Prevalence of Brucellosis in Cattle, Sheep and Goats of West Darfur State, Sudan

By

Yousif Mohammed Saleh Mustafa
B. V. Sc., University of Nyala, 2006

Supervisor

Prof. Mohammed Taha Abdalla Shigidi

A Dissertation Submitted to the University of Khartoum in Partial Fulfillment of the Degree of Master of Veterinary Science (Microbiology)

Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum

October 2010
# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter/Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of tables</td>
<td>viii</td>
</tr>
<tr>
<td>Abstract</td>
<td>ix</td>
</tr>
<tr>
<td>Arabic abstract</td>
<td>xi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
</tbody>
</table>

## Chapter one: Literature review

1.1 An overview ........................................... 5
1.2 The genus *Brucella* .................................. 6
   1.2.1 Taxonomy of *Brucella* species and biovars ....... 8
   1.2.2 Susceptibility to phages .......................... 12
   1.2.3 Susceptibility to dyes and antibiotics ........... 15
   1.2.4 Antigenic relatedness .............................. 16
1.3 Brucellosis .............................................. 18
   1.3.1 Definition ......................................... 18
   1.3.2 Transmission of the disease between animals .... 18
   1.3.3 Pathogenicity ...................................... 18
   1.3.4 The disease in cattle .............................. 19
   1.3.5 The disease in sheep and goats.................... 20
   1.3.6 Brucellosis in cattle, sheep and goats in Sudan ... 20
1.4 Diagnosis ................................................ 22
   1.4.1 Culture of samples for isolation of the causative agent... 22
   1.4.2 Demonstration of *Brucella* organisms by staining ..... 22
   1.4.3 Microscopical identification by immunofluorescence ... 22
Chapter two: Materials and methods

2.1 Samples
   2.1.1 Types and Sources of samples
   2.1.2 Collection of samples
      2.1.2.1 Serum samples
      2.1.2.2 Milk samples
      2.1.2.3 Vaginal swab
      2.1.2.4 Hygroma fluid
   2.1.3 Transportation of samples

2.2 Field investigations

2.3 Modified Zhiel Nielsen's stain (MZN)
   2.3.1 Preparation of smears
   2.3.2 Staining method

2.4 Sterilization

2.5 Culturing

2.6 Data collection

2.7 Serological tests used for diagnosis of brucellosis
   2.7.1 Rose Bengal Plate test (RBPT)
   2.7.2 Milk Ring test (MRT)
   2.7.3 Indirect ELISA (iELISA) for serum
2.7.3.1 Preparation of reagents

2.7.3.1.1 Diluting buffer

2.7.3.1.2 Wash solution

2.7.3.1.3 Substrate buffer

2.7.3.1.4 Chromogen

2.7.3.1.5 Stop solution

2.7.3.1.6 Controls

2.7.3.2 The procedure

2.7.4 Competitive ELISA (cELISA)

2.7.4.1 Preparation of reagents

2.7.4.1.1 Wash solution and diluting buffer

2.7.4.1.2 Stop solution

2.7.4.1.3 Conjugate

2.7.4.1.4 Controls

2.7.4.2 The procedure

2.7.5 Indirect ELISA (iELISA) for milk

2.7.5.1 Preparation of reagents

2.7.5.1.1 CHEKIT Wash solution

2.7.5.1.2 Control milk

2.7.5.2 The procedure

Chapter three: Results

3.1 Clinical observation

3.2 Smears

3.3 Serological tests

3.3.1 RBPT for serum

3.3.2 RBPT for hygroma fluids

3.3.3 iELISA for serum

3.3.4 iELISA for hygroma fluids

3.3.5 cELISA for serum
3.3.6 cELISA for hygroma fluids.................................46
3.3.7 Milk Ring test (MRT)........................................46
3.3.8 iELISA for milk..............................................46
3.4 Distribution of positive reactors............................46
3.5 Isolation of the organism......................................46

Chapter four:

Discussion.................................................................49
Conclusion and Recommendations.................................53
References.................................................................54
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Classification of the genus <em>Brucella</em> according to Corbel (1990)</td>
<td>13</td>
</tr>
<tr>
<td>2. Biovar differentiation of the species of the genus <em>Brucella</em> according to Alton <em>et al.</em>, (1988)</td>
<td>14</td>
</tr>
<tr>
<td>3. Differential characteristics of <em>Brucella</em> phages (Garrido-Abellan <em>et al.</em>, 2001)</td>
<td>15</td>
</tr>
<tr>
<td>4. Differential characteristics of <em>Brucella</em> from some other Gram negative bacteria (Alton <em>et al.</em>, 1988)</td>
<td>17</td>
</tr>
<tr>
<td>5. Results of brucellosis tests in West Darfur state</td>
<td>47</td>
</tr>
<tr>
<td>6. Results of brucellosis tests in El Geneina locality</td>
<td>47</td>
</tr>
<tr>
<td>7. Results of brucellosis tests in Furbranga locality</td>
<td>48</td>
</tr>
<tr>
<td>8. Results of MRT &amp; iELISA for cattle milk</td>
<td>48</td>
</tr>
</tbody>
</table>
Abstract

Brucellosis is one of the bacterial zoonotic diseases that affects both man and animal and has a considerable impact on public health and economy. However, the disease did not receive enough attention in West Darfur State, and only limited work was published. It showed that the prevalence is 10.2% in cattle, 3.5% in sheep and 5.98% in goats. This study was carried out to update information about prevalence of brucellosis in cattle, sheep and goats in the West Darfur state represented in two provinces (El-Geneina & Furbranga) for control purposes.

Three hundred blood for serum samples, 200 milk specimens and two hygroma fluid aspirates were collected. Beside 100 blood for serum samples from sheep and 100 from goats to be tested for *Brucella* antibodies using the Rose Bengal Plate test (RBPT) and Milk Ring test (MRT). The positive samples were reexamined with indirect ELISA (iELISA) and competitive ELISA (cELISA) for confirmation. The prevalence of brucellosis by the RBPT was 10.3% in cattle, 7% in sheep and 6% in goats.

When the RBPT was compared to MRT, RBPT had 95.7% sensitivity and its specificity for cattle was 87% when compared to iELISA and 90.3% when compared to cELISA, and 85.7% for sheep and 50% for goats when compared to cELISA. The specificity of MRT was 78.2% when compared to iELISA.
It could be concluded that the prevalence of brucellosis in cattle, sheep and goats in West Darfur State is similar to its prevalence reported in other parts of the country. But, the number of samples used was too small compared to the animal population in the state. Thus, further work is recommended to determine the actual situation of the disease in livestock, taking into consideration the sample size in relation to the population of cattle, sheep and goats in the state.
المستخلص

داء البروسيلا هو أحد الأمراض البكتيرية المشتركة التي تصيب الإنسان والحيوان معا والتي لها تأثير كبير على الصحة العامة والاقتصاد، مع ذلك فإنه لم يحظى بعناية كافية في ولاية غرب دارفور، حيث نشر عمل محدود وجد فيها إصابة المرض 10.2% في الأبقار، 3.5% في الأغنام و 5.9% في الماعز.

في الوقت الراهن نجد أن الكثير من الظروف قد تغيرت، هنالك حرب مسلحة اندلعت في هذه المنطقة منذ عام 2003 م لذلك فإن نظام رعاية الحيوان قد تأثرت، الإصلاح البيئي سار إلى الأسوأ و المراحي المتاحة تعرضت للرعي الجائر. لذلك فإن هذه الدراسة هدفت إلى تحديث المعلومات عن انتشار المرض في الأبقار، الأغنام والماعز في هذه الولاية والتي يمكن أن تصبح أساس لمعلومات حديثة لأهداف المكافحة.

في هذه الدراسة، جمعت 300 عينة دم للأمصال، 200 عينة لين و عينتين من سائل الورم المائي من الأبقار، بالإضافة إلى 100 عينة دم للأمصال من الأغنام و 100 عينة مماثلة من الماعز لاختبارها للأجسام المضادة للبروسيلا باستخدام اختبارات الروز ناقل الصحني و اختبار حلقة اللبن والعينات التي وجدت موجبة لهذه الاختبارين تم اختبارها مرة أخرى بواسطة اختبار الإليزا غير المباشر واختبار الإليزا التنافسي لتأكيد النتيجة. وجد انتشار المرض بواسطة اختبار الروز ناقل الصحني 10.3% في الأبقار، 7% في الأغنام و 6% في الماعز.

عندما تم مقارنة اختبار الروز ناقل باختبار حلقة اللبن وجد أن نسبة حساسيته 95.7% و نسبة تخصصيته في الأبقار 87% عند مقارنته باختبار الإليزا غير المباشر و 90.3% عند مقارنته باختبار الإليزا التنافسي، و 85.7% في الأغنام و 50% في الماعز عند مقارنه باختبار الإليزا التنافسي. كذلك وجد أن نسبة تخصيصية اختبار حلقة اللبن 78.2% عند مقارنه باختبار الإليزا غير المباشر.
بناءً على هذه الدراسة يمكن أن نستخلص أن انتشار مرض البروسيلات في الأبقار والأغنام والماعز في ولاية غرب دارفور مثل تلك التي وجدت في أجزاء من القطر، لكن عدد العينات التي فحصت قليلة مقارنة بأعداد الحيوانات المنزلية الموجودة في الولاية لذلك توصي الدراسة بإجراء دراسات أخرى لتوضيح نسب الانتشار الحقيقية آخذين في الاعتبار حجم العينة مقارنة بعداد الأبقار، الأغنام والماعز في الولاية.
Introduction

Brucellosis has been an emerging disease since the discovery of *Brucella melitensis* by Bruce in 1887. Subsequently, an increasing pattern of strains has emerged with the identification of *Brucella spp.* that infect a wide range of terrestrial animals. More recently, types infecting marine mammals. Because each type has distinctive epidemiologic features, with each new type, the complexity of the interaction with humans has increased. Because new strains may emerge and existing types adapt to changing social and agricultural practices, the picture remains incomplete.

