Sudan University of Science and Technology (SUST)

College of Graduate studies

A Study on Molecular Epidemiology of *Brucella* Species Isolated in Sudan and Exploring Knowledge, Attitudes and Practices Related to Acquisition of Brucellosis

دراسة الوبائية الجزيئية لميكروب البروسيلا المعزولة في السودان مع دراسة المعرفة والموقف والممارسات المرتبطة بداء البروسيلوزيس

By

Adil Abdel Rahman Ali Ismail

B.Vet.Sc (1998);MSc (2007) University of Khartoum.

A thesis Submitted in Fulfilment of the Requirements of the Degree of Doctor of Philosophy (PhD) in Veterinary Preventive Medicine (Molecular Epidemiology)

Supervised by

Professor Dr. Tamador El-Khansaa Elnour Angara

Co-supervisors

Professor Dr. Mohamed Abdel Salam Abdalla

Dr. Enaam Mohamed El Sanousi

Septemper 2019

Sudan University of Science & Technology

College of Graduate Studies

بسم الله الرحمن الرحيم

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

كلية الدراسات العليا

Approval Page

(To be completed after the college council approval)

Name of Candidate: Adil | Abdelrahman | Ali | Ismail

Thesis title: A study on Molecular Epidemiology of Brucella species Isolated From Sudan and Exploring Knowledge, Attitude and Practices Related to Acquistion of Brucellosis -

Degree Examined for: Ph.D Veterinary Preventive (Molecular epidemiology)

Approved by:

1. External Examiner

Name: Khitma Hassan Elmalik

Signature: ..... Date: 18/7/2019

2. Internal Examiner

Name: Sihan Elias Sulaiman Mohammed

Signature: ..... Date: 18/7/2019

3. Supervisor

Name: Tamador Elkhansaa Elnour Angara

Signature: ..... Date: 18/7/2019

DEDICATION

I dedicate this work to my parents, my wife and my daughter, my brothers and sisters for their encouragement, assistance and love.

ACKNOWLEDGEMENTS

I cordially acknowledge the endless support and guidance from my supervisor Prof Tamador El-Khansaa Elnour Angara. Without her sincere and unconditional support, along with my co-supervisors, this work would have not been achieved. My thanks and gratitude goes to the personnel of the Department of Veterinary Tropical Diseases (DVTD, South Africa, Pretoria), particularly prof. Henriette van Heerden, Betty Ledwaba, Barbara, Ayesha Hassim, Dr. Nicola Collins, Bossie Bosman for training, samples processed and analysis. Likewise, many thanks to the personnel of the Brucella Unit of the Central Veterinary Research Laboratories (Maha Khogali, Salah, Maha Mohamed, Nadia) and Perret LL (UK) for assistance in isolation and biotyping of isolates. I would like to praise the acceptance of farmers and workers in the visited farms to participate in this study. The financial support from the Ministry of High Education is highly appreciated. This work is a part of a research project entitled "Compliance with World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures by formulating a long term Animal brucellosis control strategy in Khartoum State" sponsored by Sudan University of for Science and Technology (Deanship of Scientific Research).

Special gratitude goes to people owning or working in cattle dairy farms in the study area, who generously volunteered to participate and give hand and wishing this research to contribute to solve the problem of brucellosis in Sudan.

ABBREVIATION

AMOS: Brucella AbortusMelitensisOvisSuis

Bruce-Ladder: Brucella species specific PCR

CVRL: Central Veterinary Researches Laboratories

DNA: Deoxyribonucleic acid

FAO: Food and Agriculture Organization of the United Nations (UN)

GT(s): Genotype(s).

IS: Insertion sequence.

ITS: interspacer insertion sequence

KAP: Knowledge, attitude and practices.

MLVA: multilocus variable number tandem repeats (VNTR) analysis.

OIE: World Organization for Animal Health.

PCR: Polymerase chain reaction.

PFGE: pulse-field gel electrophoresis.

RNA: ribonucleic acid

rRNA: ribosomal RNA

SNPs: single nucleotide polymorphism.

SPSS: statistical package for the social science.

VNTR (s): Variable number tandem repeat(s).

ABSTRACT

The objectives of this study were to identify biotypes and genotypes of *Brucella* organisms isolated from Sudan and to assess participants' knowledge, attitude and practices (KAP) related to the transmission of brucellosis to animals and humans in Khartoum state.

Convenient sampling was performed (March-December, 2015) to select the study subjects based on sero-positivity for brucellosis and consent. Fourteen *Brucella* cultures were isolated from 541 milk of sero-positive cows representing 127 dairy herd from Khartoum state. Further six *Brucella* strains (three *Brucella abortus* and biovar 1, 3, 6 three *Brucella melitensis* biovar 1, 2 and 3) identified at the Brucella Unit/ Central Veterinary Research laboratories (CVRL) –Soba in (2005-2014)were inculded in the study. Brucellae from this study along with the CVRL strains were used to identify the genotypes of *Brucella* in Sudan. The study also involved filling a structured-questionnaire by interviewing 150 farmers and workers about their KAP related to brucellosis.

Molecular characterization of *Brucella* organisms was performed using the *Brucella* species-specific Bruce-ladder PCR. Seventeen *B. abortus* strains and three *B. melitensis* strains were confirmed, while the AMOS-PCR, among all, identified the three *B. melitensis* strains and only two of the *B. abortus* strains as S19 vaccine strain. These 20 *Brucella* strains in this study were genotyped by the multiple-locus variable number tandem repeats (VNTR) analysis (MLVA16) scheme. Five genotypes (GTs) within panel1 (MLVA8) and six GTs within panel2A (MLVA11) and thirteen GTs within the MLVA16 were revealed including ten *B. abortus* GTs and three *B. melitensis* GTs. The *B. abortus* strains from Sudan (n=17) grouped in closely related small clusters of identical genotypes with other strains from Africa like Nigeria, Tchad, Kenya, Uganda, Morocco, Algeria and other continents like Brazil, Portugal, Spain and Syria. *B. melitensis* bv 1 strain in this study clustered with the American group of *B. melitensis*, and *B. melitensis* bv 2 and 3 clustered with the East Mediterranean group of *B. melitensis*

and all Sudan strains were new genotypes. *B. abortus* GTs circulating in livestock from Sudan in the current study are closely related to GTs from neigbouring countries, which indicate possible spread of brucellosis between Sudan and these countries. Furthermore, *B. melitensis* GTs in this study sharing genetic bonds with *B. melitensis* GTs from United Arab Emirates and Somalia.

On the other hand, analysis of KAP relating to brucellosis revealed low and poor understanding of the zoonotic nature of the disease.

Based on the findings of our and previous studies, it seems that the *B. abortus* bv 6 is the predominant cause of bovine brucellosis in this country, and that brucellosis might have spread between Sudan and neighboring countriess.

The study recommends a countrywide surveillance to isolate and genotype circulating *Brucella* strains to have further insight on brucellosis in Sudan. Bruce-ladder PCR would be better option for characterization of *Brucella* in Sudan, as the AMOS PCR could not identify most of the *B. abortus* strains that predominant in Sudan. Awareness of stakeholders about brucellosis and people in contact should be raised.

We believe that the findings of this study can contribute to and promote brucellosis control in Sudan.

المستخـــلص

هدفت هذه الدراسة الي توصيف الانماط الحيوية والوراثية لميكروبات البروسيلا في السودان مع دراسة معرفة وموقف وممارسات (knowledge, attitude and practices "KAP") المشاركين في الدراسة و المرتبطة بمرض البروسيلوزيس في ولاية الخرطوم.

تم اختيار مجتمع الدراسة بناءً على الموافقة على المشاركة في الدراسة مع إيجابية الفحص المصلي للبروسيلا. حيث تم الحصول على 14عزلة لميكروب البروسيلا المجهضة من 541 عينة لبن جمعت من 127 مزرعة البان بولاية الخرطوم و6 عينات اخرى (3 بروسيبلا مجهضة عترة 1، 3، 6 و3 بروسيلا مليتنسيس عترة 1، 2 و3) تحصل عليها من وحدة البروسيلا "معمل الابحاث البيطرية-سوبا -CVRL" والتي تم عزلها خلال الفترة (2005-2014). تم تصنيف عزلات البروسيلا في هذه الدراسة على انها من نوع البروسيلا المجهضة النمط الحيوي 6غير التقليدي و استخدمت مع تلك المتحصل عليها من CVRL لتحديد الأنماط الجينية للبروسيلا في السودان. شملت الدراسة أيضًا ملأ 150 استمارة للمزارعين والعمال عن KAP المرتبطة بداء البروسيلات.

تم إجراء التوصيف الجزيئي لميكروبات البروسيلا باستخدام اختبار البلمرة المتسلسل - Bruce ladder PCRالخاصة بأنواع البروسيلا حيث أكد ان سبعة عشرة عزلة هي بروسيلا مجهضة وثلاثة بروسيلا مليتنسيس، بينما اختبار AMOS - PCR (AbortusMelitensisOvisSuis) الخاص بالبروسيلا ايضأ تمكن من التعرف على الثلاثةعترات بروسيلا مليتنسيس وعترتين فقط من البروسيلا المجهضة والتي صنفنها بانها عترة اللقاح S19. تم اجراء التوصيف الجيني لهذه الـ 20 عينة من ميكروبات البروسيلا بواسطة مخطط تحليل التكرار المتزامن (VNTR) متعدد المواقع (MLVA). حيث كشف التحليل عن وجود خمسة أنماط وراثية ضمن الحزمة 1 (MLVA8) و 6 نمط وراثي ضمن الحزمة 2أ (MLVA11) وثلاثة عشرة نمط وراثي ضمن الحزمة MLVA16، ويشمل ذلك عشرة انماط وراثية من البروسيلا المجهضة وثلاث انماط وراثية من البرويلا مليتنسيس. اوضح هذا التحليل بأن عينات البروسيلا المجهضة من السودان (ن = 17) تجمعت في مجموعة واحدة تضم مجموعات صغيرة من الأنماط الوراثية المتماثلة عزلت في دول من افريقيا مثل نيجيريا وتشاد وكينيا وأوغندا والمغرب والجزائر وقارات أخرى مثل والبرازيل والبرتغال واسبانيا وسوريا. لقد تجمعت عينات بروسيلا مليتنسيس النمط الحيوي 1 من السودان مع المجموعة الأمريكية بينما تجمعت البروسيلا مليتنسيس النمط الحيوي 2 و 3 مع المجموعة الشرق اوسطية وجميع عينات بروسيلا مليتنسيس من السودان هي انماط وراثية جديدة. تؤكد نتائج الدراسة الحالية أن الانماط الوراثية للبروسيلا المجهضة المتناقلة بين قطعان الثروة الحيوانية في السودان ترتبط ارتباطًا وثيقًا بانماط وراثية من بلدان مجاورة، مما يشير إلى احتمال انتشار داء البروسيلات بين السودان وهذه البلدان. بالمقابل فإن عترات البروسيلا مليتنسيس في هذه الدراسة ترتبط جينياً بانماط وراثية من بروسيلا مليتنسيس في الإمارات العربية المتحدة والصومال.

من جانب اخر كشفت نتائج تحليل بيانات المعرفة والموقف والممارسات المرتبطة بداء البروسيلا عن ضعف المعرفة بطبيعة المرض كونه مرض مشترك بين الحيوان والانسان.

بناءا على نتائج هذا البحث وابحاث سابقة خلصت هذه الدراسة الى ان النمط الحيوي 6 من البروسيلا المجهضة هو المسبب الاساسي لداء البروسيلا البقري في السودان والدول المجاورة.

توصي الدراسة بإجراء دراسات على المستوى القومي لعزل عترات البروسيلا الموجودة في السودان لتكوين رؤية أكثر وضوحا عن داء البروسيلات في السودان. كما يعتبراختبار الحمض النووي Bruce-ladder PCR خيارًا أفضل لاجراء التوصيف الجزيئي لميكروبات البروسيلا في السودان بالمقارنة مع الاختبارات الاخرى. توصي الدراسة ايضاً برفع وعي أصحاب المصلحة والاشخاص المحتكين بالحيوان باهمية داء البروسيلا.

نعتقد أن مخرجات هذه الدراسة يمكن أن تسهم بفعالية في البرامج المستقبلية لمكافحة داء البروسيلا في السودان.

Table of contents

List of tables

Table of figures

INTRODUCTION

Brucellosis is a worldwide zoonotic disease affecting mainly sexually mature animals. The disease name "brucellosis" is a collective term that refers to the disease syndromes caused by bacteria of the genus Brucella, commonly characterised by epizootic abortions, chronic endometritis, infertility and arthritis in females, orchitis and epididymitis in males (Constable *et al*., 2017). In humans, the disease has protean manifestations with variable fevers (spiking and accompanied by rigors, intermittent or undulant "relapsing" or mild), malodorous perspiration (pathognomic) and localised chronic infections frequently manifested by peripheral arthritis, spondylitis, sacroiliitis, hepatomegaly, splenomegaly and lymphadenopathies (Pappas and Papadimitriou, 2007).

Although brucellosis was eradicated from parts of the world (Australia, New Zealand, Japan, and some north European countries), the disease remained endemic in Mediterranean area, Africa, South and North America (McDermott *et al*., 2013). Eradication of brucellosis is a costly intervention that explains the endemicity of the disease in most developing countries including Sudan.

Bovine brucellosis (the most globally occurring brucellosis) principally caused by *B. abortus*, and caprine/ovine brucellosis primarily caused by *B. melitensis* and *B. ovis* (Constable *et al*., 2017). Although infection often associated with host preference, some strains of *B. abortus* found to infect other animal species in Sudan such as camels (Musa *et al*., 2008) and sheep (Gumaa *et al*., 2014), while *B. melitensis* was also isolated from camels, cattle (Musa *et al*., 2008) and Man (Osman *et al*., 2015). Currently there are twelve *Brucella* spp. described. These, beside *B. abortus* and *B. melitensis*, include B. suis "pigs, reindeer, caribou, hares", *B. canis* "dogs", *B. neotomae* "desert wood rat", *B. microti* "common vole and foxes", *B. pinnipedialis* and *B. ceti* "marine mammals", B. inopinata "unknown source" and *B. papionis* "Baboons; babio sp" (Whatmore, 2016).

Although brucellosis expected to have been exiting in Sudan for unknown period, "maybe when man contacted with animals" nevertheless, the first isolation of *B. abortus* was reported in 1943 (Bennet, 1943) from the north province and *B. melitensis* in 1957

from El Gazira area “Central Sudan”. Later, several studies reported the occurrence of brucellosis from various regions in Sudan (Elnasri, 1960, Abdulla, 1966; Habiballa *et al*., 1977; Bakheit, 1981; McDermott *et al*., 1987; Musa *et al*., 1990; Musa and Jahans, 1990; Agab *et al*., 1994; Musa and Shigidi, 2001; Abbas and Agab, 2002; Ismail, 2007; Musa *et al*., 2008; Angara *et al*., 2014 and 2016; Omran and Musa, 2015; Abdalla and Baleela, 2017). According to the available/published data, *B. abortus* and *B. melitensis* were the only species currently found circulating among livestock populations in Sudan.

Human brucellosis exists where the disease is enzootic. In Sudan, human brucellosis in this country was reported earlier in 1908 (Haseeb, 1950). The author stated that the annually reported number of human brucellosis was 50 patient mostly from Kassala province (eastern state). Most reported cases of human brucellosis in Sudan were mainly through survey studies conducted among at high-risk people (i.e. veterinarians, animal owners, animal health workers, slaughter house workers…etc.), while very few studies have addressed the disease at the community level. Mustafa and Hassan, (2010) surveyed the disease among patients with febrile illness seeking health care at the main hospitals in Khartoum state and reported 8.9% seropositive reactors for brucellosis. Earlier on, Mohd (1989) reported 76% prevalence of human brucellosis from the Gazira area however; the author referred the high prevalence to the small sample size (29 participants).

Before 2011, Sudan and South Sudan consisted of one country. Nevertheless, Sudan is still among the largest African countries owing 1 765 048 km2 with 14 636 770 people (of whom 35.3 % are urban) (Worldometers, 2018). Sudan livestock population estimate in 2017 was 107 555 thousand heads consisted of 40 612 sheep, 31 481 goats, 30 632 cattle and 4 830 camels (WAHID, 2017). This estimate is the second largest livestock inventories in Africa, next to Ethiopia.

The great number of all livestock animals comes from smallholders and migratory producers. The production systems range from pure nomadic to more settled system for milk production in the urban areas (Philipsson, 2000). Livestock sector estimated to contribute to around 55% to the agricultural value added (FAO, 2005). The farming

system in terms of rural population includes transhumance, nomadic and sedentary agriculture comprising over 90% of animal population (FAO, 2005). These facts emphasize the importance of livestock as a crucial component for Sudanese economy and that it has contributed significantly to food security and rural employment. In addition, beside their economic value, domestic animals also have played an important role in many socio-cultural traditions.

Livestock movement is an important vehicle for disease transmission. Sudan trades live animals and animal products with neighbouring countries and beyond, through unofficial cross-borders (though not well documented) animal movement (IGAD, 2013). Livestock animals are reported to be imported by Sudan from New Zealand, Netherlands, Saudi Arabia, United Arab Emirates, Egypt, France, Jordan, Brazil, Turkey, Portugal and China while exported mainly to Saudi Arabia, United Arab Emirate, Egypt, France, Jordan and Turkey in 2015 (Worldometers, 2018).

Economic losses resulting from bovine brucellosis are mainly due to abortions, infertility, calf mortality and a drop in milk production (McDermott and Arimi, 2002). Domenech *et al*., (1983) estimated in Central Africa the economic impact of the disease amount at 5.8% of the income per animal. In Sub-Saharan Africa, additional milk and meat offtake potential for livestock keepers, after elimination of brucellosis estimated to lie in the range of US$ 2.6 -US$ 12.9 per animal/year in smallholder dairy system and in the range of US$ 0.70 - US$ 4.5 per animal/year in traditional livestock system (Mangen *et al*., 2002). In Mongolia, a scenario of 52% reduction of brucellosis transmission between animals achieved by mass vaccination resulted in a net value of US$ 18.3 million and an average benefit-cost ratio for society of 3.2 (Roth *et al*., 2003). Furthermore, Angara *et al*., (2016) reported economic loss due to bovine brucellosis in Khartoum state to be little more than $US 7 million as annual loss. The authors concluded that the disease constitutes a serious economic burden to the economy of the state and the producer in the absence of a formal control strategy.

Isolation and identification of brucellae is the gold standard approach in the way forward to understand the epidemiology and eventually control and/or eradicate

brucellosis in a given setting. In Sudan, some *B. abortus* and *B. melitensis* biovars were previously identified, however, until the moment of writing this report there is no unanimous countrywide survey performed to isolate all possibly circulating biovars.

Contrary to conventional microbiological (biotyping) methods, the use of PCR-based and advanced molecular tools have received much acceptance to be used for characterization and to conduct epidemiological investigation of brucellosis. These tools have the merits of high sensitivity and specificity superseding biotyping approach. In addition, the can provide epidemiological information relating isolates to geographic location of outbreak(s) (Le Fleche *et al*., 2006; Al Dahouk *et al*., 2007). In the current study, we applied both microbiological and molecular approaches to serve achieving the objectives set in this research.

Eradication of bovine brucellosis has been achieved in many rich developed countries such as Australia, Canada, Israel, Japan and New Zealand and in many European countries (Gul and Khan, 2007). However, the disease remained uncontrolled in some areas such as Africa, the Middle East and Asia (McDermott *et al*., 2013), where the disease is still endemic. In these settings, where the disease is still endemic, factors such as low awareness, poor understanding of brucellosis and absence of control policies along with limited resources could be the main culprits. The awareness and understanding of brucellosis among relevant stakeholders is an important asset for the success in this business such as prospective brucellosis control strategy. Therefore, this research involved an approach to investigate the extent of knowledge, attitude and practices related to brucellosis among people owning or working in dairy cattle farms in study area. We believe that the findings of our study will contribute to brucellosis control programs and one-health interventions in the future in Sudan.

