *B. abortus* are high incidences of abortions, arthritic joints and retained after-birth. There are two mechanism enhance growth of *Brucella*. The first is due to erythritol, which can promote infections in the fetus and placenta. The second is due to the lack of anti-Brucella activity in the amniotic fluid, but abortion is due to placentitis and necrosis of foetal membrane, obstruction of umbilical cord lead to death of foetus. Males can also harbor the bacteria in their reproductive tracts, namely seminal vesicles, ampullae, testicles, and epididymides.
2.4.1. - Nature of the disease:

Bovine brucellosis causes abortions, the birth of weak or dead calves, infertility and as a consequence, reduced milk production. All ages of cattle are susceptible and infection can last for many years. In females, abortion is the major clinical sign, typically occurring between five and seven months of gestation. After an abortion or following the birth of a weak or dead calf, it is common for the placenta to be retained and the uterus to become infected. The animals are most infectious at the time of an abortion or birth of a calf. Infected bulls develop infection characterized the swelling of testicles, lameness as well as infected bursae.

2.4.2. - Aetiology:

Brucellosis in cattle is primarily caused by the *Brucella abortus*, which is one of the ten species of the genus *Brucella* (Hubalek *et al.* 2007; Scholz *et al.* 2008). Seven biovar have been identified, all of which are intracellular, Gram-negative short rods. Brucellae have a wide host range but cattle are the preferred host of *B. abortus*. Other species of Brucella cause significant disease in domestic livestock. *B. ovis* causes significant reproductive disease in sheep. *B. suis* and *B. melitensis* causes a serious disease in pigs and sheep/goats, respectively. Corbel (1997) noted that in some areas in South America, *B. suis* has become established in cattle, and subsequently became more important than pigs as a source of infection. *B. canis* is associated with abortion and testicular infection in dogs and was recorded in many countries. Strains isolated from marine mammals form separate group and are *B. ceti* and *B. pinnipedialis*.

2.4.3. - Susceptible animal species:

Infection with *B. abortus* has been recorded in most species of domestic livestock, as well as in dogs, cats and humans. However, these species have not
been found to be significant in spreading the disease to cattle. Horses can become infected with *B. abortus*, but in this case it has a preference for bursae, tendons, muscles and joints and is commonly found in cases of fistulous withers. *Brucella* species can infect humans and cause significant disease (‘undulant fever). The most important brucellosis disease in humans is ovine/caprine brucellosis caused by *B. melitensis* (Corbel 1997). However, *B. abortus*, *B. suis* and (rarely) *B. canis* are also human pathogens. *B. ovis* has not been demonstrated to cause an overt disease in humans; it has also been confirmed *Brucella* isolated from marine mammals cause human disease.

2.4.4. - Clinical manifestation:

2.4.4.1. - Cattle:

The primary clinical symptom in female cattle is a significant number of late-term (5–7 months) abortions. In a population that has not been exposed to the disease before; these may appear as an (abortion storm) with many cows aborting over a short period. Geering *et al.* (1995) reported that 30–80% abortions in fully susceptible herds. Many cases of endometritis and retained placentae also occur. However, such overt clinical evidence may not be seen in dry areas (where conditions are unfavourable for survival on pasture) or in large, extensively managed herds. In bulls, clinical signs include inflammation of the testes (orchitis) and lameness due to bursitis, which is typically seen in infected bulls and occasionally in cows. Sexually immature cattle do not usually show any signs but may remain sub-clinically infected until maturity and pregnancy. There is little information available on the clinical signs in domestic animals other than cattle, including dogs and cats, and feral animals such as deer. However, eradication programs have been successfully completed without involvement of these species. (AUSVETPLAN, 2005)
2.4.4.2. - Humans:

Brucellosis in humans most commonly occurs during occupational contact with infected animals and their discharges, particularly at calving, but also during slaughtering if the uterus is broken. Infection can also occur by consumption of unpasteurised milk and dairy products from infected animals, by inhalation, through cuts and abrasions or by droplet infection of the eyes. In endemic areas, veterinarians are particularly prone to brucellosis infection and are also at risk of exposure to organisms from live vaccines. Acute brucellosis in humans usually begins with intermittent fever, weakness, chills, sweating, headaches, muscle and joint aches and malaise. Human infections can also cause behavioural changes. Characteristically, the fever spikes each day, giving rise to the term ‘undulant fever’. Undulant fever may be chronic and may also persist for many years.

2.4.5. - Pathology:

2.4.5.1. - Cows:

In cows, the main sites of infection are the endometrium of the uterus and the foetal placenta. The uterus appears normal externally but the endometrium is invariably infected. The intercotyledonary areas of the placenta are generally thickened with yellow gelatinised fluid and may be ulcerated, appear like leather and have mucoid or fibrino-purulent deposits on the surface. Placental cotyledons are hyperaemic and may have areas of yellow–grey necrosis and are covered with sticky brown exudates. When examined microscopically, the membranes and cotyledons contain many mononuclear cells with some neutrophils and the chorionic epithelial cells packed with the bacteria. An abnormally firm attachment of the chorionic villi of the placenta results from
necrosis and enlargement of the maternal villi and the presence of inflammatory exudates.

2.4.5.2. - Foetus:

The foetus is usually swollen, with blood-tinged fluid found subcutaneously and in the body cavities; the umbilical cord may be thickened and swollen. The most important lesion is a catarrhal or fibrinous pneumonia. Microscopic examination of the lungs shows scattered foci of bronchitis and bronchopneumonia.