Brucellosis is a disease of both public health and economic importance and it is of world wide distribution. Losses of animal production due to Brucellosis include diminution of milk and meat, abortion, infertility, longer inter calving intervals and higher culling rates (Blood *et al.*, 1983). The disease is transmitted by many routes mainly ingestion and is characterized by contagious abortion in animals and febrile illness in man.

Brucellae are Gram-negative, facultative, intracellular bacteria that can infect many species of animals and man. It is named *Brucella* in the honor of David Bruce who is the first to isolate *Brucella melitensis* from a human spleen in 1887, but the first isolation of *Brucella abortus* was done by Bang in 1897. Six species are recognized within the genus *Brucella*: *Br. abortus*, *Br. melitensis*, *Br. suis*, *Br. ovis*, *Br. canis* and *Br. neotomae* (Corbel and Brinley-Morgan, 1984). This classification is mainly based on differences in
pathogenicity and host preference (Corbel and Brinley-Morgan, 1984). Distinction between species and biovars is currently performed by differential tests based on phenotypic characterization of lipopolysaccharide antigens, phage typing, dye-sensitivity, CO₂ requirement, H₂S production and metabolic properties (Alton et al., 1988). The main pathogenic species worldwide are *Br. abortus*, responsible for bovine brucellosis; *Br. melitensis*, the main aetiological agent of ovine and caprine brucellosis; and *Br. suis*, first isolated form swine. These three *Brucella* species may cause abortion in their hosts which results in huge economic losses. *Br. ovis* and *Br. canis* are responsible for ram epididymitis and canine brucellosis, respectively, while *Br. neotomae* has only been isolated from desert rats. However, *Brucella* strains have also been isolated from a great variety of wildlife species such as bison, elk, feral swine, wild boars, foxes, hares, African Buffalo, reindeer and caribou (Davis et al., 1990). The broad spectrum of *Brucella* isolates has recently been enlarged to include marine mammals. A number of recent reports have described the isolation and characterization of *Brucella* strains from a wide variety of marine mammals such as dolphins, seals, cetaceans, otter and whales (Clavareau et al., 1998, Ewalt et al., 1994, Foster et al., 1996, Jahans et al., 1997, Ross et al., 1994, Ross et al., 1996). These strains were identified as brucellae by their colonial and cellular morphology, staining characteristics, biochemical activity, agglutination by monospecific antisera, susceptibility to lysis by *Brucella* specific bacteriophage and metabolic profiles. However, their overall characteristics
were not assimilable to those of any of the six recognized *Brucella* species. Therefore, it was suggested that they comprise a new nomen species to be called ‘*Br. maris’*, based on the current classification system (Jahans *et al.*, 1997), which is further sub-grouped into *Br. pinnipediae* (for seal isolates) and *Br. cetaceae* (for cetacean isolates), (Cloeckaert *et al.*, 2001).

It has been shown, on the basis of DNA–DNA hybridization studies, that the genus *Brucella* is a highly homogeneous group (> 90% DNA homology for all species) (Verger *et al.*, 1985, Verger *et al.*, 1987). Several techniques have been employed to find DNA polymorphisms which would enable the molecular typing of the *Brucella* species and their different biovars (Allardet-Servent *et al.*, 1988, Bricker and Halling, 1994, Cloeckaert *et al.*, 1995, Cloeckaert *et al.*, 1996, Fekete *et al.*, 1992, Ficht *et al.*, 1990). The genes coding for the major outer membrane proteins (OMPs) (*omp25*, *omp31*, *omp2a* and *omp2b* genes) have been found to be particularly useful for this purpose because they exhibit sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars (Cloeckaert *et al.*, 1995, Cloeckaert *et al.*, 1996, Ficht *et al.*, 1990, Ficht *et al.*, 1996, Vizcaino *et al.*, 2000, Vizcaino *et al.*, 1997).

In Sudan the first isolation of *Br. abortus* was made by Bennet in 1943 from a Friesian herd at Bulgravia dairy farm. But the first isolation of *Br. abortus* from local cattle was from a cow which aborted at Juba dairy farm (Dafalla, 1962).
Animal brucellosis in Sudan was suspected as early as 1904 and was first reported by Bennet (1943) in a dairy herd in Khartoum. Since then it was studied in cattle, sheep, goats, camels and wild animals.

The prevalence of cattle brucellosis in Sudan has been studied by many researchers, 23.1% in Khartoum state (Hatim, 2006), 8.7% in El-Gezira state (Dafalla, 1962), 14.2% and 66.7% in North Kordofan (Ibrahim and Habiballa, 1975), 5.7% and 8.7% in Blue Nile state (Mustafa and Hassan, 1969), 3%, 1.7% and 1.5% in North Sudan (Abdella, 1964), 6.2% (Raga, 2000) and 13.9% (Musa, 1995) in Darfur states, 5% in Kassala state (El-Ansary et al., 2001) and 14.6%, 18% in Southern Sudan states (El-Nasri, 1960). The prevalence rates of sheep and goats brucellosis has been reported in many parts of the country by many researchers, in Southern Sudan, El Nasry (1960) reported 3.5% and 6.6% respectively, in the Gezira state, Dafalla (1962) reported 4.2% and 2.5% respectively, in North Sudan, Abdalla (1964) reported 1.7% and 1.5% respectively, and Fayza et al. (1990) reported 0.01% and 0.13% respectively.

Western Sudan is known to posses over 20% of the live stock in the country. Problems of animal health in this area received less attention from researchers, therefore, this study was conducted with the following objectives to:

1. Study the prevalence of brucellosis among cattle, sheep and goats.
2. Isolate and characterize the causative agent of brucellosis in cattle, sheep and goats.
Chapter one

Literature review

1.1 An overview

Brucellosis remains a major zoonosis worldwide. Although many countries have eradicated *Br. abortus* from cattle, in some areas *Br. melitensis* has emerged as a cause of infection in this species as well as in sheep and goats. Despite vaccination campaigns with the Rev 1 strain, *Br. melitensis* remains the principal cause of human brucellosis. *Br. suis* is also emerging as an agent of infection in cattle, thus extending its opportunities to infect humans. The recent isolation of distinctive strains of *Brucella* from marine mammals has extended its ecologic range. Molecular genetic studies have demonstrated the phylogenetic affiliation to *Agrobacterium*, *Phyllobacterium*, *Ochrobactrum*, and *Rhizobium* (Deley et al., 1983). Polymerase chain reaction and gene probe development may provide more effective typing methods. Pathogenicity is related to production of lipopolysaccharides containing a poly N-formyl perosamine O chain, Cu-Zn superoxide dismutase, erythulose phosphate dehydrogenase, stress-induced proteins related to intracellular survival, and adenine and guanine monophosphate inhibitors of phagocyte functions. Protective immunity is conferred by antibody to lipopolysaccharide and T-cell-mediated macrophage activation triggered by protein antigens. Diagnosis still
centers on isolation of the organism and serologic test results, especially enzyme immunoassay, which is replacing other methods. Polymerase chain reaction is also under evaluation. Therapy is based on tetracyclines with or without rifampicin, aminoglycosides, or quinolones. No satisfactory vaccines against human brucellosis are available, although attenuated purE mutants appear promising.

1.2 The genus Brucella

It is generally accepted that, the genus Brucella consist of small, none motile, none sporing, Gram negative cocci, coccobacilli or short rods. 0.5-0.7 μm in diameter and 0.6-1.5 μm in length. It occurs singly, in pairs (less frequently), short chains or small groups. It is aerobic (carboxyphilic), possessing respiratory type of metabolism and has cytochrome based electron transport system with oxygen or nitrate as terminal electron acceptor. Many strains require supplementary CO₂ for growth especially on primary isolation. Brucella does not grow under strict anaerobic conditions. It is catalase positive, usually oxidase positive but negative strains occur, reduce nitrate, produce H₂S and hydrolyze urea. Production of indole, acetyl methyl carbinol and methyl red test and utilization of citrate are negative. They do not lyse erythrocytes and do not liquefy gelatin or inspissated serum. Colonies on primary isolation on serum dextrose agar (SDA) or other clear media are usually 0.5-1.0 mm in diameter, transparent, raised, and convex with a circular outline and an entire edge. The
colonies have shinny surfaces and appear clear pale yellow (honey like in colour) by transmitted light; while in reflected light colonies have smooth glistening surfaces and appear bluish grey. Non smooth variants of the smooth species occur, but there are also stable non-smooth species with distinctive host range. The optimum temperature is 37ºC and growth occurs between 20-40ºC and the optimum pH is 6.6-7.4 (Hirsh and Zee, 1999). They are chemoorganotrophic. Most strains require complex media containing several amino acids, thiamin, nicotinamide and magnesium 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 but hemin (X-factor) and nicotinamide adenine dinucleotide (NAD: V-factor) are not essential. Acid production does not occur from carbohydrate on conventional media, except for Br. neotomae. The organisms posses characteristic intracellular antigens specific for the genus. They are intracellular parasites transmissible to a wide range of animal species including man.