The aims of the current study were to characterize *Brucella* spp. isolated during this study and previous studies (2005-2015) using microbiological and molecular techniques and to investigate epidemiological relationships among *Brucella* spp. isolated from Sudan and elsewhere.

Brucellosis is endemic in Sudan and there is no currently applied control program at national level along with the lack of comprehensive data on brucellosis reflecting the situation in the whole country. Therefore, a nation-wide survey to investigate the circulating biovars and genotypes of *Brucella* spp. in Sudan, though not conducted in this study, is required. The available data showing the limited utilization of molecular techniques to characterize *Brucella* to the genus and species level, which proofed useful and fast compared to conventional biotyping approach (Bricker *et al*., 2003). Until the time of writing this report, there is only few genetic data (only two strains reported in two different studies), on *Brucella* species and biovars isolated in Sudan and their genetic bonds with strains at the regional and global level. In this study, we would like to dig deep and provide more insights on the epidemiology of brucellosis in Sudan following microbiological and molecular approaches. Further, the awareness and understanding of brucellosis among farmers and farm workers is indeed very important for control and eradication policies. Therefore, qualitative research is required to explore the extent of knowledge, attitude and practices (KAP) relating to brucellosis among farmers and people working in close contact with animals.

Research Objectives

1. To isolate and identify *Brucella* organisms to species and biovar level using microbiological methods.
2. To identify *Brucella* isolates (in this study and those isolated previously in Central Veterinary Research laboratories “CVRL” since 2005) using genus-specific PCR assays (such as ITS-PCR), species-specific/biovar (such AMOS, Bruce-ladder) and recommend suitable options for Sudan.
3. To identify the circulating *Brucella* genotypes within isolated strains in this study and those from the CVRL collection since 2005, and to explore genetic relationships with other *Brucella* spp. using published database of the Multi locus Variable Number Tandem Repeats analysis (MLVA) genotyping system for *Brucella*.

4. To explore the KAP related to brucellosis among farmers and farm workers using structured questionnaire.

Research hypothesis

The current research hypothesizes the following:

1. *Brucella abortus* biovar 1 or 6 are the predominant biovars causing animal brucellosis in Sudan.
2. Circulating *Brucella* genotypes in livestock herds in Sudan share genetic bonds with those from neighbouring countries that trade animals with Sudan.
   Farmers and farm workers have low knowledge about brucellosis.

CHAPTER ONE

1 LITERATURE REVIEW

1.1 Historical Background

Brucellosis is a worldwide zoonotic ancient disease. Although the disease might have existed since human-animal contact begun, however, it was only known late in the nineteenth century when the bacteria responsible for the infection were identified. This discovery in the last decade of the 19th century created awareness of the disease in the western world, which indeed a credit to Sir David Bruce who isolated the agent responsible for "Malta" or "remittent" fever (Corbel and Banai, 2005).

Recent advances in paleoepidemiology and paleopathology have presented evidences of brucellosis far long before the work of the 19th century. This evidences were obtained by investigation of debris engulfing skeletal remains of the Roman residents of Herculaneum (Napes, Italy) killed by catastrophic volcanic eruption of Mt. Vesuvius in late August AD 79 (Capasso, 2002). These discoveries revealed vertebral bone lesions typical of brucellosis in more than 17% of the residents. Scanning electron microscopy of recovered cheese provided a likely explanation for the high incidence of the disease. Moreover, buried carbonized cheese made from sheep's milk found with bones revealed the presence of cocco-bacillary forms that were morphologically similar to *Brucella* spp. (Capasso, 1999; Capasso, 2007).

The breakthrough achieved by Sir David Bruce remarked by isolation of *micrococcus melitensis* "now *Brucella melitensis*" from spleen of a British soldiers (who died from febrile illness "now Malta Fever" common among military personnel stationed on Malta "an island not far from Herculaneum") occurred in eighteen centuries later after the Mt. Vesuvius volcanic event (Godfroid *et al*., 2005). A draft genome sequence of a ~ 700 years-old strain of *B. melitensis* from human remains from Sardinia (Italy) was found to genomically cluster with *B. melitensis* biovars 3 Ether (ATCC 23458), a strain currently circulating in Italy (Kay *et al*., 2014). The second species in

the genus, *Brucella abortus*, which was isolated in 1897 by a Danish veterinarian Bernhard Bang from abortion materials of dam and named it *Bacillus abortion*.

Despite the relatively early recovery of *Brucella* spp. from humans by Bruce in 1887, however; the zoonotic nature of disease only accidentally demonstrated in 1905 by isolating *Brucella melitensis* from goat's milk used for the production of soft cheese in Malta (Nicoletti, 2002; Godfroid *et al*., 2005). Although at that time goats were not believed to be the source of infection since they do not become ill, when inoculated with *Brucella* cultures. The discovery that healthy goats could be carriers of the disease has been termed one of the greatest advances ever made in the study of epidemiology of brucellosis and marking the first knowledge on the zoonotic nature of the disease (Wyatt, 2005). The group *Brucella* was acknowledged and came to existence when Evans *et al*. (1918) demonstrated the close relationship between the *Micrococcus melitensis* and Bang's *Bacillus*, and brought them in the genus named *Brucella* to honour Sir David Bruce (Sriranganathan *et al*., 2009).

1.2 Economic Importance Of Brucellosis

Brucellosis is consistently ranked among the most economically important zoonoses globally. It is a 'multiple burdens' disease with economic impacts attributable to human, livestock and wildlife disease (McDermott *et al*., 2013). The epidemiology and economic impact of brucellosis vary by geography and livestock system. In many high-income countries, brucellosis has been successfully controlled or eliminated in livestock populations. Where it persists, wildlife populations have become the main reservoirs "e.g. bison and elk in North America" (Zinsstag *et al*., 2007). Economic impacts vary depending on the main livestock species, management systems, and on the capacity of the country's veterinary and medical systems (Zinsstag *et al*., 2005) mentioned that brucellosis can be a serious economic disease is unquestioned. Losses due to abortions or stillbirths, irregular breeding, loss of milk production and reduced human productivity are economic consequences. The reduced human productivity can hardly be measured in medical care. He referred to Shepherd *et al*., (1979) estimates of US$ 3,206 for each case. He also argues that quantitative estimates of the effects of disease

on productivity of livestock are essential for justification of organized programs. He concluded that there was an internal rate of 10.27% return on costs of program to eradicate cattle brucellosis. These data were based upon assumption that infected non-aborting dairy cows produced at 10% below potential and aborters at 20%. He further estimated that 10-35% of infected cows abort each year. The economic loss from brucellosis in developed countries arises from the slaughter of cattle herds that are infected with brucellosis. In developing countries loss arises from the actual abortion of calves and resulting decreased milk yield, birth of weak calves that die soon after birth, retention of placenta, impaired fertility and sometimes arthritis or bursitis (Constable *et al*., 2017). It is difficult to estimate the financial loss caused by brucellosis, as it depends on the type of cattle farming, herd size, and whether it is an intensive or extensive cattle farm. Furthermore, although it is very difficult to estimate the financial loss incurred by human brucellosis although there is no doubt that it is substantial (Robinson, 2003).

1.2.1 Brucellosis in sudan

In Sudan, brucellosis was proved to be enzootic since 1943, when *B. abortus* was isolated for the first time by Bennet (1943) from a dairy farm in Khartoum province. The disease diagnosed in humans earlier than in Berber, northern part the Sudan, since 1904 (Haseeb, 1950). Previous estimates of economic losses caused by brucellosis have been based on a combination of common sense and limited information. The greatest prevalence in Sudan is found in dairy cattle. The highest losses in terms of decreased milk yield account to about 50%, late abortion causes a reduction of about 20-30% and even infected cows which appear to calve normally suffer a reduction of about 7-10% (Dafaalla, 1962). More recently, based on weighted average of 25.1% prevalence, assessment of the financial loss due to bovine brucellosis in Khartoum state was estimated along with quantifying the cost of reduced reproduction, production and veterinary intervention (Angara *et al*., 2016). Based on estimates of USD 434.3/cow/year due to bovine brucellosis and USD 48.1 per person, the disease constitutes a serious economic burden to the economy of the State and producers in the absence of a formal control strategy. Accordingly, a national-wide control strategy for

brucellosis for the benefit of both agriculture and health sectors is becoming unavoidable.

1.3 Bioterrorism Of Brucellae

Bioterrorism is a form of terrorism caused by intentional release of biological agents resulting in social distress, economic burden, and heavy loss to humans, animals and plants. Bioterrorism is caused by biological agents (bacteria, viruses and other germs) or derived toxins, which can be divided into three categories: anti-personal, anti-animal and anti-plant. The use of biological agents is also classified according to the way they are used: biological warfare and bioterrorism. In 1972, the United Nations held a Biological Weapons Convention. As a result of this conference, International Leaders signed a Treaty that completely prohibits the development, production and stockpiling of bacteriological and toxin weapons in any part of the world (Cirincione *et al*., 2005). *Brucella* was one of the first agents to be used in the development of biological weapons, in particular *B suis*, which had been weaponized in the shape of particle-filled bombs, allegedly by the United States, and possibly by other countries more than 50 years ago (Christopher *et al*., 2005).

Given the ease of aerosol transmission of *Brucella* species, researchers attempted to develop it into a biological weapon and it became the first agent weaponized by the old US offensive biological weapons program. By 1955, the US was producing *B. suis*-filled cluster bombs for the US Air Force at the Pine Bluff Arsenal in Arkansas. Development of brucellae as a weapon was halted in 1967, and President Nixon later banned development of all biological weapons on November 25, 1969 (Sriranganathan *et al*., 2009). Although the *Brucella* munitions were never being used against human targets, the research performed resulted in concern that *Brucella* species someday may be used as a weapon against either military or civilian objectives. *Brucella* spp. has a high probability for use in biologic terrorism and are highly infectious via the aerosol route. It is estimated that inhalation of only 10-100 bacteria is sufficient to cause disease in man (Doganay and Doganay, 2013). The relatively long and variable incubation period (5-60 days) and the fact that many infections are asymptomatic under natural

conditions along with its low mortality has made it a less desirable agent for weaponization, although large aerosol dosage may shorten the incubation period and increase the clinical attack rate (Doganay and Doganay, 2013).

Several countries have been suspected of studying brucellae as a biological weapon, but to date, no use of *Brucella* in a bioterrorist attack has been reported. In one hand, although *Brucella* is highly contagious, as only 10-100 bacterial cell would be sufficient to produce a contaminating spray for humans. In the other hand, the extent of the risk of *Brucella*, notably *B. melitensis* and *B. suis*, are unlikely to be used as biological weapons. This is because they undergo long incubation period, the majority of infections are asymptomatic and mortality is low. However, the morbidity of this agent should not be underrated since it leads to chronic and disabling pathologies (Guihot *et al*., 2004)

1.4 Taxonomy And Etiology

The genus *Brucella* belongs to the order *Rhizobiales* within the class α-proteobacteria, family Brucellaceae that in addition to *Brucella* consists of the genera *Mycoplama* and *Ochrobactrum* (De Ley *et al*., 1987). *Brucella* spp. are gram negative, aerobic, non-spore forming, facultative intracellular cocco-bacilli (Alton *et al*., 1988). Recent years have seen the beginning of an expansion of the genus *Brucella* beyond the six classically identified species (*Brucella abortus* "cattle", *B. melitensis* "sheep and goats", *B. suis* "pigs, hares, and reindeer", *B. canis* "dogs", *B. ovis* "sheep", and *B. neotomae* "rodents"). New six additional species were described, these are: *B. microti "*voles", *B. pinnipedialis* "pinnipeds", *B. ceti* "cetaceans", *B. papionis* "baboons", *B. vulpis "*foxes" and *B. inopinata "*isolated from a human case, but natural host unknown" (Whatmore, 2009).

The added six species group were described as "atypical" reflecting their genetic separation from classical species. A number of other isolates that await formal taxonomic description originating from sources as diverse as humans, frogs, fish, and additional rodents will likely extend diversity within both the classical group and newly emerging "atypical" *Brucella* in the near future.

Of the classical species; *B. melitensis, B. abortus, and B. suis* and to lesser extent *B. canis*, are the most significant in terms of being zoonotic and can cause the considerable socio-economic impacts (Whatmore *et al*., 2016). The first three species also were sub classified into biovars: seven biovars are recognized for *B. abortus* (1-6, 9), three for *B. melitensis* (1-3) and five for *B. suis* (1-5). The remaining species have no biovars (Whatmore, 2009).

1.4.1 Taxonomic controversies

Molecular genetic studies have indicated that the genus contains only a single species differentiated into a number of biovars, with certain host preferences. DNA–DNA hybridisation studies carried out within the genus revealed a high degree of homology (>90%) between the six classical species (Hoyer and McCullough, 1968a and 1968b; Verger *et al*., 1985) and, on this basis, it was proposed that *Brucella* should constitute a monospecific genus (Verger *et al.*, 1985 and 1987). The Subcommittee on the Taxonomy of Brucella supported this proposal at that time with *B. melitensis* becoming the sole representative species and the other species being considered biovars of *B. melitensis* (Corbel, 1988). However, recognising the likelihood for confusion, the reclassification of the genus as monospecific was accompanied by a recommendation that 'the existing vernacular names for the nomen species *B. melitensis*, *B. abortus*, *B. suis* ...etc. can be retained for non-taxonomic purposes to avoid confusion.

The taxonomic validity of this viewpoint has been accepted but the proposed new nomenclature, which would identify all members of the genus as biovars of *B. melitensis*, has been met with opposition on practical ground and the genus retained its previous nomenclature for practical reasons (Corbel, 1997).

1.5 General Characteristics

1.5.1 Morphology

Brucellae are Gram-negative coccobacilli or short rods (0.6-1.5 mm - 0.5-0.7 mm) arranged singly and rarely in pairs or small groups. The morphology of *Brucella* spp. is constant except in old cultures, where pleomorphic forms may occur. Brucellae are non-motile and do not form spores, and flagella, pili, or true capsules are not produced. They

usually do not show bipolar staining and resist decolorization by weak acids (Corbel and Brinley-Morgan, 1984).

1.5.2 Culture and growth characteristics

Members of the genus *Brucella* are aerobic, but many strains require an atmosphere containing 5-10% added CO_2 for growth. The optimum pH for growth varies from 6.6-7.4. The optimal growth temperature is 36-38 C^0, but most strains can grow between 20-40 C^0. Growth in liquid media favours dissociation of smooth-phase cultures to non-smooth forms and is usually poor unless the culture is vigorously agitated. On suitable solid media, colonies are visible after 2 days of incubation. After 4 days of incubation, the colonies are round, 1-2mm in diameter, with smooth margins, translucent, and a pale honey color when plates are viewed in the daylight through a transparent medium. When viewed from above, the colonies appear convex and pearly white. Later, the colonies become larger and slightly darker. Smooth *Brucella* spp. cultures have a tendency to undergo variation during growth, especially with subcultures, and dissociate to rough (R) forms, and sometimes mucoid (M) forms (Corbel and Brinley-Morgan, 1984).

1.5.3 Biochemical characteristics

Brucella spp. metabolic activity is oxidative, and cultures show no ability to acidify carbohydrate media in conventional tests. They are catalase-positive and usually oxidase-positive, and reduce nitrates to nitrites (except *B. ovis* and some *B. canis* strains). *Brucella suis* biovar 1, *B. neotomae* and biovars 1-4 and 9 of *B. abortus* produce H_2S from sulfur-containing amino acids. Urease activity varies from fast to very slow. Indole is not produced from tryptophan, and acetylmethylcarbinol is not produced from glucose (Corbel and Brinley-Morgan, 1984). Differential characteristics of brucellae were summarized in appendix 1.

1.5.4 Antigenic characteristics

The naturally occurring smooth forms of *Brucella* spp. strains show complete cross-reaction with each other, but not with non-smooth variants, in agglutination tests with unabsorbed polyclonal antisera. Cross-reactions between non-smooth strains can be demonstrated as well with unabsorbed anti-R sera. Lipopolysaccharide (LPS) comprises

the major surface antigens of the corresponding colonial phase involved in agglutination. The S-LPS molecules carry the A and M antigens, which show different quantitative distribution among the smooth *Brucella* spp. strains. Serological cross-reactions have been reported between smooth brucellae and various other Gram-negative bacteria, and especially *Yersinia enterocolitica* O:9, which can induce significant levels of antibody cross-reacting with S-LPS *Brucella* spp. antigens in diagnostic tests (Alton *et al*.,1988).

1.5.5 Susceptibility to phages and antibiotics

Lysis by specific phages is a useful test to confirm the identity of *Brucella* spp. since more than 40 phages have been reported to be specifically lytic for *Brucella* spp. The phages mainly used for *Brucella* spp. typing are Tbilisi (Tb), Weybridge (Wb), Izatnagar1 (Iz1), and R/C). Susceptibility to Dyes and Antibiotics Susceptibility to the dyes thionin and basic fuchsin, is one of the routine typing tests of *Brucella* spp. On primary isolation, all brucellae are usually susceptible *in vitro* to gentamicin, rifampin, and tetracyclines. Most strains are also susceptible to ampicillin, chloramphenicol, cotrimoxazole, erythromycin, kanamycin, novobiocin, spectinomycin, and streptomycin, nevertheless, variation in susceptibility may occur. *In vivo*, most strains are resistant at therapeutic concentrations to amphotericin B, bacitracin, lactamins, cephalosporins, clindamycin, cycloheximide, lincomycin, nalidixic acid, nystatin, polymyxin, and vancomycin (Garin-Bastuji *et al*., 2016).

1.5.6 Resistance and survival

The brucellae are intracellular pathogens unable to multiply outside the host cells. The ability of *Brucella* spp. to persist outside its mammalian host is relatively high as compared with most other non-spore-forming pathogenic bacteria, under suitable conditions. Thus, when conditions of pH, temperature, and light are favourable, that is, pH >4, cool temperature, high humidity, and absence of direct sunlight, brucellae may retain infectivity for several months in aborted fetuses and fetal membranes, faeces and liquid manure, water, wool, and hay, and on equipment and clothes (Garin-Bastuji and Blasco, 2016). Brucellae are able to withstand drying particularly in the presence of

extraneous organic material and will remain viable in dust and soil. Survival is prolonged at low temperatures, especially when freezing. The persistence of brucellae in milk and dairy products is related to a variety of factors including the type and age of product, humidity level, temperature, changes in pH, moisture content, biological action of other bacteria present, and conditions of storage (Garin-Bastuji, 2011). The results of several studies are presented in. At low numbers in liquid media, brucellae are heat-sensitive. Thus, dilute suspensions in milk are readily inactivated by pasteurization (high-temperature short time or flash methods) or prolonged boiling (10 min). Brucellae do not remain viable for prolonged periods in ripened fermented cheese. The optimal fermentation time to ensure safety is not known but is estimated at 3 months. However, in normally acidified soft cheese, the strictly lactic and short-time fermentation and drying increase the survival of *Brucella*. Pasteurization of milk or cream is the only means to ensure safety of these products. Brucellae are fairly sensitive to ionizing radiation and are readily killed by normal sterilizing doses of gamma rays, under conditions that ensure complete exposure, especially in colostrum. In contrast to dairy products, the survival time of brucellae in meat is extremely short, due to acidic fermentation of the meat except in frozen carcasses where the organism can survive for many years. Therefore, meat consumption is less likely to be a source of infection. Direct contamination of abattoir workers and carcasses by milk and utero-vaginal secretions is prevented by a proper and hygienic removal of mammary glands, reproductive organs, and lymph nodes, which are the most heavily contaminated organs. Most commonly available disinfectants readily kill brucellae at normally recommended concentrations (phenol 10 g, formaldehyde, xylene 1ml), except in the presence of organic matter or at low temperature, which drastically reduce their efficacy. Where possible, decontamination should be carried out by heat treatment, especially for surfaces. Diluted hypochlorite solutions, ethanol, iodophors, or isopropanol, and optimally substituted phenols, but not the alkyl quaternary ammonium, are effective for decontamination of exposed skin (Garin-Bastuji, 2011).