2.4.5.3. - Bulls:

*B. abortus* causes infection and swelling of the testicles that may not be obvious, but increasing pressure results in necrotic foci that grow and coalesce and may lead to total testicular necrosis with sequestration by inflammatory thickening of the tunica. *B. abortus* may also infect the accessory sex glands. Brucellae in cattle including bulls may localise in the carpal and other bursae, where hygromas containing large numbers of bacteria may be found (AUSVETPLAN, 2005).

2.4.6. - Pathogenesis:

When brucellosis is introduced into a susceptible herd, it spreads easily because of the environmental contamination that occurs following an abortion. In cattle, infection with *B. abortus* is usually due to ingestion of infected material. The bacteria penetrate the mucosal epithelium of the gastrointestinal tract and are transported, either free or within phagocytic cells, to regional lymph nodes. If these bacteria do not remain localised or are not killed, they can spread to other organs: joints and bursae. This bacteraemic phase is subclinical and may take several weeks to some months. The bacteria then localise in the pregnant uterus and udder of cows, and the testicles and accessory sex glands of bulls. In pregnant cows, the chorioallantoic membrane becomes inflamed and ulcerated,
and bacteria can spread via the blood to the foetus and placenta. The preference of the bacteria for these sites is thought to be due to the presence of the sugar alcohol erythritol, which is a foetal product concentrated in the chorion, cotyledons and foetal fluids. In mature, nonpregnant cows, the bacterium localises in the udder. Infection of the udder is often clinically inapparent, with no gross lesions. *Brucella* localise and replicate primarily in macrophages, in mammary secretions or in phagocytes; they form an important source of organisms for periodic reinfection (and potentially for infection of calves and humans via the milk). Hence, if the cow later becomes pregnant, the uterus can become infected during a subsequent bacteraemic phase. (AUSVETPLAN, 2005)

2.4.7. - Specimens required for diagnosis:

Specimens of milk from each quarter of the udder, and whole aborted foetuses or spleen, lung and stomach contents and foetal membrane cotyledons, should be hygienically collected from each animal that aborts (Geering *et. al.*, 1995). Vaginal swabs collected in the six-week period following calving or abortion may also be useful. Blood samples for serum should be collected from all animals that have recently calved or aborted. Care must be taken to protect samples from extremes of heat during collection, storage and transport to the laboratory.

2.4.8. - Diagnostic tests:

2.4.8.1. - Laboratory diagnosis:

Diagnostic tests for brucellosis can be classified into those that identify the organism, those that demonstrate specific immunoglobulins and those that demonstrate a specific allergic response. In the Sudan, the diagnosis of
brucellosis can generally be made by the Central Veterinary research Laboratories (CVRL), Soba. Khartoum South, Sudan.

2.4.8.2. - Microscopic examination:

Microscopic examination of smears stained with the Stamp's modification of the Ziehl-Neelsen method can be used for a presumptive diagnosis. Organisms may be found in abortion products, vaginal discharges, milk, semen or various tissues. *Brucella* species are not truly acid-fast, but they are resistant to decolourization by weak acids, and stain red against a blue background. *Brucella* is a coccobacillus or a short rods, usually arranged singly but sometimes in pairs or small groups. This test is not definitive. Other organisms such as *Chlamydophila abortus* and *Coxiella burnetii* can resemble *Brucella*. Direct examination may not detect the small numbers of organisms present in milk and dairy products (OIE, 2009).

2.4.8.3. - Culture:

*Brucella* species can be recovered from numerous tissues and secretions, particularly fetal membranes, vaginal secretions, milk (or udder secretions in nonlactating cows), semen, arthritis or hygroma fluids, and the stomach contents, spleen and lung from aborted fetuses. Blood cultures are often used to detect *B. canis* in dogs.

2.4.8.3.1. - Basal media:

Direct isolation and culture of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for enrichment purpose. A wide range of commercial
dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)–soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* biovar 2, blood agar base (Oxoid) or Columbia agar (BioMérieux), with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol dextrose agar, can be used. Serum-dextrose agar is usually preferred for observation of colonial morphology. A non selective, biphasic medium, known as Castaneda’s medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk (OIE, 2009).

### 2.4.8.3.2. - Selective media:

All the basal media mentioned above can be used for preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*. The most widely used selective medium is the Farrell’s medium, which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymyxin B sulphate, bacitracin, natamycin, nalidixic acid, nystatin and vancomycin. A freeze-dried antibiotic supplement is available commercially (Oxoid). Nalidixic acid and bacitracin, at the concentration used in Farrell’s medium, have inhibitory effects on some *B. abortus* and *B. melitensis* strains. Therefore the sensitivity of culture increases significantly by the simultaneous use of both Farrell’s and the modified Thayer–Martin medium. As the number of *Brucella* organisms is likely to be lower in milk, colostrum and some tissue samples than in abortion material, enrichment is advisable in the case of milk. Results are also improved by centrifugation and culture from the cream and the pellet (OIE, 2009).
2.4.8.4. - Serology:

In the Sudan, detection of immunoglobulins is based on the Rose Bengal plate test (RBPT), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) on serum, and the milk ring test (MRT) on milk. Two ELISAs are mentioned in the OIE (2008): an indirect ELISA specific for IgG1; and a competitive (inhibition) ELISA using monoclonal antibodies. The value of ELISA testing is that it is relatively unaffected by the condition and age of the blood samples and should minimise the need to resample cattle whose serum samples are unsuitable for testing by the CFT. The USA Department of Agriculture, Animal and Plant Health Inspection Service have also reported a new serologic test for detection of *Brucella* antibodies, termed the rapid automatic presumptive (RAP) test. It uses a computer reader and recorder device to assess and report test results. This minimises subjectivity and has enhanced laboratory-to-laboratory uniformity. Cross-reactions to other organisms may cause some diagnostic problems. Several authors have reported serological reactions to the presence of *Yersinia enterocolitica*. In New Zealand, 35% of deer in a large export consignment reacted to the *B. abortus* serum agglutination test (SAT). This reaction was later considered to have been caused by previous exposure to *Yersinia enterocolitica* (Hilbink *et. al.*, 1995). The diagnostic tests currently used in the Sudan are shown in table 2.
Table 2: Diagnostic tests currently used by the (CVRL) in Sudan for bovine brucellosis:

<table>
<thead>
<tr>
<th>Test</th>
<th>Specimen Required</th>
<th>Test detects</th>
<th>Time taken to obtain a result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture and identification of B. abortus</td>
<td>Tissue</td>
<td><em>Brucellae</em></td>
<td>6 days</td>
</tr>
<tr>
<td>RBPT</td>
<td>Serum</td>
<td>Antibody</td>
<td>4 minutes</td>
</tr>
<tr>
<td>CFT</td>
<td>Serum</td>
<td>Antibody</td>
<td>4 hours</td>
</tr>
<tr>
<td>cELISA and iELISA</td>
<td>Serum</td>
<td>Antibody</td>
<td>2 hours</td>
</tr>
<tr>
<td>SAT</td>
<td>Serum</td>
<td>Antibody</td>
<td>24 hours</td>
</tr>
<tr>
<td>MRT</td>
<td>Milk</td>
<td>Antibody</td>
<td>1 hour</td>
</tr>
</tbody>
</table>
2.4.8.5. - Differential diagnosis:

There are many potential causes of abortion in cattle. Endemic infectious causes of abortion include viral diseases such as infectious bovine rhinotracheitis and mucosal disease; and infections with other organisms such as *Trichomonas foetus*, *Neospora caninum*, *Campylobacter foetus*, *Listeria monocytogenes*, *Sarcosporidia*, various *Leptospira* species and fungi. Exotic viral diseases causing abortion include Rift Valley fever and Wesselsbron disease (in sheep). There are also a range of potential non infectious causes resulting from nutritional and toxic factors. Generally bovine brucellosis can be differentiated from these conditions due to its pathology, its presentation and the excellent range of laboratory diagnostic methods (AUSVETPLAN, 2005).

2.5. - Resistance and immunity:

2.5.1. - Innate and passive immunity:

Establishment of infection by *Brucella spp* depends on the number and virulence of organisms and the relative resistance of the host animal, as determined by innate and acquired immune mechanisms. Sexually mature cows, pregnant heifers and bulls are the most susceptible to infection with *B. abortus*. A small proportion of crossbred cattle appear to be innately resistant due to the ability of macrophages to limit the replication of *B. abortus*. This innate resistance is inherited as a dominant trait. Sexually immature cattle are quite resistant to exposure to *B. abortus*, with susceptibility increasing with sexual development and pregnancy. Calves may acquire infections in utero or by ingestion of contaminated milk (Nicoletti, 1980). There is also a tendency for males to become infected at a younger age than females; they may acquire infection during calfhood and retain it into adult life (Rankin, 1965).
2.5.2. - Active immunity:

The rate of production of antibody depends on the type of stimulus received. The immunoglobulins produced following natural infection are different from those produced following vaccination; this difference is used to discriminate between them. The cell-mediated response generally appears at least one week before the appearance of agglutinating antibodies. Because the bacterium is an intracellular, facultative organism, attenuated ('live') vaccines have been far more successful than inactivated vaccines (AUSVETPLAN, 2005).

2.5.3. - Vaccination:

Effective vaccines have played an important role in reducing the incidence of brucellosis in many countries.

2.5.3.1. - Strain 19:

The most widely used vaccine for the prevention of brucellosis in cattle is prepared from B. abortus strain 19. It is an attenuated ('live') vaccine and is normally given to female calves aged between three and six months as a single subcutaneous dose of viable organisms. A disadvantage of strain 19 is that it causes vaccinated animals to produce antibodies that on standard diagnostic tests are indistinguishable from the antibodies produced by animals infected with Brucella. A reduced dose of organisms can be administered to beef or dairy cattle aged 4–12 months, but 5–10% of the animals will develop persistent antibody titres (Beckett and MacDiarmid, 1985).

The USA Center for Disease Control and Prevention (CDC) recommends concomitant regimens of doxycycline and rifampin for human post-exposure prophylaxis against the strain 19 vaccine.
2.5.3.2. -Strain RB51

The RB51 vaccine strain is an attenuated, genetically stable, rough morphology mutant of *B. abortus* that was approved for use in the USA in 1996. Vaccination with RB51 does not result in measurable antibody titres to *B. abortus*. The RB51 is protective at doses comparable to those used for strain 19 when given to calves at 3–10 months of age. RB51 can infect the placenta and uterus in the pregnant animal. Unpublished reports by the vaccine manufacturers in the USA indicated that vaccination with a reduced dose (1 × 10⁹) of strain RB51 can lead to abortion in 0.5% of vaccinated animals. An experimental dot blot assay used for serological measurement of RB51 post-vaccination titres has been evaluated under experimental and field conditions in cattle, but this assay has not been validated by using human serum. Veterinarians and other animal health-care personnel should be made aware of the possible risk of infection associated with the veterinary use of RB51 (Centres for Disease Control and Prevention).

2.5.3.3. -*B. suis* biovar 1 strain 2:

Since 1971, a smooth strain of *B. suis* biovar 1 strain 2 has been used as an oral vaccine to control brucellosis in cattle, sheep, goats and pigs in China. This vaccine protects cattle against *B. abortus*, is safe if administered orally, and does not induce persistent antibody titres.