The mole %G+C of the DNA is 55-58. Type species is Br. melitensis (Hughes, 1893; and Meyer and Shaw, 1920). Brucella is not truly acid fast, but the organism retains certain dyes including basic fuchsin in the presence of dilute acids or alkalis and this has been used as the bases of differentiating staining methods (cited by Corbel, 1989). This method is not specific for Brucella and other organisms with similar host and tissue preference including Chlamydia psittaci and Coxiella burnetti show similar staining reactions.
Compared with non-pathogenic bacteria, *Brucella* has a substantial capacity to survive and persist in the environment under suitable conditions. 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 protected from sun light it may retain infectivity for years. The organism is sensitive to heat and is killed by pasteurization or by exposure to 60ºC for 30 minutes. It is readily killed by UV or Gamma rays under complete exposure. It has no plasmids and resistance to certain antibiotics has been transferred following phage infection.

In the genus *Brucella* ten members are currently known, these are *Br. melitensis* (Hughes, 1893; and Meyer and Shaw, 1920); *Br. abortus* (Meyer and Shaw, 1920); *Br. suis* (Huddleson, 1929); *Br. neotomae* (Stonner and Lackman, 1957); *Br. ovis* (Buddle, 1956); *Br. canis* (Carmichael and Bruner, 1968); *Br. pennipedialis*; *Br. ceti* (Cloeckaert et al., 2001); *B.microti* (Hubalek et al., 2007; Scholz et al., 2008) and *B.inopinala*.

### 1.2.1 Taxonomy of *Brucella* species and biovars

Considering their high degree of DNA homology (> 90 % for all species), brucellae have been proposed as a monospecific genus in which all types should be regarded as biovars of *Br. melitensis* (Verger et al., 1985). Since this proposal has not yet met with complete agreement, the old classification of the genus (and relevant nomenclature) into six species, *i.e.* *Br. mliitensis, Br. abortus, Br. suis, Br. neotomae, Br. ovis and Br. canis* (Corbel and Brinley-Morgan, 1984), in
addition to the recently identified *Br. pennipedialis* and *Br. ceti* from marine mammals (Cloeckaert *et al.*, 2001), and more recently *B.microti* and *B.inopinala* is the classification used world-wide. The first 4 species are normally observed in the smooth form, whereas *Br. ovis* and *Br. canis* have only been encountered in the rough form. Three biovars are recognized for *Br. melitensis* (1-3), seven for *Br. abortus* (1-9), and five for *Br. suis* (1-5).

Species identification is routinely based on lysis by phages and on some simple biochemical tests such as catalase, oxidase, urease, nitrate and H₂S production. For *Br. melitensis*, *Br. abortus* and *Br. suis*, the identification at the biovar level is currently performed by five main tests, *i.e.* carbon dioxide (CO₂) requirement, production of hydrogen sulphide (H₂S), dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M anti-sera and lysis with *Brucella*-specific phages (Alton *et al.*, 1988) (Table 2). Moreover, a recently developed co-agglutination test, using latex beads coated with a pair of monoclonal antibodies directed against the rough lipopolysaccharide (R-LPS) and the 25 kDa outer membrane protein (*Omp* 25), respectively (Bowden *et al.*, 1997), makes it possible to accurately differentiate *Br. ovis* from *Br. canis* and the occasional rough isolates of the smooth *Brucella* species. *Br. melitensis* biovar 3 appears to be the most frequently biovar isolated in Mediterranean countries. The precise recognition of biovar 3, especially its differentiation from biovar 2 appears sometimes equivocal. Due to the use of insufficiently
discriminating monospecific sera, a number of strains identified initially as biovar 2 were later confirmed as biovar 3 by expert laboratories.

Intermediate strains are occasionally found due to the instability reported for some of the phenotypic characteristics used for the current classification of *Brucella* such as H₂S production. This situation sometimes impedes the identification of the species and their biovars. Therefore, the identification of stable DNA-specific markers is considered a high priority for taxonomic, diagnostic and epidemiological purposes. Several methods, mainly PCR-RFLP and Southern blot analysis of various genes or loci, have been employed to find DNA polymorphism which would enable the molecular identification and typing of the *Brucella* species and their biovars (Allardet-Servent *et al.*, 1988; Ficht *et al.*, 1990, 1996; Halling and Zehr, 1990; Halling *et al.*, 1993; Fekete *et al.*, 1992b; Grimont *et al.*, 1992; Bricker and Halling, 1994, 1995; Cloeckaert *et al.*, 1995, 1996c; Mercier *et al.*, 1996; Ouahrani *et al.*, 1993; Ouahrani-Bettache *et al.*, 1996; Vizcaino *et al.*, 1997). Among these methods, detection of polymorphism by PCR-RFLP is considered to have an advantage over Southern blotting, since it is easier to perform and is less time-consuming when applied to large numbers of samples.

Of all the DNA sequences investigated by PCR-restriction, the major outer membrane protein (*omp*) genes of *Brucella* are the most interesting as they exhibit sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars (Cloeckaert *et al.*, 1996d). Studies of the
RFLP patterns of two closely related genes, *omp2a* and *omp2b*, encoding and potentially expressing the *Brucella* spp. major omp of 36 kDa (Ficht *et al.*, 1988, 1989), showed that the type strains of the six *Brucella* species could be differentiated on this basis (Ficht *et al.*, 1990). More recently, Cloeckaert *et al.*, (1995) using PCR-RFLP and a greater number of restriction enzymes detected *Brucella* species, biovar, or strain-specific markers for the *omp25* gene, encoding the *Brucella* 25 kDa major omp (de Wergifosse *et al.*, 1995), and for the *omp2a* and *omp2b* genes. The *omp31* gene (Vizcaino *et al.*, 1996), encoding a major outer-membrane protein in *Br. melitensis*, is also an interesting gene for the differentiation of *Brucella* members. Using a combination of *omp31* PCR-RFLP patterns and Southern blot hybridization, profiles of *Brucella* species were differentiated with the exception of *Br. neotomae* which was indistinguishable from *Br. suis* biovars 1, 3, 4 and 5. It was also shown that *Br. abortus* lacks a large DNA fragment of about 10 kb contained in *omp31* and its flanking DNA (Vizcaino *et al.*, 1997).

More highly conserved *Brucella* genes may also be useful for taxonomic and epidemiological purposes, even if they contain less polymorphism than the OMP genes. In this respect, the *dnak* locus which allows the identification of *Br. melitensis*, the main *Brucella* pathogen for sheep, is of particular interest. All *Br. melitensis* biovars showed a specific PCR-RFLP pattern with EcoRV, consistent with the presence of a single site instead of two for the other *Brucella* species (Cloeckaert *et al.*, 1996c).
Taxonomic knowledge of *Brucella* has progressed a great deal since the techniques of molecular biology have been applied to these bacteria. A number of molecular tools (nucleic acid probes, primers...) are now available which make the elaboration of a more objective and reliable classification of the genus possible. Judging by the emergence of new *Brucella* types from marine mammals, the genus is far from being completely identified. In the near future, efforts should be concentrated on the harmonization of these tools to propose the most suitable method for the molecular identification and typing of *Brucella*.

### 1.2.2 Susceptibility to phages

Over 40 Brucella phages have been reported to be lytic for *Brucella* members. All phages are specific for the genus *Brucella*, and are not known to be active against any other bacteria that have been tested. Thus, lysis by *Brucella* phages is a useful test to confirm the identity of *Brucella* spp. and for speciation within the genus. The *Brucella* phages currently used for *Brucella* typing are: Tbilisi (Tb), Weybridge (Wb), Izatnagar1 (Iz1) and R/C. The three former phages are used for differentiation of smooth *Brucella* species. R/C is lytic for *Br. ovis* and *Br. canis* (WHO Report, 1986, Garrido-Abellan *et al.*, 2001) (Table 3).
<table>
<thead>
<tr>
<th>Proposed taxonomic biovar designation</th>
<th>Nomen species biovar</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Growth on media containing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thionin 20µg/ml</td>
</tr>
<tr>
<td><strong>Br. melitensis bv. melitensis</strong></td>
<td><strong>Br. melitensis</strong></td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Br. melitensis bv. abortus</strong></td>
<td><strong>Br. bortus</strong></td>
<td>1</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3*</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>6*</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Br. melitensis bv. suis</strong></td>
<td><strong>Br. suis</strong></td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Br. melitensis bv. ovis</strong></td>
<td><strong>Br. ovis</strong></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><strong>Br. melitensis bv. canis</strong></td>
<td><strong>Br. canis</strong></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>Br. melitensis bv. neotomae</strong></td>
<td><strong>Br. neotomae</strong></td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

*More differentiation on *Brucella abortus* biovar 3 and six is using thionine at 40µg/ml biovar 3 = + and biovar 6 = -.

** Some strains are inhibited by basic fuchsin. *** Some isolates are resistant to basic fuchsin. (+) Most strains positive. (-) Most strains negative
Table 2: Biovar differentiation of the species of the genus *Brucella* according to Alton *et al.*, (1988)

<table>
<thead>
<tr>
<th>Species</th>
<th>Biovar</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Growth on dyes</th>
<th>Agglutination in sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thionin</td>
<td>Basic fuchsin</td>
</tr>
<tr>
<td><em>Br. melitensis</em></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Br. abortus</em></td>
<td>1</td>
<td>C+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+c</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+c</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+c</td>
<td>+</td>
<td>-</td>
<td>D+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>+or-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Br. suis</em></td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>e-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>f-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Br. neotomae</em></td>
<td></td>
<td></td>
<td></td>
<td>g-</td>
<td>-</td>
</tr>
<tr>
<td><em>Br. ovis</em></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>f-</td>
</tr>
<tr>
<td><em>Br. canis</em></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>f-</td>
</tr>
</tbody>
</table>

a = dye concentration, 20µg/ml in serum dextrose medium (1:50000)
b = A=A mono-specific antiserum; M=M mono-specific antiserum; R=rough brucella antiserum.
c = usually positive on primary isolation  d = some strains do not grow on dyes.
e = some strains are resistant.
f = negative for most strains. g = growth at 10µg/ml (1:100000 thionine).
Table 3: Differential characteristics of Brucella phages (Garrido-Abellan et al., 2001).