1.6 Transmission

Transmission of *B. abortus* is very likely to occur via the oral route because cattle tend to lick aborted fetuses and the genital discharge of an aborting cow (Cunningham, 1977). Congenital infection can occur in new-born calves as a result of in-utero infection and the infection may persist in a small proportion of calves which may also be serologically negative until after their first parturition or abortion (Constable *et al*., 2017). Exposure to brucellae is also likely to occur when calves born to healthy dams and fed on colostrum or milk from infected dams (Catlin and Sheehan, 1986). It has been established that brucellosis in bulls does not always result in infertility, although semen quality may be affected. Bulls that remain fertile and functionally active will shed *Brucella* organisms with the semen during the acute phase of the disease. Shedding, however, may cease or become intermittent (McCaughey *et al.*, 1973). In contrast to artificial insemination, bulls used in natural service may fail to spread the infection, as the infected semen is not deposited in the uterus (Ray, 1979). While indirect exposure to *Brucella* organisms could be mediated by wildlife, birds and waterways (contaminated with uterine discharge or slurry from aborting cattle). It seems that only dogs carry pieces of placentae or aborted fetuses from one place to another causing direct exposure (Forbes, 1990). Contamination of a cowshed or pasture takes place when infected cattle abort or have full-term parturition. Although it is generally accepted that *B. abortus* is not excreted for any considerable time before abortion occurs, excretion in the vaginal discharges of infected cattle may occur as early as 39 days after exposure (Philippon *et al.*, 1970). A massive excretion of brucellae starts after abortion and may continue for 15 days. Once the fetal membranes are expelled the uterine discharges diminish and the number of *Brucella* organisms excreted decreases rapidly (Nicoletti, 1981). Although the infectious materials from the genital tract usually clear after 2-3 months, some infected cattle become carriers of *Brucella* and excrete it intermittently for many years (Philippon *et al.*, 1970). Infected udders are clinically normal but they are important as a source of re-infection of uterus, infection for calves or human drinking the milk.

Transmission of infection to humans occurs through breaks in the skin, following direct contact with tissues, blood, vaginal discharges, aborted fetuses or placentas. Food-borne infection occurs following ingestion of raw milk and other dairy products, but rarely from eating raw meat from infected animals. Occupational airborne infection in laboratories and abattoirs has also been documented. Accidental inoculation of live vaccines (such as *B. abortus* Strain 19 and *B. melitensis* Rev.1) can also occur, resulting in human infections (Robinson, 2003). Direct person-to-person spread of brucellosis is extremely rare. Mothers who are breast-feeding may transmit the infection to their infants. Sexual transmission has also been reported. Uncommon transmission may also occur via contaminated tissue transplantation (Geoffrey *et al*., 2002).

1.7 Pathogenesis

Although epidemiological evidence suggests that *B. abortus, B. melitensis* and *B. suis* show distinct host preferences, this only marks a general trend and the organisms are capable of establishing infection in a wide range of host species, including humans. *B. neotomae, B. canis and B. ovis* in contrast, show much greater host specificity, and with the exception of occasional *B. canis* infections in carnivores and in humans, it seems they have little capacity to spread beyond their usual hosts (Corbel, 1997).

Typically, in all host species *Brucella* grows intracellularly in the macrophages. Abortion is a frequent consequence of infection in the pregnant female, and orchitis and epididymitis can result in the male. Sexually immature animals are often less susceptible to the disease. *Brucella* spp. has a predilection for the pregnant uterus, udder, testicle and the accessory male sex glands, lymph nodes, joint capsules and bursa. Erythritol, a substance produced by the fetus and capable of stimulating the growth of *Brucella spp.* occurs naturally in greatest concentration in the placental and fetal fluids and is probably responsible for localization of infection in these tissues. In the adult, non-pregnant cow, localization occurs in the udder, and the uterus, if it becomes gravid, is infected from periodic bacteraemic phases originating in the udder. When the invasion of the gravid uterus occurs, the initial lesion is in the wall of the uterus and spread to lumen of the uterus soon follows, leading to a severe ulcerative endometritis of the inter-cotyledonary

spaces. The allantochorion, fetal fluids and placental cotyledons are next invaded and the villi destroyed. Abortion occurs principally in the last trimester of pregnancy, the incubation period being inversely proportional to the stage of development of the fetus at the time of infection (Constable *et al*., 2017).

In humans brucellae progress from the portal of entry, via lymphatic channels and regional lymph nodes, to the thoracic duct and the blood stream, which distributes them to the parenchymatous organs. Granulomatous nodules that may develop into abscesses from lymphatic tissues, liver, spleen, bone marrow, and other parts of the reticuloendothelial system. In such lesions, the brucellae are principally intracellular. Osteomyelitis, meningitis, or cholecystitis also occasionally occurs (Cutler *et al*., 2005). The main histological reaction in brucellosis consists of proliferation of mononuclear cells, exudation of fibrin, coagulation necrosis and fibrosis. The granulomas consist of epithelioid and giant cells, with central necrosis and peripheral fibrosis (Farrell, 1996). The four brucellae that infect humans have apparent differences in pathogenicity. *B. abortus* usually causes mild disease without suppurative complications; non-caseating granulomas of the reticuloendothelial system are found. *B. canis* also causes mild disease. *B. suis* infection tends to be chronic with suppurative lesions; caseating granulomas may be present. *B. melitensis* infection is more acute and severe. Persons with active brucellosis react more markedly (fever, myalgia) than normal persons to injected *Brucella* endotoxin. Sensitivity to endotoxin thus may play a role in pathogenesis (Cutler *et al*., 2005). In addition, single cases of human infections, caused by recently published new species (*B. ceti*, *B. pinnipedialis*, *B. inopinata*), have been reported (Foster *et al*., 2007; Scholz *et al*., 2010).

1.7.1 Bovine brucellosis

Infected animals usually develop granulomatous inflammatory responses often located within lymphoid tissues and organs with a prominent reticuloendothelial component. There is a predilection for selected body sites such as reproductive organs, udder, and supramammary lymph nodes, and sometimes joints and synovial membranes. The localization and persistence of brucellae in these organs and tissues

follow in the wake of a widespread distribution of *Brucella* during a generalized stage of infection. During this first stage of infection, the major clinical symptom is abortion however other signs may be observed (e.g., orchitis, epididymitis, hygroma, arthritis, metritis, subclinical mastitis). However, numerous animals develop self-limiting infections or they become asymptomatic latent carriers. The second stage is characterized by the elimination of brucellae or by a persistent infection of the mammary glands and supramammary and genital lymph nodes, with a constant or intermittent shedding of the organisms in the milk and genital secretions. Animals generally abort once, from 5-8 months of gestation, but reinvasion of the uterus occurs in subsequent pregnancies through shedding of the microorganism in fluids and membranes. The pregnancy can also be full-term. Vaginal discharges after abortion or normal calving are the main source of contamination of congeners, other animal species, and man (Constable *et al*., 2017). The inter-herd spread of infection generally follows the movement or gathering of infected animals. Persistent infection of mammary glands is associated with constant or intermittent shedding of the organisms in the milk in succeeding lactation periods and a drop in milk production estimated at 10%. The number of brucellae excreted in milk is relatively low and does not allow transmission through direct contact, except through the milker's hands. In the male, localization in the reproductive organs generally results in brucellae being shed in the semen. Congenital infection is of major epidemiological significance, since 2–20% of heifer calves born to infected cows may be persistently infected. Other calves fed with infected milk usually become infected, but most recover from these infections (Garin-Bastuji, 2011).

1.7.2 Brucellosis in small ruminants (specific features)

The main causative agent of brucellosis in sheep and goats is *Brucella melitensis*, nevertheless some cases due to *B. abortus* have been reported. *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle in terms of Pathological and epidemiological characteristics. The excretion from the vagina is more abundant and prolonged than in the case of cows and last in goats for at least 2-3 months. In goats,

about 2/3 of acute infections acquired naturally produce infection of the udder and the organisms will be shed in the milk during the next lactation. Excretion may cease during a lactation period. Infection reduces milk production more drastically than in cattle (Garin-Bastuji, 2011).

1.7.3 Human brucellosis

Man is accidentally infected and often represents a dead-end host of Brucellae. The disease is primarily an occupational hazard in professionals who work with animals and their products, namely, veterinarians, farmers, laboratory technicians, abattoir workers, and others (Pappas *et al*., 2006a and 2006b). The infection frequently direct or indirect contact through the skin or mucous membranes as the primary route of transmission, however other routes could be through consumption of contaminated fresh dairy products. People are susceptible mainly to *B. abortus*, *B. melitensis*, and *B. suis*. *Brucella melitensis* and *B. suis* often give rise to the most severe form of infection. After an average 8-20-day (up to several months) incubation period, illness occurs in different forms.

The asymptomatic form is frequent and mainly due to *B. abortus*, and is characterized by serologic evidence in persons with no symptoms consistent with brucellosis. The acute form is also common and symptoms include lethargy, headache, and muscular or joint pain, and drenching sweats, especially at night, are characteristic. The manifestations of brucellosis are sometimes most pronounced in/or limited to a specific system organs. Complication occurs in the course of acute infection, and localized brucellosis occurs in the absence of other signs of systemic illness (spondylitis and peripheral arthritis, especially of the hip, knee, and shoulder, epididymo-orchitis). Nervous, genitourinary, hepato-splenomegaly, and cardiovascular complications may be observed as well (Garin-Bastuji, 2011). Chronic brucellosis includes one or more of the signs described above and persists or recurs over a period of 6 months or more.

Brucellosis diagnosis is frequently based on the detection of high or rising titers in serological tests such as serum agglutination test (SAT) and the Rose Bengal test (RBT)

as screening tests, and Coombs' or complement fixation tests, or ELISA for confirmation.

1.8 Diagnosis of Brucellosis

In the absence of pathognomonic signs, the specific diagnosis of brucellosis can only be made on the basis of laboratory testing, especially in domestic animals.

1.8.1 Bacteriological methods

There is no single test by which a bacterium can be identified as *Brucella* spp. A combination of growth characteristics and serological and bacteriological methods is usually required.

1.8.2 Staining

Stamp's modification of the Ziehl Neelsen method is the usual procedure for the examination of smears of organs or biological fluids. However, this method shows a low sensitivity on milk and dairy products where brucellae are often present at low numbers and interpretation is frequently impeded by the presence of fat globules. Furthermore, staining methods are not specific, and other organisms causing abortion, for example, *Chlamydophila abortus* (formerly *Chlamydia psittaci*) or *Coxiella burnetii*, are very difficult to differentiate from *Brucella* spp. organisms. The results, whether positive or negative, should be confirmed by culture (Whatmore *et al*., 2009).

1.8.3 Culture

Direct isolation and culture of *Brucella* spp. are usually performed on solid media that enable the developing colonies to be isolated and recognized clearly, and limit the establishment of non-smooth mutants and overgrowth of contaminants. However, the use of liquid media may be recommended for specimens where brucellae may be in small numbers. A wide range of commercial dehydrated basal media is available, for example, *Brucella* medium base, Tripcase or Trypticase soy agar, and Bacto-tryptose. Addition of 2-5% bovine or equine serum is necessary for the growth of strains like *B. abortus* biovar 2, and many laboratories systematically add serum to the basal media, with excellent results. Other media such as serum dextrose agar or glycerol dextrose agar can be used satisfactorily. A nonselective, biphasic medium, known as the

Castaneda medium, is recommended for the isolation of *Brucella* spp. from blood and other body fluids or milk, where enrichment culture is usually advised (Alton *et al*., 1988).

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added in order to suppress growth of organisms other than *Brucella* spp. The selective medium most widely used is Farrell's medium (Farrell, 1974), which is prepared by the addition of six antibiotics to a basal medium. A freeze-dried antibiotic supplement is available commercially. A selective biphasic medium made of the basal Castaneda medium with the addition of antibiotics to the liquid phase is sometimes recommended for isolation of *Brucella* spp. in milk. These media allow the isolation of most strains of *Brucella* spp.; however, some strains of *B. melitensis* may be partially inhibited by bacitracin, included in the supplement. Sensitivity increases significantly by the simultaneous use of both Farrell's and the modified Thayer-Martin's medium (Corbel and Banai, 2005).

Brucellosis is one of the most easily acquired laboratory infections; hence, safety precautions for sampling, and shipping, handling, and processing of the samples are extremely important, and work should only be carried out under level 3 containment (biosafety) conditions and by personnel adequately trained and made aware of the risks.

Samples of milk have to be collected aseptically after washing and drying of the whole udder and disinfection of the teats. It is essential that the samples contain milk from all quarters, and 10–20ml of milk should be taken from each teat, avoiding contact of milk with the milker's hands. The first few streams are discarded and the sample is directly milked into a sterile vessel. Milk specimens should be cooled immediately after they are taken and sent to the laboratory by the most rapid route. If they are to spend more than 12 h in transit, they should be treated with boric acid (0.1%), or preferably frozen. On arrival at the laboratory, samples are frozen if they are not to be cultured immediately. Then, milk is centrifuged at 5-700 g for 15 min, and cream and deposits are spread on solid selective medium, separately or mixed. Brucellae are usually present

in low numbers in bulk tank samples, and isolation from such specimens is very unlikely (Alton *et al*., 1988).

Dairy products are likely to contain small numbers of organisms, and enrichment culture is advised. Sampling methods are those classically recommended for bacteriological examination of dairy products and adapted to each sort of product. Specimens need to be carefully homogenized before culture, after they have been ground in a tissue grinder or macerated and pounded in a stomacher or an electric blender, with an appropriate volume of sterile phosphate-buffered saline.

The superficial strata (rind and the underlying parts) and core of the product should be cultured. Brucellae grow, survive, or disappear more or less rapidly according to the local physicochemical conditions linked to specific process technologies, and their distribution among the different parts of the product varies. A previous inoculation into guinea pigs or mice may sometimes provide the only means of detecting the presence of *Brucella* spp., especially when the specimens are heavily contaminated or likely to contain a low number of brucellae. Spleen is then cultured and, if possible, a serum sample is subjected to specific tests (Garin-Bastuji, 2011).

The most valuable other specimens include aborted fetuses (stomach contents, spleen, and lung), fetal membranes, vaginal secretions, semen, and arthritis or hygroma fluids. On animal carcasses, the tissues preferred for culture are those of the reticuloendothelial system (i.e., head, mammary and genital lymph nodes, and spleen), the pregnant or early post parturient uterus, and the udder. Identification and typing Species identification is routinely based on lysis by phages and on simple biochemical tests (oxidase, urease, etc.). For *B. melitensis*, *B. abortus*, and *B. suis*, the identification at the biovar level is currently performed by four main tests: carbon dioxide requirement, production of hydrogen sulfide, dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M antisera (Garin-Bastuji, 2011).

The polymerase chain reaction (PCR), including the real-time format, based on selected sequences of the *Brucella* spp. genome, provides an additional means of *Brucella* detection and identification, which is unaffected by the colonial phase. A

number of other methods including a multilocus sequencing scheme and several typing schemes based on the use of multiple locus VNTR analysis (MLVA), which can add useful epidemiological information allowing isolates to be linked to geographic location (Le Fleche *et al*., 2006).

1.8.4 Serological diagnosis

Diagnosis of *Brucella* spp. infection often has to be based on serological methods, in situations where bacteriological examination is not practicable. In routine veterinary tests, anti-*Brucella* antibodies are detected in serum and milk. The most widely used and recommended serum testing procedures are (1) buffered *Brucella* antigen tests (BBAT), that is, card test and the RBT, or buffered plate agglutination test (BPAT), (2) complement fixation test (CFT), and (3) indirect ELISA tests. The milk ring test (MRT) or indirect ELISA performed on bulk tank samples have great usefulness for locating infected herds or flocks. These tests are also of great interest to identify infected animals. The World Health Organization, the World Organization for Animal Health (OIE), the US Department of Agriculture, and the European Union have adopted specific recommendations for standardization of performance of the tests and interpretation of the results for all the different methods mentioned above. In small ruminants, RBT and CFT are the most effective and the most widely used methods (OIE Terrestrial manual, 2018).

1.8.5 Allergic tests

Delayed-type hypersensitivity reactions associated with cell-mediated immunity may be induced by either infection or immunization with living or adjuvant killed vaccines. Thus, a number of skin tests have been developed. Antigens free of S-LPS, such as Brucellin-INRA, preferred to crude preparations that interfere with serological diagnosis. Reactions are specific to the genus *Brucella*. Allergic skin test is used for non-vaccinated cattle, sheep, and goat herd surveillance, as a complementary test (Garin-Bastuji, 2011).

1.9 Methods For Differentiating Vaccine Strains

In addition to the enhanced AMOS and the recently developed 'Bruceladder' and SNPs typing approaches described above there are a number of stand-alone molecular assays that have traditionally been used to differentiate vaccine isolates. The resistance of *B. melitensis* Rev1 to streptomycin is attributed to a point mutation in *rpsL* and this is the basis of a PCR-RFLP method to differentiate this vaccine from field strains (Cloeckaert *et al*., 2002). Differentiation of *B. abortus* RB51 from field isolates uses a differential PCR based on the insertion of an IS711 copy into the *wboA* gene (Vemulapalli *et al*., 1999). Differentiation of *B. abortus* S19 from field strains relies on a differential PCR based on a 702 bp deletion within the *eryCD* locus (Sangari and Aguero, 1994), even though this deletion is apparently not conserved in all S19 variants (Mukherjee *et al*., 2005).

1.10 Treatment of Brucellosis

1.10.1 Human brucellosis

The World Health Organization (WHO) guidelines from 1986 are still considered the gold standard for the treatment of brucellosis, suggesting either the combination of doxycycline and rifampicin for 6 weeks, or the combination of doxycycline for 6 weeks with streptomycin for 2 or 3 weeks (Corbel, 2006). Relapse rates approach 10% within the accepted regimen (Pappas *et al*, 2005). Rifampicin usually is added to doxycycline for a full 6-week course. In patients with spondylitis or sacroiliitis, doxycycline plus streptomycin was found to be more effective than the doxycycline/rifampicin combination. Streptomycin currently is favoured over rifampicin for combination therapy of any significant infection. In paediatric patients older than 8 years, doxycycline plus gentamicin was the recommended therapy. For children younger than 8 years, trimethoprim/sulfamethoxazole (TMP-SMZ) and gentamicin were more safe and effective. TMP-SMZ also was effective in treating pregnant women, either as a single agent or in combination with rifampicin or Gentamicin (Corbel, 1997). The most effective and the least toxic chemotherapy for human brucellosis is still undetermined (Oguz Karabay *et al.*, 2004).

1.10.2Bovine brucellosis

Antibiotic therapy is rarely employed in the treatment of bovine brucellosis. Nevertheless, in case of genetically valued animals or herds, treatment may be performed to control spread of the disease. Monotherapy by tetracyclines or aminoglycosides is very unsuccessful. While the combinations of oxytetracycline with streptomycin were found successful in stopping of milk shedding of the organisms and prolonged treatment with this combination found to have 100% of success (Radwan *et al.*, 1993).