2.6. - Epidemiology:

The most significant feature of bovine brucellosis epidemiology is the shedding of large numbers of organisms during the 10 days after abortion or calving of infected cows and the consequent contamination of the environment. The movement of infected cattle into a herd can result in transfer of the disease when cattle ingest the bacteria from aborted foetuses, placenta, and discharge
from cows that have aborted or contaminated pasture or water (AUSVETPLAN, 2005).

2.6.1. - Incubation period:

The length of the incubation period in an individual animal is influenced by sexual maturity, state of pregnancy at the time of infection (inversely proportional), size of the challenge dose and previous exposure to infection or vaccination. For example, the average incubation period is 67 days for cows infected at six months of pregnancy. The minimum incubation period is about one month. There is experimental evidence that localised foci of viable organisms remain in an unknown proportion of calves born of infected dams that have been serologically negative for considerable periods. There is a danger that such a focus may breakdown at a later stage in life and causes active disease (Lapraik et. al., 1975). In humans, the incubation period for the disease is 5–30 days or longer.

2.6.2. - Persistence of B.abortus:

Under ideal conditions, B. abortus can persist in organic materials such as faeces, abortion fluids and milk for up to six months. It may survive up to eight months in an aborted foetus in the shade (Geering et al., 1995).

2.6.3. - Modes of transmission:

2.6.3.1. - Live animals:

B. abortus is usually transmitted by ingestion of contaminated feed or water or by licking an infected placenta, calf or foetus, or the genitalia of an infected cow soon after it has aborted or calved. Inhalation and direct contact, especially with abraded skin or mucous membranes, may be a factor (Nicoletti, 1980). Heifer calves infected in this manner may not be detected by serological testing and
will be a source of infection after puberty. Transfer into a free population is primarily by importation of cows and heifers that are latently infected.

2.6.3.2. - Infected cows:

The large numbers of *B. abortus* shed by an infected cow at the time of calving or abortion are the main source of infection. Infected females may also intermittently shed organisms in colostrum and milk. Faeces, urine and hygroma fluid may be involved but these are of minor importance. Genital discharges may continue to contain high numbers of organisms for several weeks following normal parturition or abortion. Congenital transfer from an infected cow to a foetus occurs infrequently.

2.6.3.3. - Infected bulls:

Bulls usually only become infected when there are abortions due to *B. abortus* in the herd. Once infected, the organisms tend to localise in the testes; large numbers may be excreted in semen during the acute phase, making semen a potentially important source of infection. Bulls may also excrete *B. abortus* in faeces, urine and hygroma fluid (AUSVETPLAN, 2005).

2.6.3.4. - Artificial breeding:

Natural service by the bull is unlikely to transfer infection. However, there is a real risk of transferring infection through artificial insemination, given the method used and the delivery point of semen in the reproductive tract of the dam (Manthei *et. al.*, 1950). The risk of introducing the disease through embryos is negligible provided the embryos are properly handled between collections and transfer (Anon, 1998).
2.6.3.5. - Animal products and by-products:

*B. abortus* is sensitive to pasteurisation temperatures. Yoghurt is presumed to be safe because of its low pH.

2.6.3.6. - Equipment and personnel:

Mechanical transfer from milking machines contaminated by infected milk is a possible, though unlikely, source of spread. Generally, removal of infected animals from contaminated premises for one month is sufficient to prevent infection, provided the facilities have been sufficiently disinfected. Due to the fragility of the bacteria in the environment, fomites are not considered a likely source of infection.

2.6.3.7. - Vectors:

Reservoirs of infection have been reported in a wide range of domestic animals, birds and carnivores such as dogs. They may move infective material between properties; however, their role is limited. The transmission of brucellosis by ticks, fleas or mosquitoes from an infected herd to a non-infected herd has never been proved.

2.6.4. - Factors influencing transmission:

Given that environmental survival of the organism depends on favourable temperatures and thus low temperature is favourable to survival. The concentrated husbandry of dairy herds and seasonal calving provide ideal conditions for transmission within a herd should an infected cow abort following introduction. Many factors affect the epidemiology of bovine brucellosis; the most important are herd size and mobility, contiguity to infected herds, concentration of cattle and nature of production (dairy herds are more susceptible than beef cattle) (AUSVETPLAN, 2005).
2.7. - Manner and risk of introduction to Sudan:

The greatest risk of introduction of bovine brucellosis would be with animal’s movements across the open borders with neighbouring countries with endemic infection; however, this method of introduction is unlikely. The disease could also be introduced with imported semen but this risk is minimised by effective import controls. Because the bacterium is intracellular, it may exist without being detected by serological methods. Herds should be considered free only after a series of tests have confirmed the absence of the bacterium.

2.8. - Treatment of infected animals

Treatment with antibiotics is not normally used in bovine brucellosis eradication programs.

2.9. - Brucellosis in the Sudan:

Brucellosis was first reported from human cases as early as 1908 (Haseeb, 1950). *B. abortus* was first isolated from a dairy farm in Khartoum (Bennett, 1943) while *B. melitensis* was isolated from a goat milk among British residents in the Gazira area in 1953 (Daffalla and Khan, 1958).