<table>
<thead>
<tr>
<th>Species</th>
<th>Lysis by phages (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tb</td>
</tr>
<tr>
<td>Br. melitensis</td>
<td>-</td>
</tr>
<tr>
<td>Br. abortus</td>
<td>+</td>
</tr>
<tr>
<td>Br. ovis</td>
<td>-</td>
</tr>
</tbody>
</table>

(1) At the routine test dilution

1.2.3 Susceptibility to dyes and antibiotics

Susceptibility to the dyes, thionin and basic fuchsin (20 µg/ml), which varies between biovars, is one of the routine typing tests of Brucella. Br. melitensis grows in the presence of both dyes.

On primary isolation, brucellae are usually susceptible in vitro to gentamicin, tetracyclines and rifampicine. Most strains are also susceptible to the following antibiotics: ampicillin, chloramphenicol, cotrimoxazole, erythromycin, kanamycin, novobiocin, spectinomycin and streptomycin, but variation in susceptibility may occur between species, biovars and strains. Most strains are resistant to ß-lactamins, cephalosporins, polymyxin, nalidixic acid, amphotericin B, bacitracin, cycloheximide, clindamycin, lincomycin, nystatin and vancomycin at therapeutic concentrations. Penicillin is used for the routine differentiation of the vaccinal strain Br. abortus strain 19, and streptomycin for
Br. melitensis strain Rev.1, the vaccines widely used for immunization of cattle and small ruminants, respectively, from the virulent field strains by virtue of their different sensitivity to these antibiotics (Alton et al., 1988).

1.2.4 Antigenic relatedness

The genus Brucella is characterized by means of having the O-chain polysaccharide antigens which have recently been characterized at the molecular level in Br. abortus by Perry et al. (1986). The structural characteristic (N-acetyl 4-amino, 4, 6-dideoxy-D-manose repeating units in the O-chain) also exist with the O-chain of some other Gram-negative bacteria which allow antibody cross-reactions. The known cross-reacting species or strains are Yersinia enterocolitica serogroup 0:9; Salmonella serotype of Kuffman-white group N: 30; Escherichia coli 0:157 and 0:116 serotypes; Pseudomonas maltophilia; Francisella tularensis and Vibrio cholerae. This potential for cross-reaction complicates the use of anti LPS serum as a diagnostic agent unless the presence of the other known cross reacting species can be ruled out on other grounds (Nielsen and Duncan, 1990). However, DNA homology studies have shown that members of the genus Brucella lack homology with other microorganisms having similar guanine+cytocine ratios like Serratia marcescens, Escherichia coli, Agrobacterium tumefactions and the phenotypically similar species Francisella tularensis and Bordetella bronchiseptica (WHO report, 1986).
Table 4: Differential characteristics of *Brucella* from some other Gram negative bacteria (Alton *et al.*, 1988)

<table>
<thead>
<tr>
<th>Test</th>
<th>Brucella</th>
<th>Bordetella bronchiseptica</th>
<th>Campylobacter fetus</th>
<th>Moraxella</th>
<th>Acinetobacter</th>
<th>Yersinia enterocolitica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Small coccobacilli</td>
<td>Small Coccobacilli</td>
<td>Comma</td>
<td>Diplococoid</td>
<td>Diplococoid</td>
<td>Rod</td>
</tr>
<tr>
<td>Motility at 37°C</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility at 20°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lactose fermentation on Mac Conkey agar</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>V⁺</td>
<td>V</td>
<td>-</td>
</tr>
<tr>
<td>Acid production on agar containing glucose</td>
<td>- b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Haemolysis on blood agar</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td>V</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+ c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+ d</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+ e</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>V</td>
<td>-</td>
</tr>
<tr>
<td>Agglutination with: S-Brucella Antiserum</td>
<td>+ f</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R-Brucella antiserum</td>
<td>+ g</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a: Positive and negative species within the genus  

b: *Br. neotomae* may show some fermentation  
c: Except *Br. ovis, Br. neotomae* and some strains of *Br. abortus*  
d: Except *Br. ovis* and some strains of *Br. abortus*  
e: Except *Br. ovis*  
f: Except *Br. ovis, Br. canis* and R-forms of other species  
g: *Br. ovis, Br. canis* and R-forms of other species.
1.3 Brucellosis

1.3.1 Definition

It is a contagious bacterial disease of animals, which is transmitted to man (anthropozoonosis) (Carpenter and Hubbert, 1963).

1.3.2 Transmission of the disease between animals

According to Buxton and Fraser (1977) the disease is transmitted from infected animals or contaminated materials to susceptible ones through mucous membrane of the alimentary and respiratory tracts, conjunctiva, abraded and intact skin, artificial insemination and through the vagina in some species. Insects could also act as vehicles of infection (Corbel, 1989). In man, infection is by inhalation, ingestion, through conjunctiva and skin.

1.3.3 Pathogenicity

Brucellae are facultative intracellular parasites of the reticuloendothelial system. The virulence of Brucella varies considerably according to species, strain and the size of infecting inoculum. Host susceptibility is also variable and is associated with the reproductive status. Thus, in the field, all intermediate stages between typical acute infection and complete resistance may be observed. In addition, vaccinal immunity may modify the parasite-host relationship. The symptoms, which have been described in cattle are abortion, hygroma, orchitis, retention of placenta, weak or still births and long calving intervals (Blood et al., 1989 and Musa et al., 1990), while in other animals the symptoms are variable.
Pathogenically, *Br. melitensis* infection in sheep and goats is similar to *Br. abortus* infection in cattle. Nevertheless, differences are significant, and each species of *Brucella* causes a different disease (OIE Manual, 1996). In man it is caused by direct or indirect contact with infected animals and the infection usually cause severe or chronic illness.

### 1.3.4 The disease in cattle

It has a world-wide occurrence. Cattle are the most important source of infection with *Br. abortus* but other bovidae can be of local importance (Corbel, 1989). The disease is characterized by abortion (most frequently), hygromas, orchitis, placentitis and infertility (Blood *et al.*, 1989). The disease in cattle is widely distributed, and has been recorded in 120 out of the 175 (68.8%) countries of the world (Nielsen and Dunkan, 1990). It has been reported in 101 countries (WHO report, 1992). In Europe, bovine brucellosis has not been reported in some countries (Corbel, 1989). In USA the disease was eradicated from most areas and reduced in some. In Asia, Japan is free from the disease but it has been reported in India. Bovine brucellosis was eradicated from Australia. In Africa, bovine brucellosis has been reported in most African countries. In Arab countries, the disease has been reported from Syria, Saudi Arabia, Iraq, Yemen and all the Arab countries in Africa except Morocco (Thimm, 1982). Both *Br. abortus* and *Br. melitensis* were isolated from cattle in many countries. The organisms were isolated from various sources including milk, hygroma
fluids, vaginal swabs, semen (Chatterjee et al., 1995 and Casolinuovo et al., 1996), lymph nodes and aborted fetuses (Musa, 1995).

1.3.5 The disease in sheep and goats

Sheep and goats brucellosis (excluding *Brucella ovis* infection which is not pathogenic for humans) is a zoonotic infection with important effects on both public and animal health and production and is widespread in many areas of the world, particularly in some Mediterranean and Middle Eastern countries. *Brucella melitensis*, the main aetiologic agent of brucellosis in small ruminants, was the first species in the genus *Brucella* described. It was first isolated by Bruce in 1887 (Alton, 1990) from the spleens of soldiers dying of Mediterranean fever on the island of Malta. Bruce called it *Micrococcus melitensis*. The origin of the disease remained a mystery for nearly 20 years until it was discovered that goats were the source of infection for human populations. Brucellosis in sheep and goats is rarely caused by *Br. abortus* (Luchsinger and Anderson, 1979; Garin-Bastuji et al., 1994) or *Br. suis* (Paolicchi et al., 1993).

1.3.6 Brucellosis in cattle, sheep and goats in Sudan

Brucellosis in cattle was reported in all parts of the country and the prevalence was found to be higher in cattle compared to other animal species. The first incidence of bovine brucellosis in Sudan, was reported from a dairy herd in Khartoum where *Br. abortus* was isolated from an aborted cow (Bennett, 1943). Thereafter many investigators isolated the organism from cattle in
different localities in the country (Khan, 1956; Daffalla, 1962; Shigidi and Razig, 1971; Ibrahim, 1974; Musa and Mitchell, 1985; Khalafalla et al., 1987; Musa, Jahans and Fadalla, 1990). *Br. melitensis* was isolated from cow's milk in El-Gezira, central Sudan (Daffalla and Khan, 1958). *Brucella* was isolated from knee hygromas of cattle by many investigators (Shigidi and Razig, 1971; Khalafalla et al. 1987 and Musa, 1995). The disease in Darfur states, Western Sudan, appears to be widely spread. Musa et al. (1990) reported the prevalence of the disease in different animal species including cattle and concluded that the highest prevalence was in intensive farming systems and under nomadic conditions. Cattle were found most affected (13.9%) followed by camels (7.76%), goats (5.98%) and sheep (3.5%). The prevalence was found to range between 14-26% in South Darfur, which is known to be the richest state in animal population in the country. *Brucella* organisms isolated from South Darfur state were identified and typed as *Br. abortus* biovar 6 (Musa, 1995).