1.11Control

Control, eradication, and prevention of brucellosis require the implementation of regional programs based on vaccination and/or test and slaughter of infected animals, and general nonspecific management practices and hygienic measures that reduce exposure potential. These measures would not be effective without health education, training, and mobilization of livestock owners and others engaged in animal production, and if animal identity is not well recorded and stock movements are not well controlled (OIE Terrestrial manual, 2018).

1.11.1General measures

General nonspecific control measures help to reduce the spread of infection. Field personnel should be aware of simple safety measures to prevent human contamination and passive intra- and inter-herd transmission. Isolation of females at parturition, and incineration or deep burying of non-living products and fetal membranes are essential to limit the spread of infection. Contaminated materials and premises should be disinfected by heat treatment or by the use of the chemicals previously mentioned. All personnel handling contaminated material should wear disinfected or single-use protective clothing. Body surfaces that have been accidentally exposed to infection should be systematically washed and then decontaminated. Abattoir workers should take similar precautions, especially when handling udder and uterus, which should be systematically destroyed when infection is suspected (Garin-Bastuji, 2011).

In the laboratory, *Brucella* spp. present a very serious risk to workers handling heavily infected materials and cultures. Even when processing milk or dairy products risk exists but is lower. However, special safety precautions are not required for personnel engaged in routine serological diagnosis. All personnel regularly exposed to infection should be kept under close clinical and serological surveillance.

Currently no vaccine is efficient or safe enough to be recommended (Godfroid *et al*., 2005). In infected areas, trade in fresh milk and dairy products should be strictly controlled and limited to officially declared brucellosis-free farms. The milk produced on infected farms should be heat treated whatever its commercial purpose.

Eradication by Test and Slaughter Considering the low efficacy and the cost of antimicrobial chemotherapy in farm animals, test and slaughter of sero-positive animals is one of the two major forms of control and prevention of brucellosis. Such a strategy of eradication is justified on economic grounds when the prevalence rate of infected herds is 1% or below.

The epidemiological surveillance of brucellosis-free herds is generally based on regular control by the use of bulk MRT (in cattle only) and/or individual serological testing. All susceptible animals should be permanently identified and movements of animals closely controlled.

Eradication programs usually require an abortion notification and investigation scheme as well to detect infection. When positive results or abortions occur, safety measures should be undertaken and reactors or aborted females slaughtered. In some circumstances, for example, in free areas or in heavily infected herds, slaughter of the whole herd is advisable. Herd replacement should not subsequently occur and contaminated premises or pastures should not be used for animal housing or grazing, for 2-3 months.

1.11.2 Immunization

In high-prevalence areas or where the herds are large, or in extensive pastoral areas, it may be impossible to conduct the test-and-slaughter regime outlined above. Therefore, mass immunization is the only way to reduce the rate of infection. At present,

the most widely used vaccines are the live attenuated vaccines S19 in cattle and Rev.1 in small ruminants. These vaccines have proved to be effective in reducing the number of abortions and also limiting the spread of infection. The RB51 vaccine, usable only in cattle, has become the official vaccine for the prevention of brucellosis in cattle in some countries. However, its efficacy as compared to the reference S19 vaccine remains controversial. Vaccination cannot be expected to eradicate the disease from a herd. Furthermore, when used in adult animals, these vaccines induce long-term serological reactions and sometimes abortions. To reduce these reactions, immunization is generally restricted to young animals between the ages of 3 and 6 months and the conjunctival route is preferred to subcutaneous delivery.

When the epidemiological situation improves, a combined scheme including immunization of young animals and test and slaughter of infected adults may be applied. Then, when the prevalence rate of infected herds becomes sufficiently low, test and slaughter as outlined above may be applied.

1.12 Molecular Characteristics And Molecular Typing Tools

Historically, *Brucella* typing has been based on a range of phenotypic traits resulting in the current biovar (biotype) typing system (Alton *et al*., 1988). However, this approach is time consuming and involves handling of live pathogen. The emerge of molecular based methods for strain typing proved valuable in subtyping of *Brucella* at or below the species level. Starting in 2002, the complete genome sequences of the main *Brucella* species: *Brucella melitensis*, *B. suis* and *B. abortus* were published (DelVecchio *et al*., 2002; Paulsen *et al*., 2002 and Halling *et al*., 2005; respectively). This achievement offered the opportunity to compare genome sequences, which in turn accelerated marker discovery and several new methods. Of these, one new approach exploits the accelerated mutation rates associated with repeated sequences known as microsatellites or variable number tandem repeats (VNTRs). The mutations associated with VNTRs involved the loss or gain of complete repeat units. VNTR markers were used to genotype *Brucella* strains (Bricker *et al*., 2003) and the method called HOOF-Prints, based on independent loci consisting of tandem repeat units of eight nucleotides.

Later on, Le Fleche and colleagues (2006) developed a second multi-locus VNTR (MLVA-16) typing system. The assay designated from 16 loci markers for routine use. This MLVA-16 assay was described as high discriminatory tool identify *Brucella* strains to the species level with outstanding capacity to properly locate their geographic origin. The MLVA-16 data can easily be coded and exchanged by the repeat copy numbers for each locus and strain. International databases including genetic fingerprints of *Brucella* isolates from various geographic regions and corresponding epidemiological data as well as comprehensive histories of the strains from published researches can be queried from the international MLVA web service site: https://mlva.u-psud.fr. An advantage that could be utilized for epidemiological and trace-back investigations to identify the source of the infection in case of outbreaks (Al Dahouk *et al*., 2007).

1.12.1 DNA Polymorphism

(Due to the early knowledge about the high DNA homology of brucellae Hoyer and McCullough, 1968a and 1968b; Verger *et al*., 1985), much research over subsequent years focused on the identification of molecular markers and suitable experimental approaches to discriminate between members of the genus. The following are various techniques devised for brucellosis identification and typing

1.12.2 Pulsed-field gel electrophoresis (PFGE)

In this technique, infrequently cutting restriction enzymes are used to generate high molecular weight fragments that are then separated in a size dependant manner with the relative orientation of the gel and electric field being periodically altered to allow efficient fractionation of large fragments. PFGE-based approaches were shown to be invaluable in understanding genome structure and has not found widespread use as a routine typing tool for *Brucella*, reflecting the very limited diversity identified at the sub-species level.

1.12.3 Insertion sequence (IS) based typing

Insertion sequences (IS) are short DNA sequences that can transpose between prokaryotic genomes causing mutations and genomic rearrangements. They have

tendency to insert randomly and occupy multiple genomic locations, therefore, IS elements can be used as probes for discrimination between isolates. In *Brucella* an insertion sequence IS711 (Halling *et al*., 1993), also known as IS6501 (Ouahrani *et al*., 1993), appears to represent a major source of diversity in the genus. The IS711 copy number varies greatly from around six to twelve in most *Brucella* species through to >25 in *B. ovis* and marine mammal species (Ouahrani *et al*., 1993; Bricker *et al*., 2003). Two additional repeated DNA elements of 103 and 105bp have been reported in *Brucella* and designated Bru-RS1 and Bru-RS2 (Halling and Bricker, 1994).

1.12.4 PCR typing

Over recent years, a number of assays have been developed employing strain-specific targets, especially the *IS711* element described above, to differentiate *Brucella* mostly to the species level. The most widely used assay is known as the AMOS-PCR after the *Brucella* species it can identify *B. abortus* biovars 1, 2, and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1 (Bricker and Halling, 1994). The assay makes use of one common primer anchored in the *IS711* element and a species-specific primer that binds to unique sequence flanking that insertion site resulting in species discrimination due to different amplicon sizes. The assay was subsequently modified to include the vaccine strains S19 and RB51 (Bricker and Halling, 1995) a significant improvement given the critical need to distinguish field and vaccine isolates in eradication programs (Bricker *et al*., 2002) and some later modifications to improve performance (Ewalt and Bricker, 2003; Bricker *et al*., 2002). However, despite some later additions this assay still has the disadvantage of failing to detect all species or all biovars of some species (Ocampo-Sosa *et al*., 2005).

The recent availability of genome data has enabled the development of a new generation of multiplex PCR assays with wider scope. Bruce-ladder, a single tube PCR assay can *differentiate all* six classical species, *Brucella* isolates from marine mammals and the vaccine strains *B. abortus* RB51 and S19 and *B. melitensis* Rev 1, (Garcıa-Yoldi *et al*., 2006). The assay uses eight primer pairs designed based on species-specific differences to generate amplicons of different sizes resulting in a unique profile for

each species following agarose gel electrophoresis Garcia-Yoldi *et al*., 2006). A large validation exercise has recently been published examining over 600 strains and showed that the assay is very effective with the exception of *B. canis* where almost half of isolates examined were erroneously identified as *B. suis* (Lopez-Goni *et al*., 2008).

In addition to the multiplex PCRs mentioned above, a single target PCR has proven particularly useful. Such as the so-called *bp26* PCR, which utilizes a copy of *IS711*, located downstream of the *bp26* gene in marine mammal isolates to them from those associated with terrestrial mammals (Cloeckaert *et al*., 2000). This marker is for marine mammal isolates utilized in Bruce-ladder PCR, and has proven useful as a stand-alone assay to distinguish marine mammal *Brucella* (Sohn *et al*., 2003; McDonald *et al*., 2006). Additional useful PCRs applied particularly to Brucella from marine mammals are a series of four specific PCR reactions based on fragments identified by infrequent-restriction site PCR (Cloeckaert *et al*., 2003). All four PCRs are specific for *Brucella* from marine mammals but divide them into one profile specific for *B. pinnipedialis* and two profiles specific for *B. ceti.*

1.12.5 Restriction fraction length polymorphism-based approaches

One common typing approach is the use of restriction fraction length polymorphism (RFLP), applied to genes known to be polymorphic in *Brucella* (Al Dahouk *et al*., 2005a). Various outer membrane protein (omp) encoding genes have mostly targeted. This approach has become a popular method for differentiation of *Brucella* species and for descriptions of new strains (Bricker *et al*., 2002). Much work has focused on the *omp2* locus originally characterised by Ficht *et al*. (1990) and shown to consist of two genes designated *omp2a* and *omp2b*, displaying around 85% sequence identity and arranged in opposite orientations. More species-specific markers were identified within the omp encoding genes such as *omp25* and *omp31* "known to be deleted in *B. abortus*" (Cloeckaert *et al*., 1995). DNA polymorphism at the *omp2* locus was also contributory in the suggestion of the division of the new marine mammal *Brucella* isolates into two species (Cloeckaert *et al*., 2001).

1.12.6 Genome-based typing approaches

The preferred approaches are multilocus tandem repeat based or multilocus sequence-based. Data generated by such approaches are easily stored and easily compared between laboratories and available as international databases accessible via the Internet. The advantages of these typing approaches are that the use of multiple loci avoids dangers of incorrect conclusions from single loci, whose evolution may not necessarily reflect that of the genome as a whole, and that the diversity being indexed is known; however, enabling conclusions about mechanisms generating diversity to be drawn. In addition, the molecular clocks of various elements used as markers in these approaches are different giving epidemiological information at different levels. Generally, multilocus sequence approaches use markers with a slow molecular clock required to monitor evolution over many thousands of years while multilocus tandem repeat markers can have a fast molecular clock more suited to local epidemiological approaches (e.g. outbreak trace-back or identification of reactivation). However, the lack of diversity in *Brucella* has meant that, tandem repeat based approaches also appear to give meaningful information at the phylogenetic/taxonomic level (Le fleche *et al*., 2006; Al Dahouk *et al*., 2007).

1.12.7 Tandem repeat based typing

The first application of VNTR based typing to *Brucella* was the HOOF-Prints scheme “Hypervariable Octomeric Oligonucleotide Finger-Prints” published in 2003 (Bricker *et al*., 2003). The approach was based on a comparison of the newly completed genome sequences of *B. suis* and *B. melitensis* (DelVecchio *et al*., 2002; Paulsen *et al*., 2002; respectively) along with a draft *B. abortus* sequence which identified an eight base pair tandem repeat sequence at nine distinct genomic loci. Eight of the nine loci were variable among the three genome sequences allowing the development of a PCR-based method to identify the number of repeat units at each locus. The technique successfully differentiated all type strains for all species and biovars and among unrelated *B. abortus* biovar 1 field isolates, while isolates from the same herd or from short term in vitro passage showed little or no variation. However, the authors suggested

that as species-specific or biovar-specific alleles were not apparent the technique would be best used as a follow up after species/biovar identification by other methods (Bricker and Ewalt, 2005).

Later on, two additional VNTR typing schemes for *Brucella* were published. One scheme included eight of the original HOOF-Prints loci and additional 13 newly described VNTR loci to give a 21-locus scheme "VNTR-21" (Whatmore *et al*., 2006). In contrast to the original 'HOOF-Prints' scheme, this approach includes loci with a much broader range of evolutionary speeds. Thus, although the scheme retained the huge discriminatory power of 'HOOF-Prints' identifying 119 distinct genotypes when applied to a worldwide collection of 121 Brucella isolates, it also provided some resolution at the species level with species-specific alleles being identified at loci with slower evolutionary speeds. Reflecting this clustering analysis showed that, with minor exceptions, groups correspond to conventional species designations. Thus clusters corresponding to *B. abortus*, *B. ovis*, *B. melitensis,* and *B. neotomae* were identified as well as a *B. suis* cluster with subclusters corresponding to biovars 2 and biovars 1, 3 and 4. In this analysis, *B. canis* appeared closely related to *B. suis* biovars 1, 3 and 4 while *B. suis* biovar 5 appears distinct from both other *B. suis* biovars and other *Brucella* species. Reflecting this, the use of six of the more stable loci in isolation was shown to be sufficient to determine species designation. At around the same time a scheme labelled MLVA-15 was published (Le Fleche *et al*., 2006) taking a very similar approach. The authors used a comprehensive approach examining 80 tandem repeat loci in 21 reference strains and again found clusters that largely correspond to classical species (appendix3). For practical use, a subset of 15 loci that preserved this clustering was selected. These comprise eight markers with good species identification capability "minisatellites" and 7 with higher discriminatory power "microsatellites". Application to 236 clinical and reference isolates generated clustering consistent with other molecular and phenotypic characteristics and with some relationship to biovar designations of *B. suis* and, to a lesser extent, *B. abortus*.

The unprecedented level of discrimination offered by VNTR based approaches has meant they have rapidly become a method of choice in the field. Both 'HOOF-Prints' (Valdezate *et al*., 2007) and MLVA-16, a slight modification of MLVA-15, (Al Dahouk *et al*., 2007; Kattar *et al*., 2008) have been used to recognise human outbreaks that relate to a common source or to confirm relapse (Al Dahouk *et al*., 2005b). MLVA16 has also been used to confirm the source of a laboratory infection (Marianelli *et al*., 2008) as well as to demonstrate heterogeneity in profiles even in a restricted area of endemicity (Marianelli *et al*., 2007) and identify that human B. melitensis isolates from Peru form a distinct cluster from previously described European isolates (Smits *et al*., 2009).

MLVA-15 has also been used to assess the stability of a live vaccine (Garcıa-Yoldi *et al*., 2007a) and to show that wild boar and domestic pigs sharing localities can have identical *B. suis* genotypes (Garcia-Yoldi *et al*., 2007b). The "minisatellite" elements of both VNTR-21 and MLVA- 15 mean they have also found use as tools at the taxonomic level. Thus, VNTR-21 has been used to help identify at least three distinct groups within the marine mammal *Brucella* that are inconsistent with the currently accepted species (Groussaud *et al*., 2007; Whatmore *et al*., 2008).

Likewise, MLVA-15 shown to be useful for the identification of the recently described *Brucella* species such as *B. microti* and *B. inopinata* (Scholz *et al*., 2008 and 2010; respectively). The authors describing all three VNTR schemes published to date (Bricker *et al*., 2003; Le Fleche *et al*., 2006; Whatmore *et al*., 2006) as all outlined the vision of an international database of profiles available on the Internet. This would allow users to interrogate the database with their own data and add profiles, and thus offering the potential to build up a hugely powerful global database (http://mlva.u-psud.fr/brucella/).

1.12.8 Multilocus sequencing

Multilocus sequencing has become the major approach applicable to studying the global epidemiology of bacteria and is frequently used for phylogenetic studies. In its classical form multilocus sequence typing (MLST) involves the sequencing of short fragments of a number of housekeeping genes which are subject to purifying selection

and slow evolution and within which variation is nearly neutral (Enright and Spratt, 1999). While there are few polymorphic sites in individual housekeeping genes, the use of combined sequences of multiple housekeeping genes can give high discriminatory power while retaining signatures of longer-term evolutionary relationships and buffers against potentially skewed evolutionary pictures obtained by single-locus analyses (Margos *et al*., 2008). The only application of multilocus sequence analysis published to date in this field examined nine discrete genomic loci, equating to 4.396 bp from 160 *Brucella* isolates of all species and biovars known at the time (Whatmore *et al*., 2007). Overall, the study confirmed the genetic uniformity of *Brucella* with only 1.5% of sites found to be polymorphic across all 160 isolates. Can identify distinct sequence types (STs) and concatenated sequence data can be used to construct an unrooted neighbour-joining tree representing the relationships between STs (appendix 3). This showed that the four previously characterised classical *Brucella* species, *B. abortus*, *B. melitensis*, *B. ovis* and *B. neotomae* corresponded to well-separated clusters. With the exception of biovar 5, *B. suis* isolates cluster together, although they form a more diverse group than other classical species with a number of distinct STs corresponding to the remaining four biovars. *B. canis* isolates are located on the same branch very closely related to, but distinguishable from, *B. suis* biovar 3 and 4 isolates. Marine mammal isolates represented a distinct cluster.

1.12.9 Single-nucleotide polymorphism (SNPs) typing

An additional approach to typing has been developed based on multilocus sequence data for bacteria with a clonal population structure such as *Brucella*. SNPs can be used to describe the phylogenetic framework of a species. Two alternative approaches have been used both of which capable to identify any *Brucella* isolate as a member of one of the six classical species or as a marine mammal *Brucella* isolate. While an initial primer extension based approach to identify SNPs was described (Scott *et al*., 2007), two later approach based on Minor Groove Binding protein (MGB) probes applied on a real-time PCR platform were published (Gopaul *et al*., 2008; Foster *et al*., 2008). The real-time platform has certain benefits of speed and technical simplicity with the advantage that

amplification and detection occur concurrently rather than in individual steps. The PCR reaction involves competition for binding between the two probes at the target, with the one having the identical sequence binding in preference to the other. The assay can distinguish all members of the classical species.

It should be noted that no *B. suis* specific SNP has been identified to date, reflecting the phylogenetic position of *B. canis* within the *B. suis* group, however *B. suis* and *B. canis* can be differentiated as a specific *B. canis* SNP has been identified. One minor exception is *B. suis* biovar 5 which has phylogenetic position as distinct from other *B. suis* biovars. SNPs specific for this biovar have been identified and could easily be added to this assay should *B. suis* biovar 5 identified as a distinct group (Whatmore *et al*., 2007).

These assays can readily be expanded to take into account new groups or to identify relevant groups at the sub-species level. Application of such approach to *B. melitensis* and *B. abortus* may provide a fuller understanding of the genetic relationships between biovars.

1.13 Comparative Genomics

As with other bacteria, whole genome sequences (WGS) are beginning to impact greatly on understanding of the genus. The availability of WGS should sign a new era of research towards understanding the consequences of the variation observed in genome sequences.

Comparison of sequenced *Brucella* genomes revealed extensive gene similarity with the majority of genes (>90%) sharing 98–100% identity. More variable genes (<95% identity) were confined to genes encoding hypothetical genes and probable surface exposed proteins such as outer membrane proteins, membrane transporters, putative invasion and ShdA-like adhesins.

A variety of putative virulence factors were also identified including putative adhesins and haemolysins, which play a role in the pathogenesis. However, in contrast to many pathogens no obvious toxins or secreted phospholipases were identified consistent with the limited cytopathogenicity of *Brucella* (Whatmore *et al*., 2009).