In 1956 brucellosis was diagnosed at Juba, Equatoria province dairy farm after storm of abortions. Serological tests revealed about 55% positive reactors in the herd (Daffalla and Khan, 1958). In 1957, brucellosis was serologically diagnosed in western Sudan both in Elobeid and Nuba mountains and there were 155 serological positive cases (Daffalla and Khan, 1958). During the year 1958-1959, samples of sera and milk of cattle collected from Nisheshiba and Umbinein revealed 10.7% seropositive out of 1345 sera and 8.7% positive out of 104 milk samples. Also at the same period examination of experimental goats in the Veterinary Research Laboratory, yielded 3% seropositive out of 313 sera. Examination of 497 goats milk samples with the milk ring test yielded 1%
positives milk samples (Dafalla, 1962). While in the southern states, a prevalence of 14.6 to 18% was reported (Elnasri, 1960), in the Gezira state 8.7 and 10.7% (Dafalla, 1962) and at 6.5% and 22.5% in Bahr-el-ghazal state (Boumann, 1983). Thereafter, many investigators reported the disease from different parts of Sudan (Daffalla, 1962; Shigidi and Razig, 1971; Musa and Mitchell, 1985).

Shigidi and Razig (1973) isolated *Brucella abortus* from a knee hygroma of a bull. Bakhiet (1981) studied the incidence of brucellosis in cross bred and native cattle in private farms in Gezira using SAT and found the percentage of reactors between 1.2% and 22% among the native and cross-bred cattle, respectively. Shallali et. al., (1982) examined 124 milk samples from a dairy farm in the Blue Nile province and found 11 samples positive by the MRT. The highest positive numbers of samples 16.7% were reported in the central region of the Sudan, followed by Kordofan region 14.9%, Kassala region 11.0% and Khartoum 8.9% (Khalafalla, 1989).

In 1982 the disease was diagnosed in five out of twenty imported goats kept for breeding in Khartoum province (Osman and Adlan, 1986). Elwali et. al., (1983) tested sera from the southern Darfur province, using RBPT as screening test and reported 18% positive cases. In a review of the situation of brucellosis in the Sudan (Musa, 1990) cited prevalence of the disease in man, cattle, camel, sheep, goats and equines and concluded that the highest prevalence rate was encountered in the intensive farming systems and under nomadic conditions. In Darfur state, western Sudan, the prevalence of the disease in cattle was found to range between 14 and 26% in south Darfur state which is known to be the richest in cattle population nationwide (Musa, 1990).

The prevalence of bovine brucellosis in Darfur state was 13.9% (Musa, 1995), the organism was also isolated from camels in Butana area (Agab et. al.,
In northern Sudan 6.2% (Omer, 2000), in Kassala state 5% (El-Ansary et. al., 2001), in Red Sea state it was 11.9% (Mohammed, 2004), the prevalence of bovine brucellosis in Kuku Dairy Scheme, Khartoum North 24.9% (Angara, et. al., 2004), in selected dairy farms in Kosti area, central Sudan it was 12.39% (Fadul, 2006.) In Khartoum state the prevalence was 23.21% (Khalid, 2006), in Al Renk area it was 30% (Abdel-gader, 2007), In Elhuda area Gezira State it was 4.5% (Mohammed, 2009).
CHAPTER III

3. - Materials & Methods

3.1. - Study area:

The present work was performed in February 2010 in Port Sudan city, the Red Sea State, the Sudan. The Red Sea State occupies an area of 218,887 km² and is located in north-eastern Sudan, bordering Egypt to the North, Kassala State to the South, and River Nile State to the West and the Red Sea to the East. The State is sub-divided into eight mahallias (localities or districts): Port Sudan, Suakin, Gunub/Aulib, Sinkat, Hayya, Halaib and Tokar/Agig. The overall human population of the state was estimated at 846,113 (Babiker and Pantuliano, personal communication). The animal population in the state consists of camels, cattle, sheep and goats. The total number of the cattle in the state is 112,700 of which 23,151 in Port Sudan city (Administration of animal resources, 2009). These cattle are of a local zebu Halfa, Kenana and Butana types and a few Frisian crosses. The main diseases afflicting livestock are: Theileriasis, pneumonia, internal and external parasites, coccidiosis, fungal infections and nutritional disorders.

3.2. - Serum samples:

Two hundred and fifteen blood samples for serum were collected randomly from the jugular vein from three sites: northern, western and southern part of Port Sudan city. The number of serum samples was proportional to the density of cattle in each location.

The sample size was determined according to the following formula:

$$n = \frac{(1.96)^2 \times \text{pexp}(1-\text{pexp})}{d^2}$$
n = sample size

pexp = expected prevalence

d = desired absolute precision

11.9 \times 12\% = (1.96)^2 \times 0.12 \times 0.88 \times 0.5 \times 0.5 = 162.2

Sample size = 162 head of cattle.

In northern part there are 20,000 head distributed in 7 squires, in each one between 50-167 farms. 201 samples were taken from 5 large farms in different squires which contain between 80-650 head approximately.

In western part there are 1239 head distributed in 35 farms. 5 samples were taken from one farm. In southern part there are 1,900 head distributed in 57 farms. 9 samples were taken from one farm.

3.2.1. -Collection of blood:

The blood samples were taken aseptically from the jugular vein using vaccutainer tubes.

3.2.2. -Separation of sera:

Following the collection of blood samples, the vaccutainer tubes were put in rack then in a refrigerator at 4°C over night. After clotting the samples were transported to the Veterinary Research Laboratory where sera were separated by centrifugation at 3000rpm×15sec. The sera were gently poured into sterile plastic containers then tested in the same day of separation for Brucella antibodies. All serum samples were placed in sterile leak proof containers and
transported in ice (4°C). On arrival the samples were immediately frozen at -20°C until tested.

3.3. - Serological tests:

Three serological tests were used for detection of Brucella antibodies in serum; they were rose Bengal plate test (RBPT), standard agglutination test (SAT) and enzyme linked immune-sorbent assay (competitive ELISA). All equipment and antigens for these tests were available at the Brucella Department, Central Veterinary Research Laboratories (CVRL), Soba, Khartoum, Sudan.