In West Darfur state the disease was studied only by Musa, (1995) in two provinces (Wadi Saleh & Zalingi). In Zalingi, goats were found to be most affected (16.9%) followed by sheep (13.2%) and cattle (8.8%). In Wadi Saleh, the disease was studied only in cattle (12.2%).
1.4 Diagnosis of Brucellosis

Many workers used serological tests for the diagnosis of the disease, but definite diagnosis is by isolation of *Brucella* organisms from infected animals and patients. Several methods are used for diagnosis and include:

1.4.1 Culture of samples for the isolation of the causative agent.

1.4.2 Demonstration of *Brucella* organisms in suspected samples by staining with either modified Koster's method (Christofferson and Ottosen, 1941) or modified Ziel Nielsen's stain.

These methods are not specific for *Brucella* organisms, and *Coxiella burnetti* was found to be stained red as *Brucella* (Corbel, 1973).

1.4.3 Microscopical identification by immuno-fluorescence (Meyer, 1966; and Corbel, 1973).

The investigators stated that this method was specific and dependable in differentiating between *Brucella* infection and that of Q-fever.

1.4.4 Guinea pig inoculation:

This method is more successful than direct culture especially from contaminated material. Guinea pigs are injected intramuscularly and killed after 4-5 weeks of inoculation. Then their sera are tested by the Serum Agglutination test (SAT). Recovery of the organism from the spleen or positive serum agglutination test (SAT) at 1/10 serum dilution or over are taken as evidence of infection (Brinely et al., 1978).
1.4.5 Serological tests:

There are two types of serological tests. Very sensitive tests and these are used for screening, and definitive ones for confirmation of infection. Usually more than one type of test is used 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 reaction. 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 (Beh, 1974). These tests are: Rose Bengal Plate test (RBPT), Serum and tube agglutination test (SAT or TAT), Complement fixation test (CFT), Card test, Plate Agglutination test, Modified SAT, Coomb test, Enzyme Linked Immunosorbent Assay (ELISA), Milk Ring test (MRT), Whey agglutination test and Allergic skin test (AST) (Nielsen, 2002). But according to the WHO report, (1992), RBPT, MRT, ELISA and CFT are the conventional diagnostic methods which should continue in use for brucellosis surveillance until year 2000.

1.4.1.1 The Rose Bengal plate test (RBPT)

The RBPT was developed more than 20 years ago for the diagnosis of bovine brucellosis, and it is widely used as a screening test to detect reliably the presence of *Br. abortus* infection in cattle (Morgan *et al.*, 1969 and Alton *et al.*, 1975). Also it can be used as a definitive test (Nicoletti, 1967). Despite the
scanty and sometimes conflicting information available (Trap and Gaumont, 1975; Farina, 1985; Macmillan, 1990; Alton, 1990; Blasco et al., 1994a,b), this test is internationally recommended for the screening of brucellosis in small ruminants (FAO/WHO report, 1986; Garin-Bastuji and Blasco, 1997). An important problem affecting the sensitivity of the RBPT concerns the standardization of the antigen. The European Union regulations require antigen suspensions in lactate buffer at pH 3.65 ± 0.05 that are able to agglutinate at a dilution of 1:47.5 (21 IU/ml) of the International Standard anti-Br. abortus serum (ISaBS) but give a negative reaction at a dilution of 1:55 (18.2 IU/ml) of the same serum (EEC, 1964). These standardization conditions, which seem to be suitable for the diagnosis of Br. abortus infection in cattle (Macmillan, 1990), limit the sensitivity of the test resulting in reduced performance for the diagnosis of Br. melitensis infection in sheep (Blasco et al., 1994a,b). This accounts for the relatively low sensitivity of some commercial RBPT antigens when diagnosing brucellosis in sheep and goats (Falade, 1978, 1983; Blasco et al., 1994a) and for the fact that a high proportion of sheep and goats belonging to Br. melitensis-infected areas give negative results in the RBPT but positive ones in the CFT (Blasco et al., 1994a). These phenomena have raised serious questions over the efficacy of using the RBPT as an individual test in small ruminants. However, if the antigen is standardised differently to give a higher analytical sensitivity, the diagnostic sensitivity is much improved (Macmillan, 1997). Some workers claimed that, at least for sheep, the sensitivity of the RBPT
could be improved significantly when the antigens were standardized against a panel of sera from several \textit{Br. melitensis} culture positive and \textit{Brucella}-free sheep, respectively, or when the volume tested was increased from 25\(\mu\)l to 75\(\mu\)l (Blasco \textit{et al}., 1994a).

The RBPT stained antigen is buffered at pH 3.65 to inhibit non-specific agglutinins, but not those of \textit{Brucella} (Rose and Roepke, 1957). The test detects IgG1 only (Corbel, 1972). 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 easy to perform, cheap and rapid (four minutes), it is more sensitive, but less specific than SAT and CFT. Sera negative for RBPT are not tested further but the positive ones are tested by SAT and CFT (Morgan \textit{et al}., 1978). Nevertheless false negative reactions have been obtained (Miller \textit{et al}., 1973 and Lapraik \textit{et al}., 1975).

\textbf{1.4.1.2 Serum Agglutination test (SAT)}

This test is widely used in some countries and it is positive results are subjected to the definite CFT. Other than sera, the agglutination can be used for vaginal mucus and semen examinations. The antigen used in the test is a \textit{Brucella} whole cell and the antibodies detected are those directed against the surface molecules. SAT can be performed in tubes or microtitre plates and the plate test was found to be more sensitive (Herr \textit{et al}., 1982). SAT has international standardization, it is used for control programmes and in import
and export policies (Macmillan and Cockrem, 1985). The two investigators also reported that, sometimes non-specific agglutinations occurred in the test using known negative sera due to non-immune binding of bovine IgM to cells of *Br. abortus*. 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. According to reports of FAO/WHO report, (1964), the results of this test 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 animals over 30 month of age, the level should be more than 50 I.U.

SAT is modified by addition of 10% sodium chloride to the diluent and this is found to abrogate prozone phenomenon which is due to high concentration of IgG1 (Kolar, 1989). Falade, (1978) compared RBPT, SAT and MRT for the diagnosis of brucellosis in caprine and concluded that SAT offered a better serological result.

### 1.4.1.3 Complement fixation test (CFT)

The CFT is the most widely used test for the serological confirmation of brucellosis in animals. And it is used for confirming the result of the RBPT and SAT. the test was found to be more accurate for bovine brucellosis (Morgan *et al.*, 1973), while Meyer, (1979) stated that the test was superior to other tests in sensitivity and specificity, and it has found to have the highest specificity in both
non-vaccinated and vaccinated cattle when compared with standard tube agglutination test, haemolysis in gel, indirect enzyme immunoassay and buffered plate antigen tests.

As in cattle brucellosis, despite its complexity and the heterogeneity of the techniques used in different countries, there is agreement that this test is effective for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; Macmillan, 1990; Alton, 1990). When testing a limited number of sera obtained from *Br. melitensis* culture positive and *Brucella* free goats, the CFT provided the same sensitivity as the RBPT and iELISA (Díaz-Aparicio *et al.*, 1994). However, under field conditions, the sensitivity of the CFT has been reported to be somewhat lower (88.6%) than those of the RBPT (92.1%) and iELISA (100%) for diagnosing *Br. melitensis* infection in sheep (Blasco *et al.*, 1994a, b). More recently (Nielsen *et al.*, 2000), in a Pan-American and European comparative study, the results on sensitivity for the different tests were: cELISA (76.0 %), buffered plate agglutination test (77.5 %), CFT (83.1 %), iELISA (90.1 %) and fluorescence polarization assay – FPA (91.5%). On the other hand, the CFT has many drawbacks such as complexity, variability of reagents, prozones, anticomplementary activity of sera, difficulty to perform with hemolysed sera, and subjectivity of the interpretation of low titres. Therefore, while the sensitivity of RBPT is sufficient for the surveillance of free areas at the flock level, RBPT and CFT should be used together in infected flocks to obtain accurate individual sensitivity in test-and-slaughter
programmes. Moreover, an important drawback of both RBPT and CFT is their low specificity when testing sera from sheep and goats vaccinated subcutaneously with Rev.1 (Fensterbank et al., 1982; Díaz-Aparicio et al., 1994). However, when the Rev.1 vaccine is applied conjunctivally (Fensterbank et al., 1982), the interference problem is significantly reduced in all serological tests (Díaz-Aparicio et al., 1994). The test procedures were described by Morgan et al., (1978). Titres $\geq 2/4$ were considered positive, but according to the Australian Bureau a positive titre was $\geq 1/8$.

1.4.1.4 Enzyme-immunoassay (EIA) Methods

The majority of EIAs in use in brucellosis diagnosis are indirect ELISAs (iELISA). ELISAs are methods that involve the immobilization of one of the active components on a solid phase, and iELISAs are those in which the antigen is bound to a solid phase, usually a polystyrene microtitre plate so that antibody, if present in a sample, binds to the immobilized antigen and may be detected by an appropriate anti-globulin-enzyme conjugate which in combination with a chromogenic substrate gives a colored reaction indicative of the presence of antibody in the sample. It is this method that is now familiar to most diagnosticians.