Given the observed conservation of virulence-associated genes among *Bruella* species, and the pattern of species-specific gene inactivations affecting transcriptional regulators and outer membrane proteins, it was suggested that these inactivations play an important role in the establishment of host specificity and may be a primary driver of speciation (Chain *et al*., 2005). Numerous losses affecting general metabolic processes and the loss of the ability to synthesise and use storage compounds such as glycogen and polyhydroxybutyrate were considered consistent with the adaptation of brucellae to the protected, nutrient-poor, low oxygen tension environment of its intracellular niche.

Attempts were made to link genome structure with differential virulence and a large number of genomic islands noted to be absent in *B. ovis*, a species considered non-pathogenic for man. However, *B. neotomae*, the other classical species considered non-pathogenic for humans, possessed these islands. Further *B. canis* and *B. suis*, although differing in human virulence, were found to be very similar by this approach, implying that in addition to loss or gain of genetic content mechanisms involving gene inactivation or altered expression may contribute to host preference and virulence. The deletion of one genomic island, absent from *B. ovis*, resulted in a rough phenotype and attenuation of growth in macrophages and virulence in a murine model (Whatmore e*t al*., 2009).

The first full sequence of *B. abortus* S19 vaccine strain became available for which the mechanism of attenuation is unclear was published (Crasta *et al*., 2008). Comparison of this sequence with the two genomes of virulent *B. abortus* revealed consistent differences between S19 and both virulent strains. These included four major differences of over 60 bp. These included a deletion in in *eryC* and *eryD* genes, characterised previously (Sangari *et al*., 1994), but known to be insufficient or required for attenuation in a mouse model (Sangari *et al*., 1998) and in *eryF* involved in erythritol uptake. Besides these three major differences, more minor changes were possible relevance to attenuation, including lipid transport and metabolism, transcription regulation, transporter proteins, outer membrane proteins and several hypothetical

proteins. Characterisation of these changes might help unravel the basis of attenuation of S19.

Recently, a large number of additional genomes have been completed allowing for more comparisons that are extensive. Wattam *et al*. (2009) compared sequences of 10 genomes adding *B. canis*, *B. ovis*, *B. suis* biovar 2, and *B. melitensis* biovar 2 and an incomplete *B. ceti* genome to those already described above. Analysis confirmed the similar gross genomic structures with the only major exceptions being a 210 kb translocation in *B. suis* biovar 2 and a 700 kb inversion in chromosome 2 shared by all the *B. abortus* genomes. The authors noted that although genome sizes compared to *Ochrobactrum* suggest ongoing genome reduction in the number of pseudogenes, while higher than that of some other Alphaproteobacteria, is substantially lower than many other organisms. Based on this analysis a number of interesting loci including the type IV secretion system, tra genes and enzymes responsible for the LPS synthesis that gives *Brucella* its smooth phenotype appear to have been acquired horizontally. Roughness appears to have developed twice independently as different gene inactivations were postulated to be responsible for this phenotype in the naturally rough species *B. ovis* and *B. canis*. These isolates, as well as genomes of *B. suis* biovar 3 and 4 and *B. melitensis* biovar 3 isolates were also included in a recent comparative genome analysis focussing on whole genome phylogeny (Foster *et al*., 2009). This analysis produced a phylogeny roughly equating to that generated by multilocus sequencing and again pointed to a lack of evidence of recombination among Brucella species. As with multilocus sequencing this analysis showed considerable diversity among *B. suis* with biovar 2 as most basal and distantly related to other strains in the clade and *B. canis* arising from within this clade. This analysis also confirmed that *B. suis* biovars 3 and 4 are closely related despite different genome organisation with *B. canis* separated from *B. suis* biovar 4 by only 253 SNPs. It was estimated that this split occurred only 7500-22,500 years ago. In contrast to multilocus sequence data, limited diversity was apparent in *B. abortus* reflecting the limited coverage of the diversity of this species by genomes sequenced to date. This also suggested that the *B. ovis* lineage is basal to the rest of *Brucella* lineage. In the past,

some authors have suggested that *B. suis* is the closest species to the *Brucella* ancestor based on nutritional requirements (Plommet, 1991), diversity in genome structure, host preference and metabolic activities and protein cross-reactivity with Ochrobactrum (Moreno *et al*., 2002).

1.14 Future Perspectives

In light of the extreme homogeneity between the classical *Brucella* species early attempts to identify useful epidemiological markers and to understand the phylogenetics and inter-species relationships of the group advanced only slowly. However, it is now clear that the classical taxonomy based on host specificity and phenotype and which predates molecular characterization represents an astonishingly accurate picture of genetic relationships. While it has been debated for many years whether the degree of differentiation merits species status it is now becoming apparent that Brucella species are reproductively isolated and (with the exception of *B. suis/canis*) represent monophyletic lineages separated by long branch lengths (Whatmore *et al*., 2007; Foster *et al*., 2009). Thus, although the debate between pure taxonomists and clinical and veterinary microbiologists as to the validity of *Brucella* species is likely to continue, data that have emerged in the last few years appear to strengthen the argument against the monospecific genus concept. There are likely to be substantial additions to *Brucella* taxonomy in the coming years and tools are now available to ensure that any new species are justifiable on genetic, as well as ecological and phenotypic grounds. Such consistency of genetics and taxonomy would ensure that in the future appropriate rapid typing tools to identify these organisms can easily be developed.

To date there has been very limited progress in understanding the basis of host specificity and the genetic events responsible for differences in disease presentations are not understood. While whole genome sequences per se are unlikely to provide immediate answers they will surely ultimately revolutionise understanding of the relationship between genome diversity and biology by providing productive hypotheses for further research. Studies of genomes at transcriptomic and proteomic levels may also provide information on variation in biological function and host adaptation.

Forthcoming genome sequences should provide more immediate resolution to issues such as the phylogenetic relationships between and within Brucella species. Genome comparisons with some of the emerging organisms populating the Brucella - Ochrobactrum divide are likely to prove particularly interesting in understanding the emergence of the *Brucella*. Further, more detailed knowledge of the population structure of the genus and improved typing tools may lead to the identification of genotypes within species displaying enhanced pathogenic potential, something there is little evidence for to date, but which would be invaluable in unravelling the basis of virulence.

Advances in understanding of molecular diversity within the group coupled with technological advances are already providing typing tools with hitherto unimagined powers of resolution. For many years biotyping was the mainstay of Brucella typing but it provides limited epidemiological data and is not likely to prove applicable in the face of expansion of the genus. There is now a plethora of different molecular techniques available for characterization of the genus. These are increasingly rapid, reproducible and may prove directly applicable to clinical material and the most promising are amenable to adaptation for high throughput analysis and inter-laboratory comparisons. While many early molecular tools such as AFLP, RFLP and genome fingerprinting provided invaluable information on the relationships between species they were unable to resolve epidemiologically meaningful

groups at the sub-species level due to restricted genetic diversity. Indeed until very recently there was no prospect of reliably tracing pathways of transmission using molecular methods. The application of VNTR-based techniques promises to change this but challenges remain. One such challenge is the development and population of worldwide Internet-based databases as the use of standard fingerprinting approaches to compare isolates in large scale national and international studies will facilitate better

understanding of the global epidemiology, phylogenetic structure and population genetics of the group. Progress is being made in this direction; a database for VNTR profiles is already established and we are in the process of developing a multilocus sequencing database that should be available for open access in due course. For the more

local situation, and to facilitate confident epidemiological matching, methods need to be defined for inclusion or exclusion of VNTR matches backed by statistical parameters for acceptance or rejection of the null hypothesis based on improved understanding of population biology, rates of mutation and recombination.

There have been substantial advances in understanding of the diversity of Brucella in recent years and it is clear that tools are now in place that will address many of the above issues. In addition ongoing technological innovations in the bacterial typing arena are certain to provide additional tools of value in improving understanding of this important bacterial group (van Belkum, 2008).

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Khartoum state that comprised of seven localities and encompasses the major modernized dairy farms, which specialized in commercial milk production (Angara *et al*., 2016; appendix 5-6). Khartoum state is located at almost the northeast centre of the country between 15 and 16 degrees latitude north, and between 31 and 32 degrees longitude east (Wikipedia, 2018). This state marks the convergence of the White Nile and the Blue Nile, where they join to form the bottom of the leaning-S shape of the main Nile as it zigzags through northern Sudan into Egypt at Lake Nasser. The northern region of the state is mostly desert because it receives barely any rainfall, whereas the other regions have semi-desert climates. The weather is rainy in the fall, and cold and dry in the winter. The temperature in summer ranges from 25 to 40 °C from April to June, and from 20 to 35 °C in the months of July to October. In winter, the temperature declines gradually from 25 to 15 °C between March and November (Wikipedia, 2018).

Khartoum State is one of eighteen states constituting the Sudan (appendix 5-6). Livestock population estimate in Khartoum State in 2017 was 1, 369, 603 heads (cattle 249,083; camels 6,733; sheep 454, 501 and goats 659,286) as reported by the Federal Ministry of Finance (2002). As it is the most populous area, Khartoum state has the highest demand for milk and milk products. We believe that this high demand constitutes a key reason for the establishment of many private modernized cattle dairy farms. Herds raised in these dairy cattle farms are frequently infected with brucellosis and their milk or milk product present serious health hazard for in contact people as well as for the community.

2.2 Study Population

2.2.1 Animal population

Cattle raised in dairy farms in Khartoum state constitute the study subject. Lactating cows previously tested (Angara *et al*., 2016) positive for Rose Bengal plate test were selected for milk collection. During collection of milk samples, swabs from animals showing clinical signs such as , abortion, retention of placenta were collected, as well as, fluids aspirated from join hygromas for culturing of brucellae. Seropositive animals but do not comply with the above criteria were excluded from selection.

2.2.2 Human population

People working in selected dairy farms and who are farmers, workers or attendees were interviewed.

2.3 Sample Size and Data Collection

2.3.1 Collection of biological materials

541 milk samples were collected from cows previously tested positive for brucellosis (n=39566) ranging from 19-35% (Table 1; Angara *et al*., 2016) during 2014-2015 from 7 localities in Khartoum state, Sudan (Table 2, Fig 3). The sampled animals selected based on history of sero-positivity for RBPT and owner's consent. The sampled animals representing 127 herd distributed in Khartoum state municipalities. 541 Milk samples (20 ml from each selected animal) were collected after the udder was cleaned and disinfected from all functioning teats directly into a sterile 100 ml falcon tubes after discarding the first streams. The milk samples were transported on ice to the National Health laboratory, Federal Ministry of Health, Khartoum-Sudan. Stored at 4° C until processed for culture (within three days).

2.3.2 Collection of KAP data

A total of 150 participants were interviewed during milk sample collection. Verbal consent was obtained before each interview session. A questionnaire was structured to capture knowledge, attitude and practices of interviewed people with regard to acquisition of brucellosis in animals and humans. The questionnaire was divided into two parts. Part one comprised demographic characteristics, herd management practices

and knowledge about brucellosis and causes of abortion. The second part consisted of knowledge of human brucellosis, potential routes of transmission, information on practices posing risk of brucellosis acquisition in humans. Data collected on hard copy and converted into soft copy in SPSS (version 16.0) after coding and entering of them in data sheet ready for analysed.

2.3.3 Culture of brucellae

Milk samples were cultured on modified Thayer Martin medium (mTM) medium as previously described (Marin *et al*., 1996; OIE Terrestrial manual, 2018). The mTM medium was prepared as shown in appendix 8. Culture was performed as milk centrifuged at 3000 xg at 4˚C for 15 minutes and the pellets and deposits were used to inoculate mTM plates. Inoculated plates were incubated in 10% CO_2 atmosphere at 37°C and examined for *Brucella*-like colonies through 2-21 days of incubation.

2.3.4 Biotyping

Culture colonies resembling brucellae were examined with Gram's and modified Ziel-Neelsen's staining methods. Colonies revealing small gram-negative cocco-bacilli were further sent for biotyping at the Animal and Plant Health Agency bacteriology laboratory in Surry, UK (Table 2). Biotyping was performed based on CO_2 dependence; activity of urease and production H_2S, agglutination with A, B and R monospecific specific *Brucella* antisera, growth in the presence of dyes (Basic Fuchsin at 20µl/ml (1/50,000 w/v); Thionin at 20µl/ml & 10µl/ml (1/50,000 w/v & 1/100,000)) and lysis by *Brucella* specific bacteriophages.

Table 1. Information of seropositive cattle sampled for *Brucella* culturing.

Locality*	population *	Sero +ve *	(%)*	No. herds	Size*	Specimen
Karrari	8032	2217	27.6	29	58	milk
Omdurman	7846	1495	19.1	23	79	milk
Umbada	17019	3506	20.6	22	73	milk
Bahri	16188	4937	30.5	32	132	milk
Shargalnile	79777	18668	23.4	16	167	milk
Jabel Awolia	11764	4117	35.0	5	32	milk
Total	143688	39566		127	541	milk

Result summary obtained from (Angara *et al*., 2016).

2.4 DNA Extraction and PCR Assays

Genomic DNA was extracted from all *Brucella* strains from Sudan in this study and those that obtained from the culture collection of the (CVRL) Central Veterinary Research Laboratories, Soba, Sudan isolated during 2005-2015. Extraction was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics), following the manufacturer's instructions. Extracted DNA was quantified and store at -20°C until tested. Whole DNA amplification was performed for samples showing low DNA concentration using the GenomiPhi V2 DNA Amplification Kit following to the manufacturer's instruction (GE Health Care).

2.4.1 *Brucella* specific 16S-23S rDNA interspacer PCR assay

The *Brucella* specific 16S-23S rDNA interspacer region was amplified with primers ITS66 (Appendix 1): ACA TAG ATC GCA GGC CAG TCA and ITS279: AGA TAC CGA CGC AAA CGC TAC as described by Keid *et al.,* (2007). The PCR reaction was performed in 15 µl composed of 1x MyTaq mix (Bioline), 0.4 mM of each primer and 10 ng template DNA. PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing 62˚C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5 min. *Brucella* reference strains (i.e. *Brucella canis*, *B. ovis*, *B. abortus* bv 2, *B. abortus* S19 vaccine and *B. melitensis* Rev1 vaccine; Table 3) were included as controls. Amplification was

performed on a GeneAmp-PCR System 2700 thermal cycler (Applied Bio systems) and PCR products were separated by gel electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 µg/ml, Invitrogen). The DNA bands were visualized under UV-illuminator camera (Bio-Rad) and photographed.

2.4.2 AMOS PCR

The AMOS-PCR was performed as described previously (Bricker and Halling, 1994; Bricker and Halling 1995 and Ewalt and Bricker *et al*., 2000). The PCR mixture consisted of 1X MyTaq mix (Bioline), a combination of five *Brucella* species specific forward primers (0.2 µM each) and reverse IS711 (1 µM), 10 ng DNA in 15 µl reaction volume. The PCR conditions were of an initial denaturation at 95˚C for 30 min followed by 35 cycles of denaturation at 95˚C for 1 min, annealing 60˚C for 2 min and extension at 72˚C for 2 min. *Brucella* reference DNAs and water, as positive and negative controls (respectively) were included. AMOS-PCR is capable of identifying *B. abortus* bv 1, 2 and 4, *B. melitensis* (all biovars), *B. ovis* and *B. suis* bv 1 and *Brucella* vaccine strains. The primers used for this PCR assay were summerized in appendix 4.

2.4.3 Bruce-ladder PCR

Bruce-ladder PCR was performed as described previously (Garcia-Yoldi, 2006 and Lopez-Goni *et al*., 2008). Briefly, a PCR reactions was performed on 15 µl volume composed of 1X MyTaq mix (Bioline), 0.4mM of each primer (8 primer pairs) and 10 ng template DNA. PCR conditions included an initial denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 35 secs, 64°C for 45 secs and 72°C for 3 min and a final extension at 72°C for 5 min on a GeneAmp-PCR System 2700 thermal cycler (Applied Bio systems). *Brucella* reference DNAs and water, as positive and negative controls (respectively) were included. PCR products were separated by gel electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 µg/ml, Invitrogen), and DNA bands visualized under UV-illuminator camera (Bio-Rad) and photographed. The primers used for this PCR assay were summerized in appendix 4.

2.4.4 MLVA assays

The multiple locus variable number tandem repeats (VNTR) analysis (MLVA) was performed to genotype obtained *Brucella* isolates. A set of sixteen pairs of markers specific for *Brucella* were used to amplify VNTR loci. The markers, as described previously (Le Fleche *et al*., 2006), were classified into 2 panels; panel 1 which consisted of eight primers "macro satellite" and panel 2 "microsatellite" (2A three primers and 2B five primers). The primers used for this PCR assay were summerized in appendix 4.

The PCR assay was performed in 15µl reaction volume containing 10 ng of DNA template, 1X MyTaq mix (Bioline) and 0.4µM for each primer. The PCR conditions include an initial denaturation at 96˚C for 2 min followed by 30 cycles of denaturation at 96˚C for 15 secs, annealing 60˚C for 15 sec and extension at 72˚C for 15 sec and final extension at 72°C for 2 min. *Brucella abortus* bv.2 (REF86/8/59 BCCN R5) was included in each PCR run as positive control and double distilled water (ddH_20) as negative control. Amplification was performed on GeneAmp-PCR System 2700 thermal cycler (Applied Bio systems). PCR products were separated by gel electrophoresis on standard agarose gel (2% for panel 1 and 3% for panel 2 markers) stained with ethidium bromide (0.5 µg/ml, Invitrogen). Gene Ruler 100 bp plus (Thermofisher scientific; appendix 7) and low molecular weight ladders (New England BioLabs; appendix 7) were used as molecular size markers for panel 1 and panel 2; respectively. DNA bands visualized under UV-illuminator (Bio-Rad) and photographed.

2.5 Data Analysis and Management

2.5.1 KAP data.

Data gathered using questionnaire administered interview in this study were managed and analysed using Statistical Package Software for social science (SPSS 16.0). descriptive analysis mainly frequencies were used to analysed the data and outputs of analysis were presented in tables.

2.5.2 MLVA data

Gel images were managed using the BioNumerics software package version 6.6 (Applied-Maths, Belgium). PCR product size was converted into copy number of units (loci) following the published allele numbering system as previously described (Le fleche *et al*., 2006). MLVA data were analysed using the character data set within the BioNumerics software. Cluster analysis was performed using the categorical coefficient and the unweighted pair Group method using arithmetic average (UPGMA) method. A different weight was assigned to markers depending on the panel they belong to. Panel 1 markers get an individual weight of 2 (total weight for panel 1: 16), panel 2A markers a weight of 1 (total weight for panel 2A: 3), and markers of panel 2B get a weight of 0.2 (total weight for panel 2B: 1). The MLVA-constructed phylogenetic tree included *Brucella* strains from Sudan along with strains from Africa, Middle East region, as well as from the globe. The results were compared with MLVA published data to identify genetic relationships among *Brucella* strains from Sudan and those from elsewhere. Minimum spanning tree (MST) analysis was also generated using BioNumerics to further demonstrate genetic bonds within *Brucella* strains in this study and those from elsewhere.

CHAPTER THREE

3 RESULTS

3.1 Microbiological Characterization of Brucellae

Isolation of *Brucella* was attempted from 541-milk sample. Isolates from this study (n=14; SN1-14; Table 3) were all shown to be non-motile, gram-negative, oxidase positive acid-fast small rods. Likewise, biotyping of these isolates showed identical traits and hence characterized as *B. abortus* bv 6 except SN 11-12 which were doubtful as either bv 1 or bv 6 due to inconclusive growth on media plates impregnated with thionin dye.

Table 2. Microbiological characteristics of isolates (biotyping).