3.3.1.- Modified Rose Bengal Plate (mRBPT) (performed in the Regional veterinary research Laboratory, Port Sudan):

This was similar to the classic Rose Bengal test but differed in the volume of antigen used which was half or third of the serum volume (antigen to serum was 1:2). This procedure was deemed suitable for detection of weakly positive samples.

3.3.2. - Rose Bengal Plate Test: - RPBT

This test is a simple spot agglutination test using antigen stained with rose Bengal and buffered to low pH, usually 3.65±0.05. The antigen was obtained from the Central Veterinary Research Laboratory (CVRL), Soba. The test was performed according to the OIE manual (2004).

Test procedure:

The serum samples and the antigen were removed from the freezer (-20°C) and brought to room temperature (22±4°C) to thaw; only sufficient antigen for the day’s tests was taken from the refrigerator and left to warm up. An amount of 25µl of each serum sample was placed on a porcelain plate. The
antigen bottle was shaken well, but gently, and an equal volume of the antigen was placed near each serum spot. Immediately after the last drop of antigen had been added to the plate, both the serum and antigen were mixed thoroughly using disposable clean wood rod for each spot to make a circular or oval zone. The mixture was rocked gently for 4 minutes. Any visible clotting was considered positive. The positive test was compared with a control negative test to confirm it.

3.3.3. - **Standard tube agglutination test SAT**: -

- The antigen: *B. abortus* strain 19 was used in preparing the antigen.

**Equipment used:**

- Glass tube (8mm×50mm) with rim and metal agglutination boxes for carrying the tubes.

- Automatic pipette and tips, Phenol saline, Flasks and Serum samples.

**Test procedure:**

In view of the occasional occurrence of the prozone phenomenon, seven tubes were normally used for each serum under test. Using an automatic pipette amounts of 0.8 ml of phenol saline was placed in the first tube and 0.5 ml in each succeeding tone; 0.2 ml of the serum under test was transferred to the first tube and mixed thoroughly with the phenol-saline already there; 0.5 ml of the mixture was carried over to the second tube from which, after mixing, 0.5 ml was transferred to the third tube, and so on. This process was continued until the last tube, from which, after mixing, 0.5 ml of the serum dilution was discarded. This process of doubling dilutions results in the following dilutions 1:5, 1:10, 1:20, and so on, in each tube. To each tube was then added 0.5 ml of antigen at the recommended dilution and the contents of the tube are thoroughly mixed,
thus giving final serum dilutions of 1:10 to 1:640. The tubes were then incubated at 37°C for 20 hours ± 1 hour before the results were read.

**Interpretation of results:**

The degree of agglutination was assessed by the amount of clearing that had taken place in the tubes compared with the standard tubes. The tubes were examined, without being shaken, against a black background. With a source of light coming from above and behind the tubes, complete agglutination and sedimentation with water-clear supernatant was recorded as ++++, nearly complete agglutination and 75% clearing as ++, marked agglutination and 50% clearing as +, some sedimentation and 25% clearing as +, and no clearing as negative. The Standard tubes were prepared at the time of the test and incubated with them. The antigen was diluted by mixing 2 ml of antigen with 2 ml of phenol-saline, the 5 standard tubes were prepared as following: In the first tube: 1 ml phenol saline as ++++, in the second tube 0.75 ml phenol saline with 0.25 ml diluted antigen (1:2) as ++, in the third tube 0.5 ml phenol saline with 0.5 ml diluted antigen as ++, in the fourth tube 0.25 phenol saline with 0.75 ml diluted antigen as + and in the last tube 1 ml of diluted antigen as ?.

**3.3.4. Competitive ELISA: (Veterinary Laboratories Agency (VLA), 2005)**

Competitive ELISA works through competitive binding.

**Test procedure:**

1. Prepare the conjugate solution

2. Add 20 μL of each test serum per well. Leave column 11 and 12 for controls.

3. Add 20 μL of the negative control to wells A11, A12, B11, B12, C11 and C12.
4. Add 20 µL of the positive control to wells F11, F12, G11, G12, H11 and H12.

5. The remaining wells have no serum added and act as the conjugate controls.

6. Immediately dispense into all wells 100 µL of the prepared conjugate solution. This gives a final serum dilution of 1/6.

7. The plate is then vigorously shaken (on the microtitre plate shaker) for 2 minutes in order to mix the serum and conjugate solution. Cover the plate with the lid and incubate at room temperature (21°C±6°C) for 30 minutes on a rotary shaker at 160 revs/min.

8. Shake out the contents of the plate and rinse 5 times with washing solution and then thoroughly dry by tapping on absorbent paper towel.

9. Switch on microplate reader and allow the unit to stabilize for 10 minutes.

10. Immediately before use prepare the substrate and chromogen solution by dissolving one tablet of urea H₂O₂ in 12 ml of distilled water. When dissolved add the OPD tablet and mix thoroughly. This can take a few minutes; the use of a magnetic stirrer will greatly increase the speed with which it dissolves. Add 100µL to all wells.

11. Leave the plate at room temperature for a minimum of 10 minutes and a maximum of 15 minutes.

12. Slow the reaction by adding 100µL of stopping solution to all wells.
13. Remove condensation from the bottom of the plate with absorbent paper towel. Read plate at 450nm.