Another method which is gaining prominence in the publications on brucellosis diagnosis is the competitive ELISA (cELISA). (Gorrell et al., 1984; Rylatt et al., 1985; Sutherland et al., 1986; Macmillan et al., 1990; Greiser-Wilke et al., 1991; Nielsen et al., 1991; Marín et al., 1999; Nielsen et al., 2000). In this
test, *Brucella* antigen is immobilized on the plate as with the indirect ELISA. Following that, the serum under test and a monoclonal antibody directed against an epitope on the antigen are co-incubated. This anti-*Brucella* monoclonal antibody is conjugated to an enzyme, the presence of which is detected if it binds to the antigen. This will only occur if there is no antibody in the serum sample which is bound preferentially.

ELISA has proven to be specific and sensitive as the MRT and SAT in detecting *Brucella* antibodies in milk and serum (Nielsen *et al.*, 1981). ELISA results are usually in agreement with CFT (Ruppanner *et al.*, 1980; Bercovich and Taaijke, 1990). The test can be used for screening and confirmation of brucellosis in both milk and serum. However, depending on the presence of traces of colostrums in the milk, or the presence of low concentration of lactated immunoglobulin, ELISA may test false positive or false negative (Bercovich and Taaijke, 1990; Kerkhofs *et al.*, 1990). Some researchers imply that the main advantage of the ELISA when compared with the CFT lies in its relative simple test procedure (Sutherland *et al.*, 1986).

1.4.1.5 **Milk Ring test (MRT)**

The test is used for screening and diagnosis of brucellosis. The test results are influenced by factors such as mastitis, mechanical agitation and vaccination with *Br. abortus* S19 vaccine. According to WHO report, (1992) the MRT is not suitable for sheep and goats as ring formation do not readily occur.
1.5 Treatment of Brucellosis

Treatment is unlikely to be undertaken in animals. The use of long-acting oxytetracycline at 20 mg/kg body weight intramuscularly at 3-4 day intervals for 5 treatments in combination with streptomycin at 25 mg/kg body weight intramuscularly or intravenously daily for seven consecutive days was partially successful in the treatment of infected cows. The administration of oxytetracycline concurrently with vaccination may reduce the antibody response in cattle (Blood and Roddostitis, 1989). Radwan et al. (1987) pointed out that a long term treatment with a high dose of oxytetracycline (1000 mg/day per 6 weeks, I/P) had completely eliminated Br. melitensis from naturally infected sheep. In humans however, many antimicrobial agents are used such as Tetracycline or Doxycycline, Trimethoprim, Sulfamethoxazole and Streptomycin (Young and Corbel, 1989). Chloramphenicol (1 gm/100 kg body weight daily for 12-20 days) is reported as a successful treatment in infected horses (Blood and Roddostitis, 1989).

1.6 Control and eradication of brucellosis

It is difficult to cure because of the capacity of the organism to grow intracellular. Because of the tremendous effects of the disease on economy and exportation, it must be controlled and eradicated. Plommet, (1986) recommended three ways for control and prevention of brucellosis:
1- Protection of herds free from disease and areas of importation from non-free areas by restriction of animal movement.

2- Vaccination of exposed herds or animals.

3- Segregation of infected animals or herds from free ones and this is done by testing and slaughter or isolation of sero-positive animals.

The maximum control and prevention is achieved when the three ways above are combined (Nicoletti, 1980). According to WHO report, (1986), elimination by test and slaughter is carried out only in small farms under closed systems.

Immunization against bovine brucellosis is mostly preferred using *Br. abortus* strain 19 vaccine found by Buck (1930). The common age for strain 19 vaccination is 2-10 months depending upon the breed of cattle. In the usual strain 19 vaccination, at 4-6 months, the post-vaccinal response lasts 6-12 months. Strain 19 vaccination is usually administered subcutaneously (Buck, 1930) or conjunctivally (Plommet and Fensterbank 1976). The usual dose of a young calf 3-8 months was calculated to be $50-120 \times 10^9$ viable count. Recently, it was found that a dose of $3-10 \times 10^9$ viable counts in two ml produce similar protection (WHO report, 1986), while the dose in adult cattle is $3 \times 10^8$ CFU. The protection rate of S19 was said to be 65-70%.

Other vaccines are used like rough *Br. abortus* killed vaccine with an adjuvant-designated 45/20 is used for cattle 9 months or above, it is save and stable. In sheep and goats Rev.1 *Br. melitensis* live vaccine and a killed adjuvant vaccine H38 are used. Recently Br. suis strain 2 ($S_2$) vaccine was introduced by the
Chinese. Oral vaccination by S₂ was found to be better than other routes (in sheep, goats, cattle and pigs). *Br. abortus* BA 19 vaccine was produced for human vaccination (WHO report, 1992).

Another classically obtained, live attenuated but rough (S-LPS lacking) *Brucella* strain is the *Br. abortus* RB51 strain (Schurig *et al.*, 1991). The strain RB51 has been reported to be equally effective as S19 vaccine in protecting against *Br. abortus* in cattle, without inducing anti-O chain antibodies as detected by serological tests (Palmer *et al.*, 1997) therefore it does not interfere with diagnostic serologic tests.
Chapter two

Materials and Methods

2.1 Samples

2.1.1 Type and Sources of samples

A total of 300 cattle serum samples, 200 cows milk samples, 100 sheep serum samples, 100 goat's serum samples, one vaginal swab from an aborted goat and two hygroma fluids were collected randomly in El-Geneina and Furbranga localities.

2.1.2 Collection of samples

2.1.2.1 Serum Samples

Blood for serum samples from cattle, sheep and goats were taken as described by Alton et al. (1975). The skin over the jugular vein was rubbed with 70% alcohol and disinfected with tincture of iodine. About 7 ml of blood was withdrawn using a labeled vaccutainer. Samples were put in a wire basket under shade, before taken to laboratory with minimum possible shaking. These samples were kept overnight at 4°C in a refrigerator to separate the serum. Sometimes the blood samples were centrifuged at 2500 rpm for five minutes to separate the serum.

2.1.2.2 Milk Samples

Milk samples were collected from milking cows according to Alton et al. (1975). The whole udder was washed and dried and the tip of each teat was
disinfected with alcohol and wiped dry. The first stream of the milk was
discarded, and then about 10 ml of milk were taken from each cow directly into
a labeled sterile cryovial. The samples were kept in an ice box and then
transported to the laboratory. Milk samples were kept in the refrigerator until
used within 24 hours.

2.1.2.3 Vaginal Swab

A vaginal swab was taken from a goat which had aborted before 17 days,
using a sterile cotton swab which was cultured directly at the same day.

2.1.2.4 Hygroma Fluid

Fluids from knee hygromas of two different cattle were aspirated using
sterile disposable syringes and put into sterile McCartney bottles. The fluids
were immediately tested for *Brucella* antibodies using RBPT then preserved
frozen for bacteriological culture.

2.1.3 Transportation of samples

Samples were labeled, kept in ice boxes and transported to the laboratory.
Samples for bacterial isolation were cultured immediately after reaching the
laboratory.

2.2 Field investigation

About 10000 head of cattle, 5000 of sheep and 4000 of goats were
examined clinically for abortion, hygromas, orchitis, retained placenta or any
other signs of the disease. This was performed in different localities including
pasture lands around El-Geneina, the veterinary clinic, slaughter houses and the market in El-Geneina, pasture lands around Furbranga, the veterinary clinic, slaughter houses and the market in Furbranga. Blood and milk samples were collected randomly.

2.3 **Modified Ziehl Nielsen's stain (MZN)**

This is a differential stain used for staining bacteria of the genus *Brucella*.

2.3.1 **Preparation of Smears**

Smears were prepared from samples on a clean dry glass slide and allowed to dry in air then fixed by gentle flaming.

2.3.2 **Staining Method**

The staining procedures were carried out according to Barrow and Feiltham (1993) as follows:

The smears were dried and fixed by passing over a flame. Stained for ten minutes with a 1:10 carbol fuchsin (1gm basic fuchsin dissolved in ten ml absolute ethanol solution), washed with tap water, differentiated with 0.5% acetic acid for not more than 30 seconds and washed thoroughly with tap water and differentiated lightly with 1% methylene blue (20 seconds). In case of positive samples the organism will stain red with blue background.
2.4 Sterilization

Glass wares such as petri dishes, test tubes, pipettes, flasks and bottles were sterilized in the hot air oven at 160°C for 90 minutes. Tryptone soya agar was sterilized by autoclaving at 15 lb/in (121°C) for 15 minutes.

2.5 Culturing

The two hygroma fluids and the vaginal swab were cultured directly into Tryptone soya agar plates and incubated in anaerobic jar with CO₂ generated by candle at 37 ºC and checked daily for 10 days.

2.6 Data collection

Data on number of herds, herd sizes, feeding and drinking hygiene, history of abortion, and vaccination program were collected from livestock owners inside and around El-Geneina and Furbranga provinces.

2.7 Serological tests used for diagnosis of brucellosis

2.7.1 Rose Bengal Plate test

The test was performed according to Morgan et al.,(1978) by dispensing 0.03 ml of each serum to be tested to an enamel plated plate. The same amount of RBPT antigen was added to each serum sample and both were mixed together, rocked by hand for four minutes after which the test was immediately read. Only positive sera were retained for further test.
2.7.2 Milk Ring test

All milk samples were tested for brucellosis using milk MRT according to Morgan *et al.*, (1978). At the same time they were tested to demonstrate *Brucella* organism using modified Ziehl Nielsen stain. This test was done by adding 0.03 ml of stained milk ring test antigen to 1ml of milk. Both were mixed well and incubated at 37º C for one hour before the test was observed for ring formation.