SN Geog-origin	**locality**	**Growth Characteristics**				**Anti-Sera**			**Phage typing at RDT**						**Species and biovar (bv)**
		Urea	H_2S	CO_2	BF	TH	A	M	Wb	Tb	BK2	Fi	Iz	R/C	
1	K	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
2	Om	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
3	Um	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
4	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
5	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
6	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
7	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
8	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
9	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
10	Sharg	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
11	Ba	+	+	-	+	+/-	+	-	CL	CL	CL	PL	CL	NL	BA bv1/6
12	Ba	+	+	-	+	+/-	+	-	CL	CL	CL	PL	CL	NL	BA bv1/6
13	Ba	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6
14	J	+	+	-	+	+	+	-	CL	CL	CL	PL	CL	NL	BA bv 6

SN: Strain serial number; K: Khartoum locality; Om: Obadda; Um: Umdorman; Sharg: Sharelnel; Ba: Bahri; J: Jabal Awolia; BF: Basic fuchsin dye (at 20 µg, w/v); TH: Thionin dye (at 20 and 40 µg, w/v); (Wb: Weybridge; Tb: Tibilisi; BK_2: Berkingy 2; Fi: Frinzi; Iz: Izantar; R/C) *Brucella* specific bacteriophage; CL: conclusive lysis; PL: partial lysis; NL: no lysis; BA bv 6: Brucella abortus biovar 6

3.2 Molecular Characterization and Genotyping of brucellae

3.2.1 *Brucella* specific 16S-23S rDNA interspacer PCR (ITS)

Molecular characterization was performed for twenty-one *Brucella* isolates from Sudan (Table 4). These include the 14 isolates from this study and seven previously identified *Brucella* strains obtained from the CVRL collection. All 21 isolates were confirmed as *Brucella* organisms using the *Brucella* specific 16S-23S rDNA interspacer region PCR (ITS) as the 214 bp product was amplified (Fig 3). *Brucella* strain SN 8 did not amplify initially but the DNA template was increase with the GenomPhi kit (GE Health Care, USA, where after it produced PCR products). http://www3.gehealthcare.com/en.).

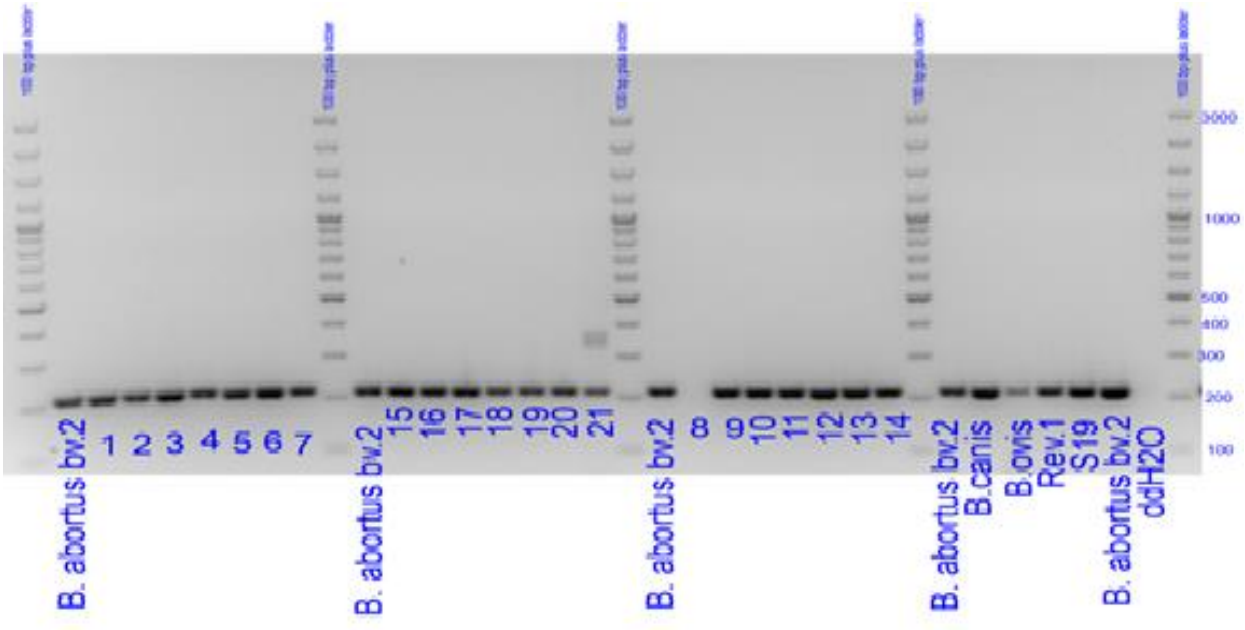


Figure 1. *Brucella* specific 16S-23S rDNA interspacer PCR profile.

Lanes M contain the Gene Ruler 100bp plus DNA marker; lanes *B. abortus* bv.2 (BCCN R5; REF86/8/59); lane *B. canis* (RM6/66); *B. ovis* (REF63/290); Rev.1 (B. *melitensis* Rev1 vaccine stain); S19 (*B. abortus* S19 vaccine strain); lane ddH_2O (negative control), lanes 1-7, 8-14, 15-21 (SN) contains DNA from *Brucella* isolates from Sudan (Table 4).

Table 3. *Brucella* strains and information used in the current study.

(SN)	Strain lab ID	GO	SP	Host	BS and biovar#
1	333_2/14	Khartoum	Milk	Bovine	B. abortus bv 6
2	333_2/15	Khartoum	Milk	Bovine	B. abortus bv 6
3	188_4/14	Khartoum	Milk	Bovine	B. abortus bv 6
4	188_4/15	Khartoum	Milk	Bovine	B. abortus bv 6
5	1/M_15	Khartoum	Milk	Bovine	B. abortus bv 6
6	2/M_15	Khartoum	Milk	Bovine	B. abortus bv 6
7	6_7_15	Khartoum	Milk	Bovine	B. abortus bv 6
8	6_8_15	Khartoum	Milk	Bovine	B. abortus bv 6
9	Braig715	Khartoum	Milk	Bovine	B. abortus bv 6
10	BtBraig715	Khartoum	Milk	Bovine	B. abortus bv 6
11	Soba9_15	Khartoum	Milk	Bovine	B. abortus S19
12	B_Soba 915	Khartoum	Milk	Bovine	B. abortus S19
13	Sarah1114	Khartoum	Milk	Bovine	B. abortus bv 6
14	Sarah1115	Khartoum	Milk	Bovine	B. abortus bv 6
15*	SO_M1_08	Khartoum	LN	Camel	B. melitensis bv 2
16*	SO_M3_05	Darfur	LN	Camel	B. melitensis bv 1
17*	SO_M2_05	Khartoum	Placenta	Bovine	B. melitensis bv.3
18*	SO_BA1_05	Darfur	LN	Camel	B. abortus bv 1
19*	SO_BA6_06	Gazira	Milk	Bovine	B. abortus bv 6
20*	SO_BA3_09	Khartoum	Milk	Caprine	B. abortus bv 3
21*	BMH_14	Gadarif	Blood	Human	B. melitensis bv 1
22**	(BCCN R18)	USA		Dog	B. canis
23**	(BCCN R17)	Australia		Ovine	B. ovis
24**	(BCCN R5)	England	Milk	Bovine	B. abortus bv 2
25**	S19	USA	Milk	Bovine	B. abortus S19
26**	Rev1				B. melitensis rev 1

SN; strain serial number, GO: geographic origin, SP; specimen, BS: Brucella species, BCCN: Brucella culture collection, Nouzilly; S19: Brucella abortus vaccine strain; Rev1: Brucella melitensis vaccine strains LN: lymph node, # Identification based on biotyping; ITS, AMOS and Bruceladder PCR assays; *: isolates obtained from the CVRL-Sudan collection; **: Reference Brucella strains.

3.2.2 AMOS PCR

AMOS-PCR results showed two isolates (out of 21), SN 11-12, identified as *B. abortus* S19 vaccine strain as the eri locus was not amplified in these samples, and three

samples, SN 15-17, identified as *B. melitensis* (Table 4). AMOS PCR did not amplify the other isolates (Fig 4).

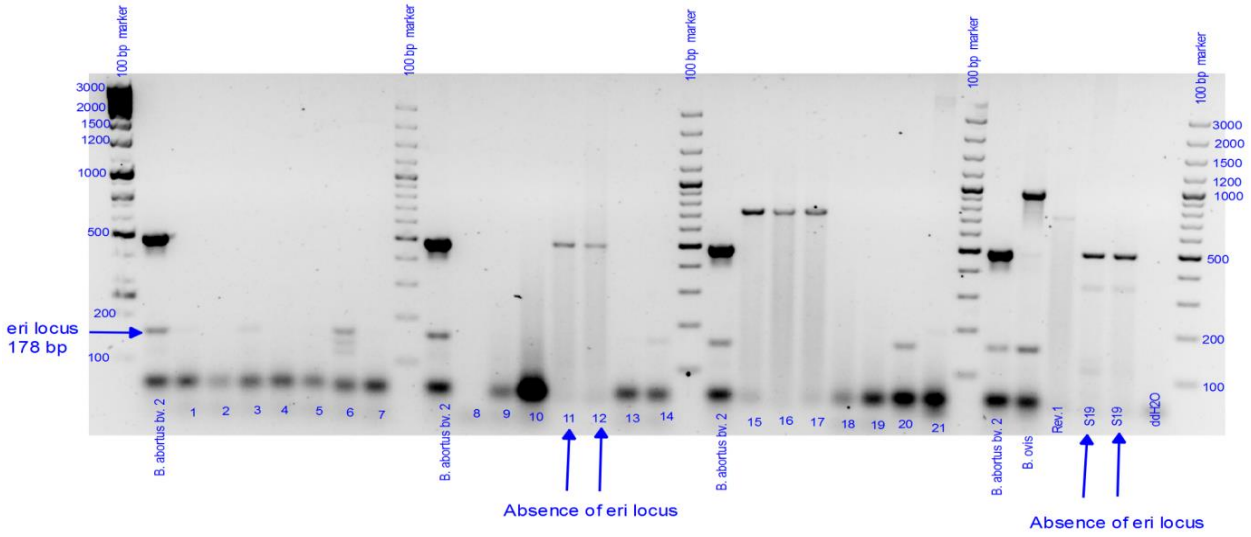


Figure 2. AMOS PCR profile

Lanes 100 bp ruletr plus contain the Gene Ruler 100 bp plus DNA size marker; lanes B. abortus bv 2 (REF REF86/8/59), lane *B. ovis* (BCCN R17) reference strain; Rev.1 vaccine (*B. melitensis* Rev. 1 vaccine stain), S19 (*B. abortus* S19 vaccine strain); lane ddH2O (negative control); lanes 1-7, 8-14, 15-21 (SN) contain DNA from *Brucella* isolates from Sudan shown in Table 3-4.

3.2.3 Bruce-ladder PCR

Bruce-ladder PCR assay confirmed 16 isolates (out of the 21) as *B. abortus* and three as *B. melitensis* (Fig 3). Samples SN 8 and 21 did not amplify due to insufficient DNA and therefore could not be characterized using this *Brucella* species-specific PCR assays.

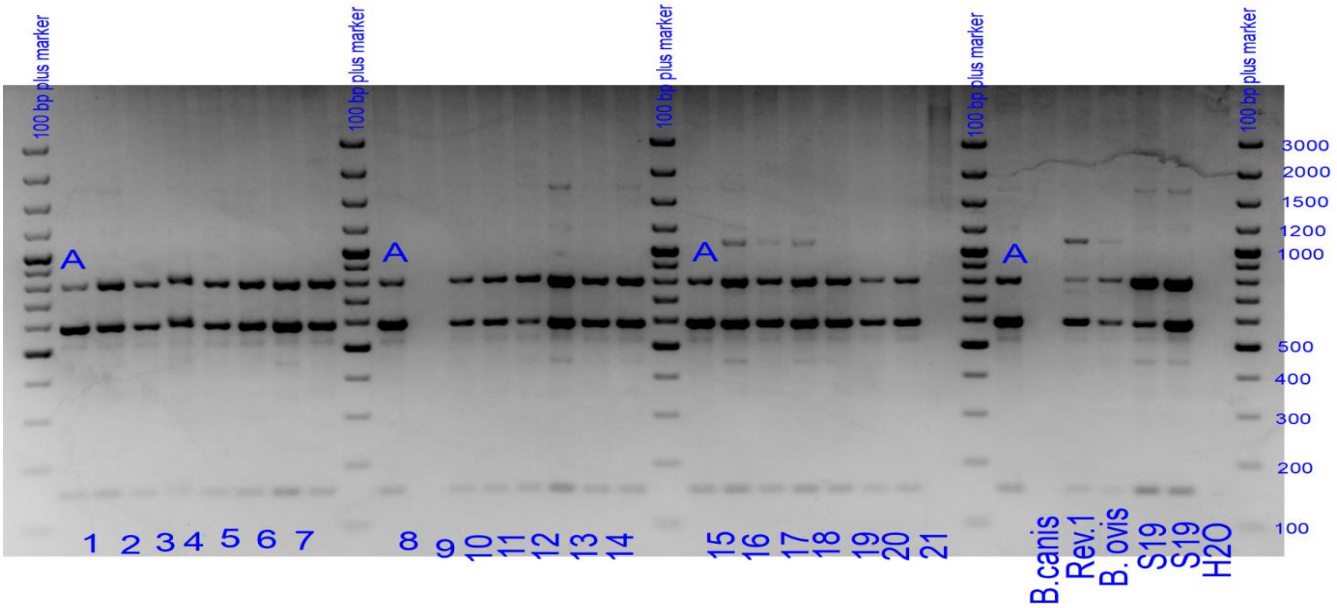


Figure 3. Bruce-ladder PCR profile.

Lanes 100 bp ladder contains the Gene Ruler 100 bp plus DNA size marker; Lanes A consisted of *B. abortus* bv2 (REF REF86/8/59, BCCN R5) reference strain, lane *B. canis* consisted of *B. canis* (RM6/66) reference strain, lane *B. ovis* consisted of *B. ovis* (BCCN R17) reference strain, lane S19 (*B. abortus* S19 vaccine strain); lane ddH2O (as positive and negative controls); lanes 1-7, 8-14, 15-21 consisted of DNA from *Brucella* isolates from Sudan shown in Table 3-4.

3.2.4 MLVA genotyping of brucellae

Beside the 14 isolates obtained in the current study (Table 3) further seven *Brucella* strains were included in this test from the CVRL collection (Table 4). DNA from *Brucella* bv 2 reference strains (Ref86/8/59 BCCN R5) was included for each MLVA PCR. MLVA PCR profile as shown in Fig (4-5) and loci (allele) copy numbers are presented in Table 5. Fig (6-7) showing the resultant phylogenetic tree consisting of *Brucella* strains from this study and from published Brucella MLVA database. The MLVA-16 genotyping system identified ten of Sudan *Brucella abortus* strains as new genotypes three new genotypes from *Brucella melitensis* strains (Fig 6-7).

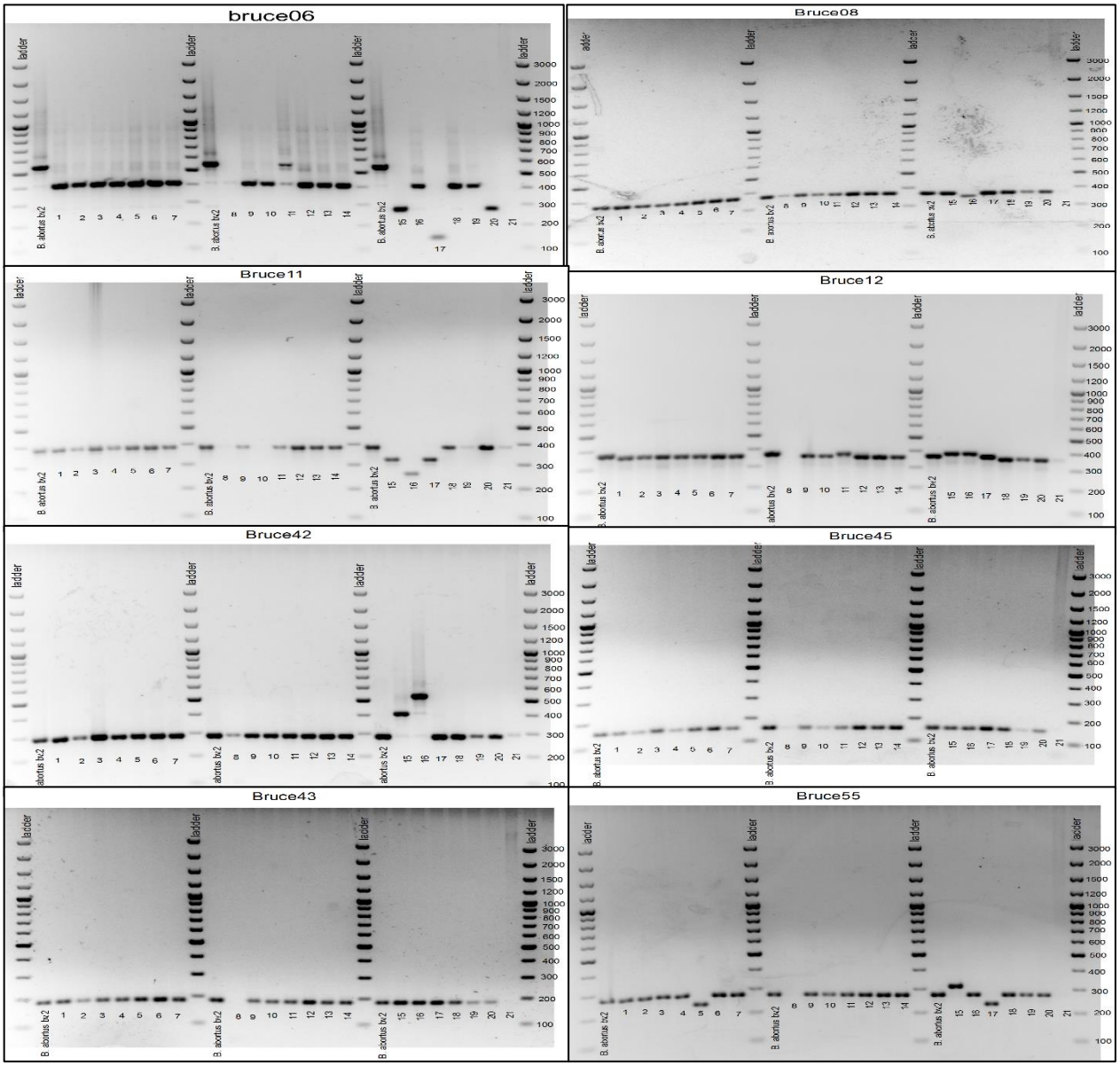


Figure 4. MLVA (Panel1)-PCR profile.