For competitive ELISA, the higher the sample antigen concentration, the weaker the eventual signal. The major advantage of a competitive ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present. Note that some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with your sample antigen (unlabeled). The more antigen in the sample, the less labeled antigen is retained in the well and the weaker the signal. Commonly the antigen is not first positioned in the well.
CHAPTER IV

4. -RESULTS

4.1. - Results of serological tests:

4.1.1. - Modified Rose Bengal plate test:

Of the 215 serum samples tested by MRBT were positive for brucella antibodies 21% (Fig.1). There was clear clot in the plates which was visible by the naked eye. The proportions of positive samples using MRBT in each part of city are shown in table ???. Of the 201 samples collected from the northern part of Port Sudan 44(22%) were positive, while in the southern part 2 out of 9 samples 22% were positive. The 5 samples from the western part of the city were all negative.

4.1.2. - Rose Bengal Plate test:

When the 215 serum samples were tested by the RBPT 27(13%) were seropositive. Of the 201 sera collected from the northern part 26(13%) were seropositive. All 9 serum samples collected from the southern part were tested with the RBPT 1(11%) were seropositive. RBPT also showed that the 5 serum samples collected from western part of Port Sudan were negative.

4.1.3. - Serum agglutination test:

Of the 27 Brucella seropositive samples by RBPT, 93% were seropositive with the SAT, the highest titre was 1488 IU/ml and the lowest was 20 IU/ml. Two sera were negative.

4.2.4. -Competitive ELISA test (cELISA):

Of the 215 serum samples collected from Port Sudan city, 120 samples were chosen randomly for examination with cELISA; the later test showed 21
seropositive cattle from the 46 positive with the mRBPT, 27 positive with the RBPT and 25 positive with SAT. Yet, one negative sample with the mRBPT proved positive show figure.2.

4.3. -Comparison between the four diagnostic techniques:

Chi square test (Chi$^2$) showed significant differences between the four techniques with respect to brucellosis seropositive cattle (chi$^2$= 71.7, d.f=3, p<0.00000). Subsequent paired comparisons showed significant differences between cElisa and the SAT test (chi$^2$, = 39.0, df = 1, p < 0000001 ), RBPT/mRBPT ( chi$^2$ = 4.3, def = 1, p < 0.04), RBPT/SAT (chi$^2$ = 66.3, df = 1, p < 00000001) and mRBPT / SAT (chi$^2$ = 42.0 , df = 1 p < 0.0000001). There were no similar significant differences between the results obtained by RBPT and cELISA (chi$^2$ = 1.6, df =1 p > 0.2) and mRBPT/cELISA (chi$^2$ = 0.1, df = 1 p > 0.7) show table4.
Table 3. Detection of *Brucella* seropositive cattle using two diagnostic tests: modified and Rose Bengal Plate test (RBPT).

<table>
<thead>
<tr>
<th>Area</th>
<th>No of Samples collected</th>
<th>Modified RBT</th>
<th>Classic RBT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baraka (North)</td>
<td>201</td>
<td>44(22%)</td>
<td>26(13%)</td>
</tr>
<tr>
<td>Assalam (South)</td>
<td>9</td>
<td>2(22%)</td>
<td>1(11%)</td>
</tr>
<tr>
<td>Ongab (West)</td>
<td>5</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>215</strong></td>
<td><strong>46(21%)</strong></td>
<td><strong>27(13%)</strong></td>
</tr>
</tbody>
</table>
Table 4. Comparison between MRBT and RBPT and SAT and cElisa using chi square test:

<table>
<thead>
<tr>
<th>Test</th>
<th>Negative</th>
<th>Positive</th>
<th>Chi²</th>
<th>Degrees of freedom</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT \ cELISA</td>
<td>5*</td>
<td>22</td>
<td>39.0</td>
<td>1</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cELISA \ modified RBT</td>
<td>98</td>
<td>22</td>
<td>0.1</td>
<td>1</td>
<td>p&gt;0.7</td>
</tr>
<tr>
<td></td>
<td>171</td>
<td>46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic RBT \ SAT</td>
<td>188</td>
<td>27</td>
<td>66.3</td>
<td>1</td>
<td>P&lt;0.00000000001</td>
</tr>
<tr>
<td></td>
<td>5*</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modified RBT \ classic RBT</td>
<td>171</td>
<td>46</td>
<td>4.3</td>
<td>1</td>
<td>P&lt;0.04</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic RBT \ cELISA</td>
<td>188</td>
<td>27</td>
<td>1.6</td>
<td>1</td>
<td>p&gt;0.2</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>22</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Modified RBT \ SAT</td>
<td>171</td>
<td>46</td>
<td>42.0</td>
<td>1</td>
<td>P&lt;.000000001</td>
</tr>
<tr>
<td></td>
<td>5*</td>
<td>22</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* = original is three
Fig.1- A Competitive ELISA plate test showing brucella seropositive wells with decrease in colour:
CHAPTER V

5. - DISCUSSION

In the present study, four types of serological tests used for the diagnosis of Brucellosis were compared, and the results showed that the mRBPT was the most sensitive of the four followed by the RBPT but were less sensitive than cELISA. mRBPT detected 46 seropositive bovine brucellosis out of 215 test sera. Thus prevalence of bovine brucellosis in Port Sudan city was 21% according to mRBPT and 13% according to Rose Bengal Plate Test (RBPT). The cELISA was used as confirmatory tests because of its high sensitivity and specificity in detection of Brucella antibodies. The cELISA confirmed 21 seropositive samples from: 46 seropositive with the mRBPT, 27 with the RBPT and 25 with the SAT. Yet one negative sample by modified rose Bengal test proved positive by cELISA. According to the OIE (2008) only samples positive with mRBPT were confirmed by the cELISA because of the more false positive samples in the test. The mRBPT was found over sensitive and this could be due to the fact that it might not have been standardized after production.