2.7.3 Indirect ELISA for Serum


2.7.3.1 Preparation of Reagents

2.7.3.1.1 Diluting buffer

The diluting buffer was prepared by adding 5 tablets of phosphate buffered saline (PBS), 0.5 ml phenol red indicator and 250µl of tween 20 to 500ml distilled water. The pH was in the range of 7.2-7.6. The buffer was stored at 4ºC until used in the next day.

2.7.3.1.2 Wash solution

The wash solution was prepared by adding the contents of the ampoule of Na₂HPO₄ and 1ml of Tween 20 to 10 litres of distilled water then stored at room temperature until used in the next day.
2.7.3.1.3 **Substrate buffer**

Substrate buffer was prepared by dissolving 1 tablet of the substrate in 120ml of distilled water. The pH was within 3.9-4.4. The substrate buffer was stored at 4°C until used in next day.

2.7.3.1.4 **Chromogen**

Chromogen was prepared by dissolving 2 ABTS tablets in 1ml sterile distilled water and stored in the dark at 4°C until used in the next day.

2.7.3.1.5 **Stop solution**

Stop solution was prepared by diluting the contents of the ampoule of sodium azide with 500ml of distilled water then stored at room temperature until used in the next day.

2.7.3.1.6 **Controls**

These were prepared by the reconstitution of the positive and negative control samples included in the kit each with 1ml sterile distilled water and allowed to stand until an even suspension was obtained then stored at 4°C until used in the next day.

2.7.3.2 **The procedure**

A primary dilution of 1/40 of all test and control sera was made by the addition of 25µl serum to 1ml of diluting buffer. The plate was prepared by addition of 80µl of the diluting buffer to all wells. A 20µl of each of the primary
diluted samples was added to all prepared wells. This gave a final dilution of 1/200. Columns 11 and 12 were left for the serum controls. A 20µl of the primary diluted positive control was added to each of the wells in column 11, and 20µl of the primary diluted negative control was added to each of the wells in column 12 except well H12 which was left without sample so as to blank the plate. The plate was then covered with the lid and incubated at 37°C for 1 hour. The content was then shaken out and the plate was rinsed 5 times with the washing solution and then thoroughly dried by tapping the plate on absorbent paper towel. The conjugate solution was then prepared by adding the content of the ampoule to 11ml of the previously prepared diluting buffer. A 100µl of the conjugate solution was added to all wells. The plate was then covered with the lid and incubated at 37°C for 1 hour. The content was then shaken out and the plate was rinsed 5 times with the washing solution and then thoroughly dried by tapping the plate on absorbent paper towel. The substrate solution was prepared immediately before use by addition of 300µl of ABTS (2, 2'-Azinobis {3-ethyl benzo thiazoline-6-sulfonic acid}-diammonium salt) chromogen to 12ml of substrate buffer plus 60µl of the substrate (hydrogen peroxide). Mixed well and a 100µl of it was added to all wells. The plate was then left at room temperature for 12 minutes. A 100µl of the stopping solution was then added to all wells. The plate was then read in the microtire plate reader at 405nm blanked on well H12. A positive/negative cut-off was calculated as 10% of the mean of the
optical density (OD) of the 8 positive control wells. Any test sample gave an OD equal to or above this value was considered as being positive.

2.7.4 Competitive ELISA


2.7.4.1 Preparation of reagents

2.7.4.1.1 Wash solution and Diluting buffer

The same as in indirect ELISA

2.7.4.1.2 Stop solution

The stop solution was prepared by adding the ampoule of citric acid to 30ml of distill water.

2.7.4.1.3 Conjugate

The conjugate was prepared by adding the content of the ampoule to 11ml of the diluting buffer.

2.7.4.1.4 Controls

Positive and negative controls were prepared by reconstituting the content of the ampoule in 1ml sterile distill water.

2.7.4.2 The procedure

A 20 µl of each test sample was added per well except columns 11 & 12 which were left for controls. 20 µl of the negative control was added to the wells A11, A12, B11, B12 and C12, and 20 µl of the positive control was added to the
wells F11, F12, G11, G12, H11 and H12. The remaining wells of columns 11 & 12 had no serum and were left for the conjugate controls. 100 µl of the immediately prepared conjugate solution was added to all wells. The plate was then vigorously shaken by the microtitre plate shaker for 2 minutes then covered with the lid and incubated at room temperature for 30 minutes on a rotary shaker at 160 revs/min. The content was then shaken out and the plate was rinsed 5 times with the wash solution and then thoroughly dried by tapping on absorbent paper towel. Immediately before use the substrate and chromogen solution was prepared by dissolving one tablet of urea H₂O₂ in 12 ml of distill water, when dissolved, one tablet of OPD was added and mixed thoroughly, and then a 100 µl of this solution was added to all wells. The plate was left at room temperature for 13 min. (the range is 10-15min.). A 100 µl of the stop solution was added to all wells. The plate was then read in the microtitre plate reader at 450nm.

2.7.5 Indirect ELISA for Milk

CHEKIT Brucellose Milk® (IDEXX LABORATORIES, Switzerland AG, Stationsstrasse 12.CH-3097 LIEBEFELD, SWITZERLAND, September 12, 2008).
2.7.5.1 Preparation of reagents

2.7.5.1.1 CHEKIT wash solution

CHEKIT-wash solution was prepared by adding 100 ml of CHEKIT-10x wash solution to 900 ml of distill water (1:10 dilution) then stored at refrigerator until used.

2.7.5.1.2 Control Milk

Positive control milk was prepared by adding 1ml of the positive control milk to 5ml sterile distill water.

2.7.5.2 The procedure

The positive control milk was diluted 1:4 in CHEKIT-wash solution. A 50 µl of CHEKIT-wash solution was dispensed to each well of the microtitre plate. The 50 µl of the diluted positive control was added to each well of the column 11, and 50 µl of the negative control milk was added to each well of the column 12 except well 12H which was used to blank the plate, 50 µl of each tested samples was added to the rest of the wells. The contents were then mixed by gentle shaking with microtitre plate shaker and then covered with the lid and incubated for 1 hr at 37 °C in a humid champer. The plate was then washed by adding 300 µl of the CHEKIT-wash solution to each well and rinsed three times then tapped on absorbent paper towel, after which a 100 µl of CHEKIT brucellose anti bovine-IgG-PO conjugate was dispensed into each well, and the plate was then covered with the lid and incubated at 37 °C for 1 hr in a humid
champer. The plate was then washed by adding 300 µl of the CHEKIT-wash solution to each well and rinsed three times then tapped on absorbent paper towel, and then a 100 µl of CHEKIT-TMB substrate was added to each well. The plate was then incubated at room temperature for 15 minutes. The reaction was then stopped by adding 100 µl of CHEKIT-Stop Solution TMB per well. The plate was then read using a photometer at a wave length of 450 nm.
Chapter three

Results

3.1 Clinical observations

Two cases of knee hygromas were observed in two cows in a slaughter house in El-Geneina province, and one case of aborted goat was observed at El-Geneina veterinary clinic, there was a history of abortions in some cattle herds years ago.

3.2 Smears

Only one out of the two hygroma fluids showed red stained short rods resembling \textit{Brucella} organisms when stained with MZN stain. Smears of the vaginal swab from an aborted goat showed no organisms resembling \textit{Brucella}. All smears from milk samples stained with MZN and Gram's stains showed no bacteria resembling \textit{Brucella}.

3.3 Serological tests

3.3.1 The Rose Bengal Plate test for serum

A total of 300 cattle sera were tested with RBPT, 31 (10.3\%) samples were positive, six (6\%) out of 100 serum samples from goats were positive, and seven (7\%) out of 100 serum samples from sheep were positive.
3.3.2 The Rose Bengal Plate test for hygroma fluids

The hygroma fluid that showed organisms resembling *Brucella* when stained with MZN stain reacted very strongly with RBPT but the other sample was weakly positive.

3.3.3 Indirect ELISA for serum

Only samples that were positive for RBPT were subjected to iELISA. 27 (9% to the total No.) out of the 31 RBPT-positive samples were positive for iELISA, and the other 4 were negative. No differences were observed between naked eye and microtitre plate reader in the reading process.

3.3.4 iELISA for hygroma fluids

Both hygroma fluids were found positive for iELISA.

3.3.5 Competitive ELISA for serum

Only samples that were positive for RBPT were subjected to cELISA. For cattle, 28 (9.3%) out of the 31 RBPT-positive samples were found positive for cELISA and the other 3 were negative. For goats, 3 (3%) out of the 6 were positive and 3 negative. For sheep, 5 (5%) out of the 7 were positive and 2 were negative.

3.3.6 Competitive ELISA for hygroma fluids

Both hygroma fluids were found positive for cELISA.

3.3.7 Milk Ring test

Of the total 200 cattle milk samples, 23 (11.5%) were positive.
3.3.8 **Indirect ELISA for milk**

Only samples that were positive for MRT were subjected for iELISA; 18 of the 23 MRT-positive samples were positive for iELISA, the other 5 were negative.

3.4 **Distribution of positive reactors**

The distribution of positive reactors to the serological tests among El-Geneina and Furbranga provinces showed slight differences, however, more seropositive reactors were found in Furbranga province. (Tables: 6, 7 and 8).