Data shown: Lanes: 1, 10, 19 and 28 contain Gene Ruler 100 bp plus (Thermofisher ltd) molecular size marker for MLVA panel 1. Lanes 2, 11, and 20 contained DNA from *Brucella abortus* bv 2 REF86/8/59 (BCCN R5) included in each PCR run as positive control, Lanes 3-9,12-18 and 20-27 consisted of DNA from *Brucella* strains in this Study

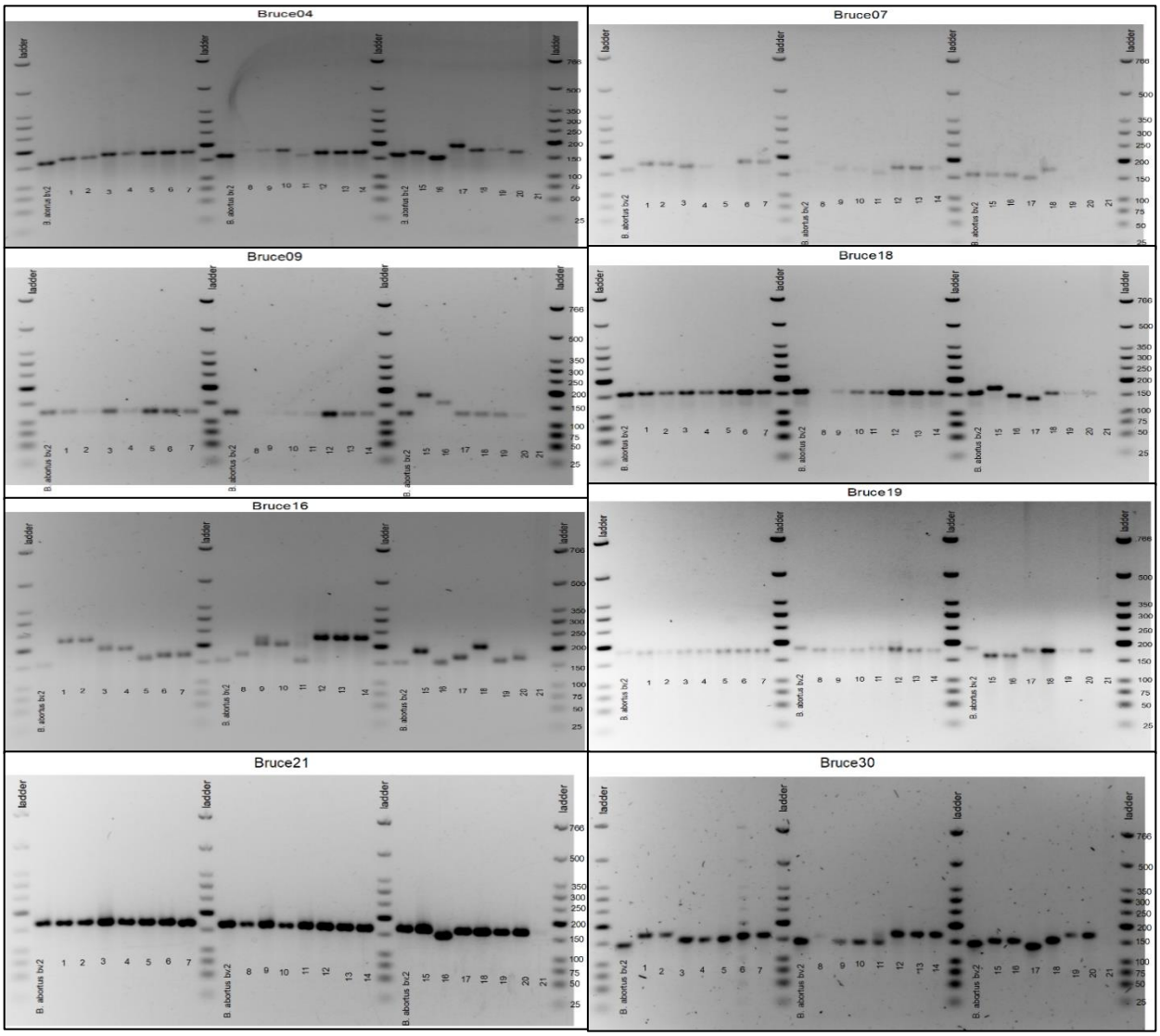


Figure 5. MLVA (panel2A&B)-PCR profile.

Data shown: Lanes: 1, 10, 19 and 28 contain low molecular weight (New England BioLabs) molecular size marker for MLVA panel panel 2. Lanes 2, 11, and 20 contained DNA from *Brucella abortus* bv 2 REF86/8/59 (BCCN R5) included in each PCR run as positive control, Lanes 3-9,12-18 and 20-27 consisted of DNA from *Brucella* strains in this Study.

Table 4. VNTRs copy number for isolates in the current study.

SN.	Panel 1 markers								Panel 2A markers			Panel 2B markers				
	Bru06	Bru08	Bru11	Bru12	Bru42	Bru43	Bru45	Bru55	Bru18	Bru19	Bru21	Bru04	Bru07	Bru09	Bru16	Bru30
1	3	5	4	10	2	3	3	3	6	44	8	4	7	3	13	8
2	3	5	4	10	2	3	3	3	6	44	8	5	7	3	13	8
3	3	5	4	10	2	3	3	3	6	43	8	5	6	3	9	6
4	3	5	4	10	2	3	3	3	6	43	8	5	6	3	9	6
5	3	5	4	10	2	3	3	2	6	43	8	5	6	3	5	6
6	3	5	4	10	2	3	3	3	6	44	8	5	9	3	6	7
7	3	5	4	10	2	3	3	3	6	43	8	5	9	3	6	7
8	3	5	4	10	2	2	3	3	6	42	8	5	9	3	6	7
9	3	5	4	10	2	2	3	3	6	42	8	5	6	3	9	5
10	3	5	4	10	2	2	3	3	6	42	8	5	6	3	9	5
11	4	5	4	10	2	2	3	3	6	43	8	3	5	3	3	5
12	3	5	4	10	2	2	3	3	6	40	8	4	7	3	13	8
13	3	5	4	10	2	2	3	3	6	40	8	4	7	3	13	8
14	3	5	4	10	2	2	3	3	6	40	8	4	7	3	13	8
15	2	5	3	12	3	2	3	4	7	35	8	4	5	11	7	6
16	3	4	2	12	4	2	3	3	5	40	8	2	5	8	3	6
17	1	5	3	11	2	2	3	2	4	40	8	7	4	3	3	3
18	3	5	4	10	2	2	3	3	6	40	8	5	7	3	9	6
19	3	5	4	10	2	2	3	3	6	40	8	5	4	3	4	10
20	2	5	4	10	2	2	3	3	6	42	8	4	7	3	5	10
24*	4	5	4	11	2	2	3	3	6	47	8	3	5	3	3	5

*: *Brucella* Bru: Bruce (marker given name)*abortus* bv 2 (REF86/8/59 BCCN R5); SN: strain serial number.

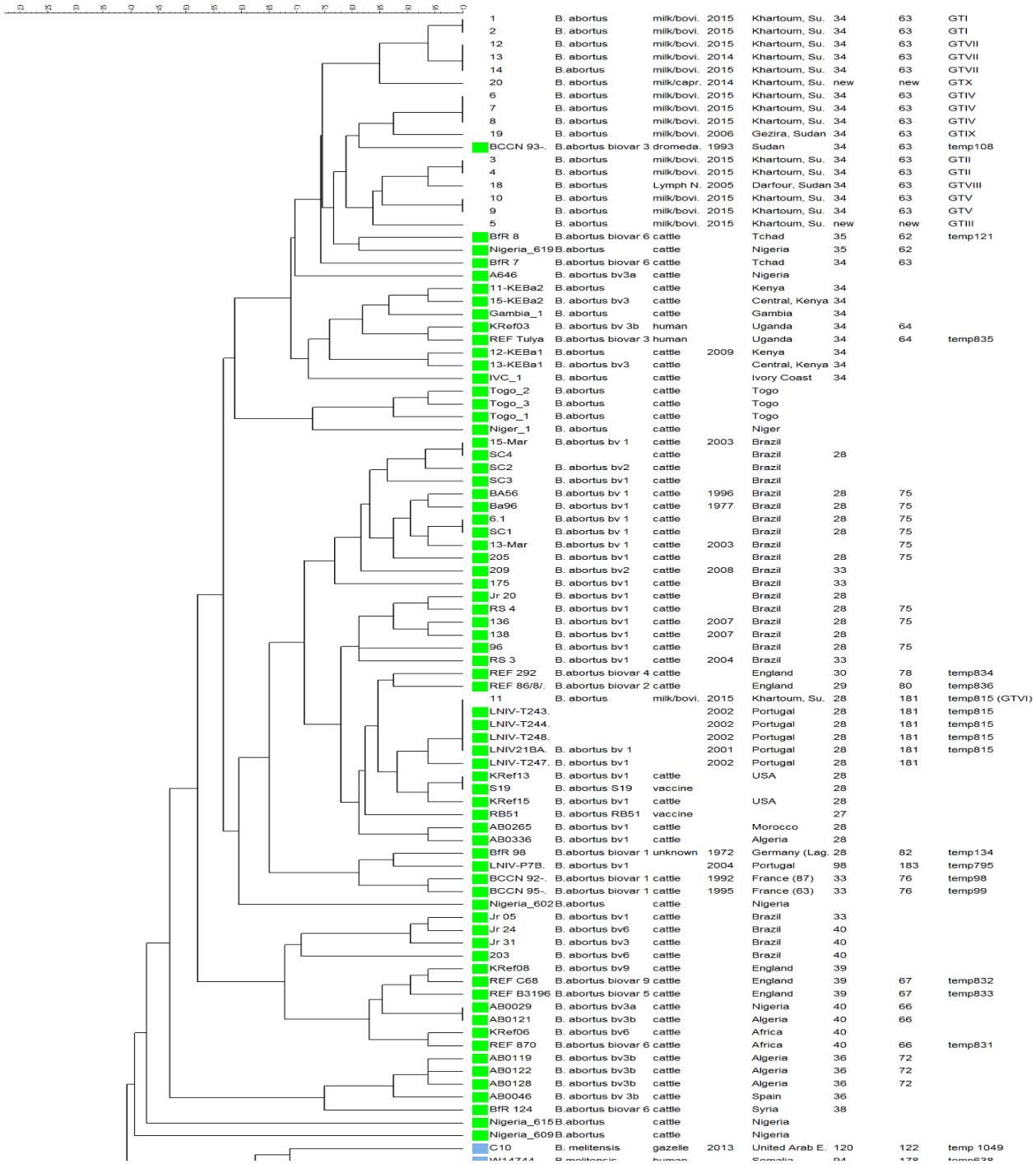


Figure 6. *Brucella abortus* section of MLVA-16 dendrogram.

Using UPGMA method of (85 out of 162) selected *B. abortus* and *B. melitensis* strains including the Sudan strains. Data shown in the columns: identification numbers, species-biovar, host species, year, geographical location, MLVA 8-, 11- and 16 genotype. The green colour indicates the *B. abortus* strains with the Sudan strains from this study indicated by white colour.

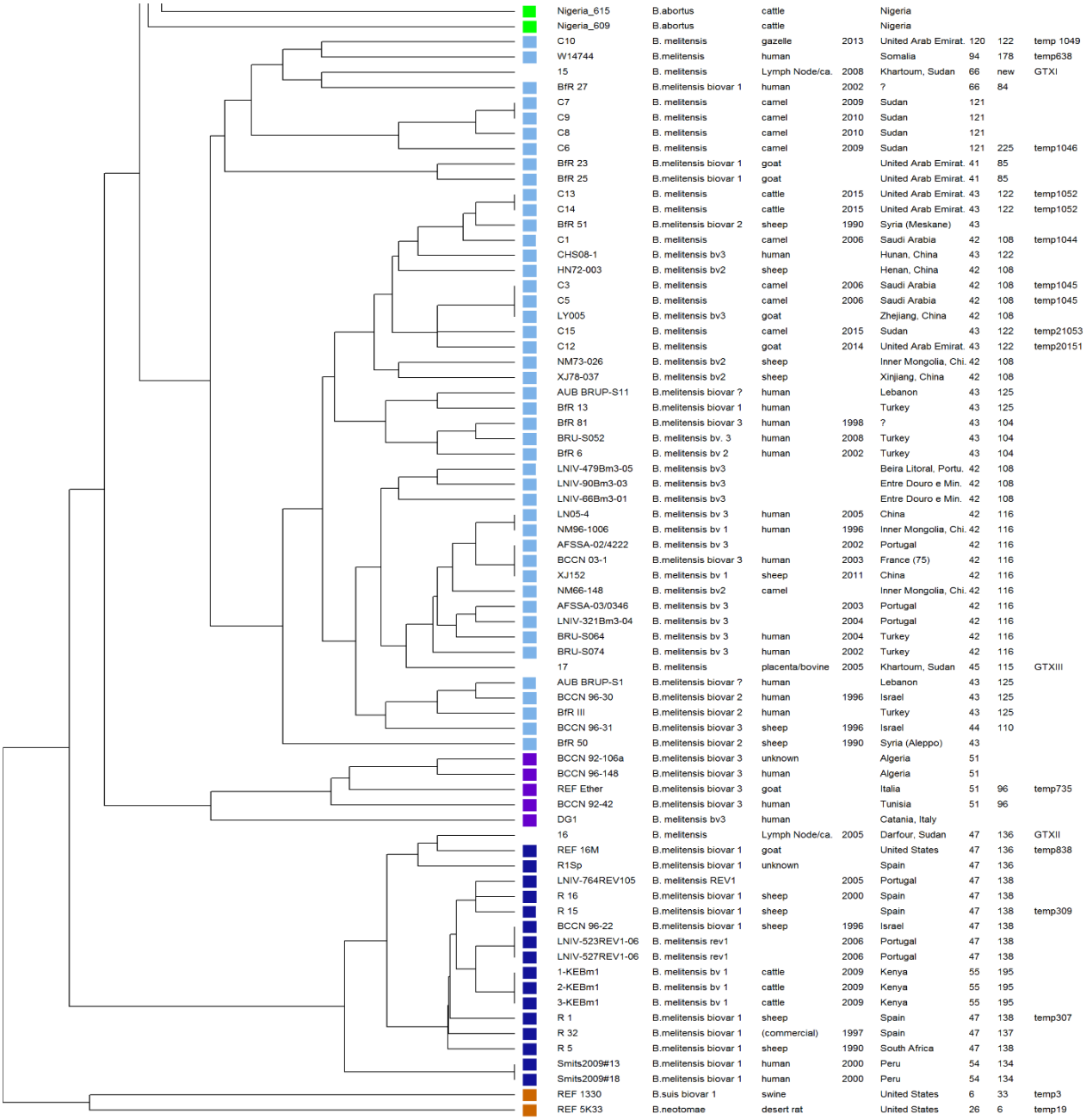


Figure 7. *Brucella melitensis* section of MLVA-16 dendrogram.

Using UPGMA method of (85 out of 162) selected *Brucella melitensis* strains including the Sudan strains. The columns show the identification numbers, species-biovar, host species, year, geographical location, MLVA 8-, 11- and 16 genotype. The dark blue, purple and light blue colour indicates the American, western Mediterranean and eastern Mediterranean *B. melitensis* strains with sudan strains in white, respectively. Reference strains for *B. suis* bv 1 (REF 1330) and *B. neotomae* (REF 5K33) in brown were included as outgroups.

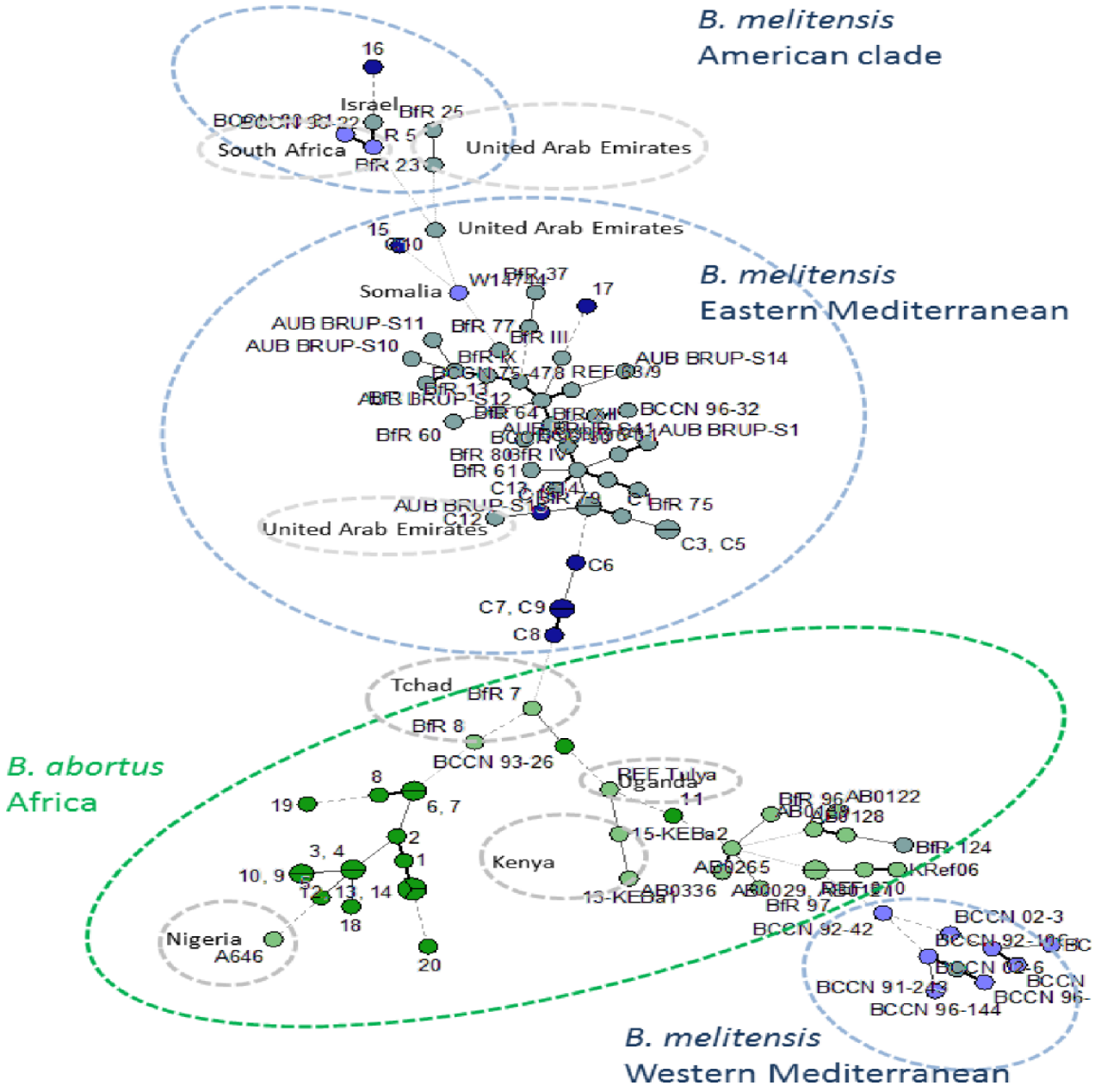


Figure 8. MLVA 16 minimum spanning tree for selected *Brucella* strains.

The different species marked by different colour, and geographic origin written. *Brucella melitensis* strains from Sudan were marked in dark blue and those from African countries were in light blue, while *Brucella melitensis* from Middle East countries in grey, *Brucella abortus* from Sudan is marked in dark green colour, *B. abortus* from other African countries were in light green colour.

3.3 Knowledge and perception about brucellosis

3.3.1 Herd management mal practices associated with animal brucellosis.

Mal practices associated with acquisition of brucellosis of brucellosis in animals were listed in Table 6 below. 85.3% (128 out 150) of respondents do not provide specific delivery space, 99.3% apply natural insemination for herd breeding (Table 5).

Table 5. Herd management risky practices associated animal brucellosis.

Practice	Responses	No.	Percentage
Type of herd	Singe breed	98	65.3
	Mixed breeds	52	34.7
Breed	Cross breed	100	66.7
	Local breed	50	33.3
Keeping animals	Mixed	40	26.7
	Age-separated	100	66.7
	Sex-separated	10	6.7
Presence of pets	Yes	97	64.7
	No	53	35.3
Breeding	NI	149	99.3
	AI	1	.7
Bull ownership	Have a bull	127	84.7
	borrow one	23	15.3
Presence of specified delivery room	Yes	22	14.7
	No	128	85.3
Cleaning of food trough	Yes	85	56.7
	No	65	43.3
In cases of abortion	use antibiotics	61	40.7
	consult a vet	59	39.3
	do nothing	30	20.0
Disposition of abortion materials	By burying	14	9.3
	Take it to remote area	130	86.7
	Nothing	6	4.0

56.7% do not clean food troughs and they make no action towards abortion cases and abortion materials (20% and 4%, respectively).