Similar findings were reported by Nielsen (2002). There is also general agreement that ELISA is more sensitive than the conventional tests used in the diagnosis of brucellosis (Gad EL-Rab and kambal, 1998). Serological diagnosis of brucellosis began more than 100 years ago with simple agglutination tests. Since then it was realized that the serological tests were susceptible to false negative and false positive reactions resulting from, for instance, exposure to cross reacting microorganisms (Nielsen, 2002). Thus cELISA has been shown to be a highly sensitive technique and suitable for large-scale screening of bovine brucellosis, but availability of the diagnostic tests might be a constraint to some laboratories.
The seroprevalence of brucellosis obtained in the present work is lower than that observed in Eretria and higher than that of Egypt. The prevalence of *Brucella* seropositive cattle in Eretria using RBPT in dairy herds kept under intensive husbandry systems around Asmara, the capital, were 8% and 35% (Omer *et. al.*, 2000). The seroprevalence of brucellosis using CRBT in Egypt was 4.98% for cattle, 3.52% for buffaloes, 4.8% for sheep, and 2.19% for goats. (Samaha *et. al.*, 2008). In the sub-Saharan Africa, the prevalence of brucellosis among animals, mainly cattle, sheep, goats and pigs, is poorly estimated or unknown. Since the economic status of most of these countries is poor, disease control has been very difficult, chronic brucellosis cases and infertility were common among the cattle and sheep and goats herds. Carcasses and abattoir products are the continuous sources of infectious among animals and humans. Outbreaks of bovine brucellosis in animals have occurred in most sub-Saharan African countries; however, no data are available from Benin, Burundi, Cape Verde, Congo, Equatorial Guinea, Rwanda, or Sierra Leone. In South Africa, more than 300 outbreaks took place each year from 1996 to 2000, with over 5,000 cases reported per year in humans. Most countries of West, East and Central Africa also had outbreaks, but the numbers of cases among animals and humans were less well defined (Memish and Balkhy Hanan, 2004).

In Darfur state, western Sudan, the prevalence of the disease in cattle was found to range between 14 and 26% in south Darfur state which is known to be density populated cattle with nationwide (Musa, 1990). The prevalence of bovine brucellosis in greater Darfur State was 13.9% (Musa, 1995). In Al Renk area, Upper Nile State, southern Sudan, the prevalence was 30% (Abdel-gader, 2007). In Khartoum state the prevalence was 23.21% (Khalid, 2006) and in Red Sea State it was 11.9% (Mohammed, 2004). In selected dairy farms in Kosti area, central Sudan it was 12.39% (Fadul, 2006.). Nationwide the highest seropositive samples for brucellosis in cattle (16.7%) were reported in the
central region of the Sudan, followed by Kordofan region (14.9%). The prevalence of bovine brucellosis in Kassala State was 5% (EL-Ansary et al., 2001), in the Northern State, northern Sudan it was 6.2% (Omer, 2000) while in the southern states a prevalence of 14.6 to 18% was reported by Elnasri (1960). Seroprevalence 6.5% and 22.5% were reported in Bahr-el-ghazal state by Boumann (1983). In Elhuda area Gezira state, 4.5% seroprevalence was reported by Mohammed (2009).

The prevalence of brucellosis in cattle in the Red Sea State was 11.9% (Mohammed, 2004). Human and animal populations in the state are exposed to brucellosis by direct contact or by consumption of animal products or both without much awareness about the disease. Records from the Veterinary Research Laboratory in Port Sudan showed that between May 1998 - March 2007 there was 54.6% Brucella positive cattle, 9.1% camels, 25.0% sheep, 31.3%, goat and 66.7% equines. In the two last years 29.0% of cattle were Brucella seropositive, 33.0% camels, 12.0% sheep, 2.5% goats and 0% equines were positive for brucellosis. The high prevalence rate of brucellosis in Port Sudan city in our study may be due to the poor management, crowding of animals in small and closed farms as well as poor hygienic measures. To date there is no annual vaccination against brucellosis in Red Sea State. However there is a traditional method of avoiding brucellosis by buying wet cows from Butana and Kassala with their calves. When these cows were dry, they were immediately disposed of whether they contracted brucellosis or not.
Conclusion & Recommendations

Conclusion:

The seroprevalence rate of bovine brucellosis in 2004 in the whole Red Sea State was 11.9% (Mohammed, 2004). In the present study the seroprevalence of brucellosis in dairy farms in Port Sudan city was 13% according to Rose Bengal Plate Test (RBPT). This result suggests an increase in Brucella infection which highlights the importance of control efforts against brucellosis in cattle.

Recommendations:

Vaccination:

Vaccination of calves with B. abortus Strain 19 or RB51 to increases the resistance to infection.

Depopulation of infected animals

Depopulation has the advantage of being quick and of allowing the country to be declared free without undue delay. Depopulation will therefore be used immediately if the disease is restricted to a few herds. The slaughtered animals will be disposed of by the most appropriate means for each situation.

Treatment of animal products and by-products

Confirmed infected cows that are close to calving or that have a vaginal discharge must not be sent to slaughter, because there is a risk of human infection. Such animals must be handled with care and destroyed on the property. B. abortus is readily destroyed by heat, and infected carcases and parts can be safely rendered. Unpasteurised milk from infected cows must not be used for human consumption.
Public awareness

1- A media campaign must emphasize the importance of cattle producers inspecting susceptible animals regularly and reporting abortions, the birth of weak or dead calves, or infertility. An abortion investigation program that relieves producers of the costs of investigation is a useful strategy. Details of any imposed movement controls need to be readily available and clearly explained to industry.

2- Given the important zoonotic implications, people at risk must be advised of appropriate occupational health and safety.

3- Additional research on *Brucella* and its vaccines should commence in the Red Sea State to formulate strategies for disease control.
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