3.5 **Isolation of the organism**

All attempts to isolate *Brucella* from the 200 milk samples, two hygroma fluids and one vaginal swab had failed.
Table 5: Results of brucellosis tests in West Darfur state

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of sample</th>
<th>No. of samples</th>
<th>RBPT (+)</th>
<th>iELISA (+)</th>
<th>iELISA (-)</th>
<th>cELISA (+)</th>
<th>cELISA (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Serum</td>
<td>300</td>
<td>31 (10.3%)</td>
<td>269 (89.7%)</td>
<td>27 (9%)</td>
<td>273 (91%)</td>
<td>28 (9.3%)</td>
</tr>
<tr>
<td></td>
<td>Hygroma fluid</td>
<td>2</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>0 (100%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Serum</td>
<td>100</td>
<td>7 (7%)</td>
<td>93 (93%)</td>
<td>-</td>
<td>-</td>
<td>6 (6%)</td>
</tr>
<tr>
<td>Goats</td>
<td>Serum</td>
<td>100</td>
<td>6 (6%)</td>
<td>94 (94%)</td>
<td>-</td>
<td>-</td>
<td>3 (3%)</td>
</tr>
</tbody>
</table>

Table 6: Results of brucellosis tests in El Geneina locality

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of sample</th>
<th>No. of samples</th>
<th>RBPT (+)</th>
<th>iELISA (+)</th>
<th>iELISA (-)</th>
<th>cELISA (+)</th>
<th>cELISA (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Serum</td>
<td>150</td>
<td>14 (9.3%)</td>
<td>136 (90.7%)</td>
<td>12 (8%)</td>
<td>138 (92%)</td>
<td>12 (8%)</td>
</tr>
<tr>
<td></td>
<td>Hygroma fluid</td>
<td>2</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>0 (100%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Serum</td>
<td>50</td>
<td>2 (4%)</td>
<td>48 (96%)</td>
<td>-</td>
<td>-</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Goats</td>
<td>Serum</td>
<td>50</td>
<td>3 (6%)</td>
<td>47 (94%)</td>
<td>-</td>
<td>-</td>
<td>1(2%)</td>
</tr>
</tbody>
</table>


Table 7: Results of brucellosis tests in Furbranga locality

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of sample</th>
<th>No. of samples</th>
<th>RBPT (+)</th>
<th>RBPT (-)</th>
<th>iELISA (+)</th>
<th>iELISA (-)</th>
<th>cELISA (+)</th>
<th>cELISA (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Serum</td>
<td>150</td>
<td>17 (11.3%)</td>
<td>133 (88.7%)</td>
<td>14 (9.3%)</td>
<td>136 (90.7%)</td>
<td>15 (10%)</td>
<td>135 (90%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Serum</td>
<td>50</td>
<td>5 (10%)</td>
<td>45 (90%)</td>
<td>-</td>
<td>-</td>
<td>3 (6%)</td>
<td>47 (94%)</td>
</tr>
<tr>
<td>Goats</td>
<td>Serum</td>
<td>50</td>
<td>3 (6%)</td>
<td>47 (94%)</td>
<td>-</td>
<td>-</td>
<td>2 (4%)</td>
<td>48 (96%)</td>
</tr>
</tbody>
</table>

Table 8: Results of MRT & iELISA for cattle milk

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Locality</th>
<th>No. of samples</th>
<th>MRT (+)</th>
<th>MRT (-)</th>
<th>iELISA (+)</th>
<th>iELISA (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>West Darfur</td>
<td>200</td>
<td>23 (11.5%)</td>
<td>177 (88.5%)</td>
<td>18 (9%)</td>
<td>182 (91%)</td>
</tr>
<tr>
<td></td>
<td>El Geneina</td>
<td>100</td>
<td>11 (11%)</td>
<td>89 (89%)</td>
<td>7 (7%)</td>
<td>93 (93%)</td>
</tr>
<tr>
<td></td>
<td>Furbranga</td>
<td>100</td>
<td>12 (12%)</td>
<td>88 (88%)</td>
<td>11 (11%)</td>
<td>89 (98%)</td>
</tr>
</tbody>
</table>
Chapter four

Discussion

This study on brucellosis in cattle, sheep and goats was carried out in West Darfur state which owns considerable numbers of livestock. Although it was the second study in this state, after the work of Musa (1995), however, it was the first one in El Geneina and Furbranga provinces. The state, as one of the Great Darfur states, has been affected by an armed conflict since 2003. Therefore, the animal husbandry systems (nomadic, semi-nomadic and sedentary) were severely affected and became very difficult to classify rearing animals according to these systems.

In this study, the overall prevalence of brucellosis in cattle, sheep and goats as detected by RBPT was 10.3%, 7% and 6% respectively. It was higher for cattle and sheep in Furbranga province (11.3% and 10% respectively) compared to that found in El Geneina province (9.3% and 4% respectively). This could be attributed to the fact that Furbranga has a very large numbers of livestock and posses one of the biggest livestock market in the country to which animals from different parts of the state are gathered. But, it has few pastures for grazing resulting in high animal concentration in them. In addition, Furbranga is close to Republic of Chad and there is a large transboundary movements of animals between the two countries and these factors facilitate spread of diseases
including brucellosis. In goats, the prevalence of brucellosis was similar (6%) in the two provinces because goats appear to be less affected by these factors.

The prevalence rate of cattle brucellosis recorded in this study (10.3%) appears to be less than that reported by Musa (1995), who found 13.9% prevalence rate in the Great Darfur state, and this could be attributed to the fact that Musa studied very large number (7375) of animals compared to the number tested in this study (300). He reported a prevalence rate of 10.2% in West Darfur state (Wadi Saleh and Zalingi provinces) which is in close agreement with that reported by this study (10.3%).

Comparable rates for brucellosis in cattle were reported in Gezira state (8.7% and 10.7%) by Dafalla (1962) and in Blue Nile state 8.7% by Mustafa and Hassan, (1969), but higher rates have been reported in Khartoum state 23.1% by Khalid (2006) and in North Kordofan state (14.2% and 66.7%) by Ibrahim and Habiballa (1975). Animals are usually kept in intensive systems in Khartoum state which increase the chances of contamination and spread of brucellosis, Kordofan state is considered to be a point of cross-transition of nomads from different parts of the country which facilitate the spread of the disease between animals.

The prevalences of brucellosis in sheep and goats in West Darfur state were 7% and 6%, respectively. For goats, the rate is comparable to that reported by Musa (1995) (5.98%), but for sheep, the rate obtained in this study (7%) was higher than that (3.5%) recorded by Musa (1995) this could be attributed to the
fact that, sheep are mostly reared in a nomadic or semi-nomadic system and due to the armed conflict they migrated and condensed inside towns which facilitate the spread of the disease, whereas goats were less affected by the conflict because they are normally kept around and inside towns and this is the situation when Musa (1995) did his study.

Prevalence of brucellosis in sheep and goats has also been reported in other parts of the country by many researchers. In the Gezira state, Dafalla (1962) reported 4.2% and 2.5% respectively, in North Sudan, Abdalla (1964) reported 1.7% and 1.5% respectively, and Fayza et al. (1990) reported 0.01% and 0.13% respectively.

In this study, four types of serological tests were used, namely RBPT, MRT, indirect ELISA and competitive ELISA. RBPT and MRT were used as screening tests for Brucella antibodies in serum and milk respectively. While iELISA and cELISA were used only to confirm the positive results obtained by the former tests.

Among the 300 cattle, 200 were sampled both for serum and milk. RBPT has detected 22 (11%) positive samples whereas MRT has detected 23 (11.5%) positive samples, this shows that MRT was slightly more sensitive than RBPT (11.5% Vs 11%) and when the RBPT was compared to MRT, statistical analysis demonstrated RBPT had 95.7% sensitivity. Similar finding was found by Nagi (2009) who found 95.2% sensitivity for RBPT when compared to cELISA.
Thirty-one bovine positive sera were detected by RBPT, when subjected to the confirmatory tests used in this study, iELISA detected 27 positive samples and cELISA detected 28 positive samples, so the RBPT had 87% and 90.3% specificity when compared to iELISA and cELISA, respectively. The RBPT was also found to have specificities of 85.7% in sheep and 50% in goats when compared with cELISA. Comparable results were reported by many researchers, but Diaz-Aparicio et al., (1994) reported 100% sensitivity and 100% specificity for RBPT in goats infected experimentally with *Br. melitensis*, the same result also reported by Marin et al., (1999) in sheep infected experimentally with *Br. melitensis*.

Twenty-three bovine positive milk samples were detected by MRT, when subjected to milk-ELISA, 18 were found positive and MRT was found to have 78.2% specificity when compared to iELISA.

Attempts to isolate *Brucella* from samples of hygroma fluids from cattle and one vaginal swab from an aborted goat had failed. This could be attributed to the delay in the isolation attempts as the samples were preserved for a long time in the freezer during which the power was unstable and might have had subjected the samples to freezing and thawing. Failure to isolate *Brucella* organisms from seropositive animals had been encountered by other researchers, for instance, Raga (2000); Hyfa (2001) and Rias (2005).
Conclusions & Recommendations

Conclusions

• According to this study, it could be concluded that, the prevalence of brucellosis in cattle, sheep and goats in West Darfur state is similar to that reported in the other parts of the country.

• Sheep were the most species affected by armed conflicts.

• Close vicinity to Republic of Chad resulted in higher incidence rates of brucellosis (Furbranga province) due to free animal movement.

Recommendations

• Numbers of samples used in this study were too small compared to the animal populations sampled, so, it's recommended that, samples sizes should be representatives in further researches.

• Due to lack of public health awareness and extension programs in this area, work should be directed to human brucellosis to evaluate the impact of the disease on the public health.

• Vaccination programs should be attempted to control the disease.

• There should be co ordinations with the related authorities in the Republic of Chad to determine the magnitude of spread of the disease in the areas around the border to adopt effective control programs in these areas.

•
References


Hughes, L. (1893). Su rune formede fievre frequente sur less Cotes de La Mediterranee. Annles de l' Institut Pasteur, paris, 628-639.