3.3.2 Risky practices associated with acquisition of brucellosis in humans

The results (as shown table 6 below) show that 94.7% of respondents do not wash their hands before and after milking, 78.7 % milk animals without protecting gloves even when they have and cuts, but fortunately only 29.3% and 38% of the participants do not consume raw meat and milk, respectively.

Table 6. Risky practices associated with brucellosis in humans.

Practice	Response		Number	Percentage
Consumption of raw milk	Yes		57	38.0
	No		93	62.0
Eating raw meat	yes		44	29.3
	no		106	70.7
Milk while having hand cuts	Yes without gloves		118	78.7
	Yes with gloves		32	21.3
Hygiene measures	Washing hands before and after milking	Yes	8	5.3
		No	142	94.7
	Cleaning and washing udder before milking	Yes	9	6
		No	141	94
	Wearing gloves before milking	yes	1	0.7
		No	149	99.3

3.3.3 Knowledge about animal brucellosis.

Regarding knowledge about animal brucellosis, the majority of participants claimed that they do not know what causes abortion; although 55.3% of them mentioned that, they heard about brucellosis. Despite hearing about brucellosis, only 20% of the participants mentioned that they are aware of brucellosis as a cause of abortion and only one participant knows that brucellosis affects the fertility of infected animals (Table 7).

Table 7. Information regarding knowledge animal brucellosis

Knowledge	Response	Number	Percentage
Do you know the causes of abortion	Yes	43	28.0
	No	107	71.3
Total		100	100%
If yes, what are the causes	Heat	2	4.7
	Cold	14	32.6
	nutrition	12	27.9
	diseases	13	30.20
	Trauma	2	4.6
	Total	43	100%
Have you heard about brucellosis	Yes	83	55.3
	No	67	44.7
What are the signs of brucellosis in animals	causes still birth	53	64
	causes fever	1	1.2
	causes abortion	17	20.4
	causes diarrhea	1	1.2
	affects man and animal	10	12.0
	causes infertility	1	1.2
	Total	83	100%
What animals affected by brucellosis	not sure	84	56.0
	cattle	47	31.3
	cattle, sheep and goats	19	12.7
How do you identify brucellosis-infected animal	do not know	105	70.0
	by abortion	30	20.0
	decrease productivity	9	6.0
	by lab testing	4	2.7
	by animal death	1	.7
	by loss of appetite	1	.7

How brucellosis is transmitted	not sure	126	84.0
	natural insemination	11	7.3
	contaminated food and water	13	8.7
How brucellosis could be prevented	Not sure	112	74.7
	artificial insemination	5	3.3
	vaccination	16	10.7
	Isolating infected animals	17	11.3

This lack of knowledge about brucellosis reflected in that 70% of the participant cannot identify brucellosis-infected animals, 84% do not know how the disease is transmitted and 74.7% do not know how brucellosis is prevented.Knowledge about human brucellosis

Regarding knowledge about human brucellosis, high percentages of respondents to the questionnaire confessed that they used to milk animals without protective clothing even if they have had hand cuts (78.7%). Moreover, they also used to consume raw milk and meat (62% and 70%%, respectively). The participants also expressed that they have no knowledge on the disease is transmitted to humans (59.3%) and 58.7% do know its signs (Table 8).

Table 8. Knowledge of participants about human brucellosis.

Feature	Response	Number	Percentage
Milking while having hand cuts	Without gloves	118	78.7
	With gloves	32	21.3
Drinking raw milk	No	57	38.0
	Yes	93	62.0
Eating raw meat	No	44	29.3
	Yes	106	70.7
	yes	69	46.0

Does brucellosis infect people	no	81	54.0
How does it transmitted to humans	By insect bit	10	6.7
	by drinking of raw milk	46	30.7
	by contact with infected animal	5	3.3
	do not know	89	59.3
What are the signs	do not know	88	58.7
	fever	28	18.7
	Back pain	17	11.3
	Night sweating	3	2
	Arthralgia	5	3.3
	Headache	9	6

CHAPTER FOUR

DISCUSSION

Brucellosis is considered one of the most important bacterial zoonosis worldwide. Therefore the disease has drawn attention of stakeholders in animal production systems and economists in many countries due to its economic impacts (McDermott *et al*., 2013). According to available data, brucellosis in Sudan is caused by biovars of *Brucella abortus* and *B. melitensis* affecting most domestic livestock species (i.e. cattle, sheep and goats, camels and equines) and humans. However; despite the fact that bovine brucellosis proved endemic in Sudan early in the nineteenth century; there is no control strategy currently being put in place. This situation is not ideal and we hope that the findings and recommendations of this study and previous ones will benefit forthcoming control programmes.

High-resolution phenotypic and molecular approaches have been developed for *Brucella* speciation, biotyping, and epidemiological trace-back (Lopez-Goni *et al*., 2008; Le fleche *et al*., 2006). To date, advanced molecular technologies have not been widely used in low-income countries where brucellosis is endemic (McDermott and Arimi, 2002;). Thus, information about the prevailing *Brucella* species, biovars, and genotypes in such areas of endemicity will provide insight on brucellae and extends knowledge on their epidemiology. Increased understanding of the *Brucella* epidemiology in local context is critical for refining control approaches in resource weak countries, where costly measures cannot be applied.

B. abortus bv 6 identified from all isolated from cattle in this study. This finding is in agreement with previous studies from various regions in Sudan reporting isolation and identification of *B. abortus* bv 6 from different animal species. For instance, *B. abortus* bv 6 isolated from cattle in western, eastern and central parts of Sudan, (Musa *et al*., 1990; Omer *et al*., 2010 ;this study; respectively). As well from camels in western and eastern provinces (Musa *et al*., 2008; Omer *et al*., 2010). From sheep in eastern

provinces (Gumaa *et al*., 2014). These findings indicate that *B. abortus* bv 6 is the predominant biovar circulating in livestock in this country.

Moreover; *B. abortus* bv 6 idetified in this study were not typical bv 6 since they showed unusual biotyping profile. Similar biovars were reported previously from cattle and camels (Musa *et al*., 2008; Gumaa *et al*., 2014). These biovars showed variable sensitivity to grow in a medium containing the thionin dye; a common feature for this biovar. It seems that the strains reported by Musa *et al*. and Gumaa *et al*.,(2008 and 2014; respectively) might have had a common origin with the strains from this study. Fuethermore, these findings give rise to the speculation that *B. abortus* bv 6 are likely to be the pricipal cause of animal brucellosis in Sudan.

Brucella abortus strains from Sudan possessing genetic bonds with strains isolated from neighbouring countries, like Tchad and Central Republic of Africa. It is valid to speculate that these *Brucella* strains might have spread between Sudan and its neighbouring countries. This could also be true, since there are some studies reported the isolation of the same *B. abortus* strains from these African countries (Domenech *et al*., 1983). Likewise, *Brucella* strains isolated from Nigeria (Bertu *et al*., 2015), Kenya and Central Republic of Africa (Behnke Roy, 2011) found to resemble Sudanese strains, emphasizing the speculation that these strains have had spread between Sudan and its neighbours through cross-border animal movement. This finding is in agreement with our research hypothesis.

Atypical strains of *B. abortus* bv 6 from Sudan were partially insensitive to thionin dye. This could be due mutation occurring due to freguent passage among animals. It is unfortunate that it was not possible to obtain DNA for atypical isolates reported earlier by other authors, and hence it was not possible to correlate their genetic inheritances with isolates in the current study.

B. melitensis previously isolated from sheep and goats in Sudan (Musa and Jahans, 1990) could have spread from sheep and goats to cattle (SN 17) and camel (SN 15 and 16). This assumption is highly likely, regarding the traditional grazing system in Sudan, where the majority of livestock herds intermix and share open grazing lands. This

assumption is inline with those speculated by Agab *et al*., 1994; Musa *et al*., 2008; Gumaa *et al*., 2014 and Omer *et al*., 2010.

Although culture and biotyping remain the gold standard approach for diagnosis and identification of brucellosis, these methods are time-consuming and difficult to interpret (OIE manual, 2018; Alton *et al*., 1988). *Brucella* species-specific PCR assays like AMOS and Bruce-ladder could be useful for identification of *Brucella* spp. in resource-limited countries like Sudan. These assays allow for rapid identification, speciation and differentiation of most *Brucella* species and biovars. In this study, *B. abortus* isolates SN 11-12 (Table 3) were doubtful in biotyping results as either *B. abortus* bv 1 or 6, were identified as *B. abortus* S19 vaccine strain by AMOS PCR (Fig 2). This was demonstrated by the absence of the eri locus that exists in other *Brucella* strains. However, AMOS could not identify *B. abortus* bv 6 and 3, which were identified previously in Sudan and in this study by biotyping and Bruce-ladder PCR (Fig 3). This indicates that Bruce-ladder, beside biotyping, are more suitable for characterization of *Brucella* spp. in Sudan. Regarding *B. abortus* vaccine strain in this study (SN 11-12), the farm owners, from where S19 vaccine strain was isolated, confessed that beside calves they usually vaccinate adult cows when neighbouring farms experience abortion storms "believed to have been due to brucellosis". The fining of isolatioo on S19 in this study is in agreement with those published by Thomas *et al*., 1981; Nicoletti, 1981.

Moreover; isolation of *B. abortus* S19 vaccine strain from milk of dairy cattle pose risk of infection for humans. Interestingly, this viewpoint is in agreement with the results of Osman *et al*., 2015, who reported cases humans infected with *B. abortus* S19 in Sudan among farm workers who used to consume raw milk while not aware of the risk of brucellosis. Human brucellosis caused by live vaccine from secretions of vaccinated animals is in agreement with our research hypothesis.

The MLVA analysis revealed the presence of thirteen genotypes (GT) comprising of *B. abortus* and *B. melitensis*. Genotypes from *B. abortus* strains in this study showed identical or close genetic profiles, indicating that these strains might have spread among farms. This is evident from strains (SN 1-10, 12-14), which share and/or cluster with

genotype (GT) 34 MLVA 8 and GT 63 MLVA 11 (Fig 6). These similarities are also supported by field information noted during sample collection that farmers trade cattle based on productivity, while many of them ignore the risk of brucellosis. Therefore, this people might have introduced new animals without being pre-tested for brucellosis. The MLVA 16 analysis revealed that the majority of *B. abortus* strains investigated in this study (Fig 6) sharing the 34 MLVA 8 and 63 MLVA 11 GT with *B. abortus* strains from neighbouring country Tchad (BfR8 *B. abortus* bv 6) and its neighbour Nigeria (Nigeria_619 *B. abortus*). This indicates that these genotypes could have spread between Sudan and these countries through animal movement.

Likewise, *B. melitensis* bv 2 (SN15) reported from camels in Sudan, clustered with *B. melitensis* strains isolates from United Arab Emirates "UAE" and Somalia (Fig 7). This indicates that these *B. melitensis* strains might have spread through trade with UAE and African countries like Somalia. This assumption is in agreement with our research hypothesis, as well as with the findings of Radwan *et al*., (1983), who reported isolation of *B. melitensis* from camels in Saudi Arabia imported from Sudan. Similarly, Gyuranecz *et al*., (2016) reported genotypes recovered from camels imported to UAE from Sudan. These findings affirm our research hypothesis.

Brucella genotypes have a worldwide distribution, which indicates the spread of these bacteria through trade with amongst countries. In one hand, the (WITs) World Integrated Trade Solutions (2018) reported that live animals have been imported to Sudan from New Zealand, Netherlands, Saudi Arabia, United Arab Emirates, Egypt, France, Jordan, Brazil, Turkey, Portugal and China. In the other hand, Sudan export animals mainly to Saudi Arabia, UAE, Egypt, France, Jordan and Turkey. Trade could be responsible for the introduction of some of these strains like SN 11 that had identical MLVA16 GT profile with *B. abortus* strains from Portugal "LNIV-T243BA1/02, LNIV-T244BA1/02 and LNIV-T248BA1/02" (Fig 6; Ferreira *et al*., 2012).

Regarding *B. abortus* bv 3 in this study (positive for urease and negative for oxidase activity), and based on MLVA genotyping system, they belonged to the 3a subgroup non-Mediterranean African *Brucella abortus* biovar 3 (including the reference strain

Tulya as reported by Ocampo-Sosa *et al.*, (2005) and co-workers. It worth mentioning that *B. abortus* bv 6 and *B. abortus* bv 3 from Sudan grouped in one cluster (Fig 6) along with *B. abortus* bv 3 (BCCN 93-26) previously reported by Khames *et al*., (2017). *B. abortus* bv 3 (BCCN 93-26) classified with the 3a non-Mediterranean African cluster. In this regard, the Sudan *B. abortus* bv 3 strains possess 3 repeats of the MLVA bruce55 marker (Table 5) similar to those published by Khames and co-workers (2017). This finding propose that these strains might belong to a third subgroup of the *B. abortus* bv 3, which was previously classified into two subgroups (Ocampo-Sosa *et al*., 2005). Khames *et al.,* (2017) concluded that, isolates of this unique biovar 3 subgroup are distinct from the European isolates and those of countries across the Sahara, giving rise to assumption that they may represent a new African lineage.

The results also revealed low knowledge, attitude and practices (KAP) regarding bovine brucellosis, and poor understanding of the zoonotic nature of the disease among smallholder dairy farms community. These findings are absolutely in line with our research hypothesis. the endemicity of brucellosis in Sudan was previous reported (Dafaalla and Khan, 1958; Musa and Jahans, 1990; Agab *et al*., 1994; Ismail, 2007; Omer *et al*., 2010; Gumaa *et al*., 2014; Wilson, 2018; and this study). This endemicity along with low knowledge and risky practice of people working animal production sector, could represent a serious health hazard to these people. Research studies considering KAP, like the current one, are limited. Such researches will facilitate better understanding of the problem, and hence, contribute to better planning for surveillance, management and control of brucellosis in the country.

Despite that more than half (56.7%; Table 8) of the study population have heard about bovine brucellosis, only 12% were aware of its zoonotic nature (Table 9). This finding could result in enormous human morbidity as farmers and workers would likely to perform risky practices in the farm level while milking or handling infectious materials. It is worth mentioning that despite 55.3% of participants who claimed have heard of bovine brucellosis, 56.7% recognized abortion as one of its clinical signs. Nevertheless, the majority of them were not sure, how the disease is transmitted (84%)

nor how it could be prevented (74.7%; Table 8). These findings completely agree with this research hypothesis. Education of people about brucellosis is very important element for an integrated brucellosis control program. It is crucial that farmers and animal workers should be educated about brucellosis if spread of the disease to both animals and humans hosts is to be avoided. A "One Health" framework applied to brucellosis should include stakeholders from farmers, the medical, veterinary, wildlife and sociological disciplines to provide their inputs as to come about all-inclusive perception of the disease (Godfroid *et al*., 2011).

Regarding knowledge about human brucellosis, the majority of participant have low knowledge and poor understanding of the disease. For instance, 62% and 60.7% (Table 9) of the participants confessed that they used to drink raw milk and eat raw meat, respectively. This attitude does not seem strange as more than half (60.7%) of the participants were not aware that brucellosis infects people. Similar study in Ethiopia showed that none of the participants in that study were aware of the zoonotic importance of brucellosis (Tesfaye *et al*., 2013). The zoonotic aspect of brucellosis is mostly favoured by the lack of awareness of the disease among pastoralists, the scarce collaboration between different sectors and the small investment in the control of the disease by governments in developing countries (Ntirandekura *et al.*, 2018).

CONCLUSION AND RECOMMENDATIONS

Conclusion

- This study emphasizes that *Brucella abortus* bv 6 is the predominant biovar circulating in livestock in Sudan.
- Brucellae in Sudan cannot be detected by the *Brucella* species-specific AMOS PCR assay; therefore, Bruce-ladder PCR is a better option since it is capable to overcome limitations associated with AMOS. Further, AMOS ERY reported by Ocampo-Sosa *et al*. (2005) seems ideal due to its ability to differentiate a wider range of all Brucella spp. including subgrouping of B. abortus bv 3.
- The *Brucella* strains between Sudan and other countries through animal movement.
- Research studies considering KAP and molecular epidemiology of brucellosis, like the current one, are limited.
- Most people working in dairy farms are illiterate, have very few knowledge on the nature of animal disease including brucellosis
- Findings of this research could contribute to brucellosis control in the country.

Recommendations

- Bruce-ladder, beside biotyping, are more suitable for characterization of *Brucella* spp. in Sudan. In addition, the modified AMOS-ERY PCR, which is "not performed in this study" capable of differentiating *B. abortus* biovar 5, 6 and 9 and the new subgroup 3b of biovar 3 as well as other *Brucella* spp. (Ocampo-Sosa *et al*., 2005).
- A national-wide surveillance to identify all existing *Brucella* spp. is a vital step forward in the course of control of brucellosis.
- Research studies considering KAP, like the current one, are highly recommended as such researches will facilitate better understanding of the problem, and hence, contribute to better planning for surveillance, management and control of brucellosis in the country.
- Implantation of strict biosafety measures on animal movement should further spread and/or exchange of this pathogen between Sudan and other countries is to be avoided.

References

Abbas, B., Agab, A. (2002). A review of camel brucellosis. *Prev. Vet. Med*, 55: 47-56.

Abdalla, Z.A.A., Baleela, R.M.N. (2017). Detection and identification of *Brucella* species infecting camels in Darfur, Sudan. *Sud. J. Sci*, 9:2. http://ejournals.uofk.edu/index.php/SJS .

Abdulla, A. (1966). Incidence of brucellosis in Wadi Halfa District. *Sud. J. Vet. Sci.Anim. Husb*, 7: 28-31 .

Agab, H., Abbas, B., El-Jack, A.H., Maoun, I.F. (1994). First isolation of *Brucella abortus* biovar 3 from camel (*Camelus dromedarius*) in the Sudan. *Rev. Elev. Med. Vet. Pays. Trop*, 47(4):361-3.

Al Dahouk, S., Fleche, P.L., Nockler, K., Jacques, I., Grayon, M., Scholz, H.C., Tomaso, H., Vergnaud, G., Neubauer, H. (2007). Evaluation of Brucella MLVA typing for human brucellosis. *J. Microbiol. Methods*, 69: 137-145 .

Al Dahouk, S., Hagen, R.M., Nockler, K., Tomaso, H., Wittig, M., Scholz, H.C., Vergnaud, G., Neubauer, H. (2005b). Failure of a short-term antibiotic therapy for human brucellosis using ciprofloxacin. A study on in vitro susceptibility of *Brucella* strains. *Chemotherapy*, 51: 352-356.

Al Dahouk, S., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W.D., Scholz, H.C., Neubauer, H. (2005a). Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *Crit. Rev. Microbiol*, 31: 191-196.

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M. (1988). Techniques for the Brucellosis Laboratory. INRA, Paris, France.

Angara, T.E., Ismail, A.A., Saeed, S.N. (2014) Seroprevalence of human brucellosis in Kuku Dairy Scheme, Khartoum State, Khartoum. *J. Life Sci*, 8: 811-814.

Angara, T.E.E., Ismail, A.A.A., Ibrahim, A.M., Osman, S.Z. (2016) Assessment of the economic losses due to bovine brucellosis in Khartoum state, Sudan. *Int. J. Tech. Res. Appl*, 7(2): 85-90.

Bakheit, M.R. (1981). Brucellosis in crossbred cattle. *Sud. J. Vet. Sci. Anim. Husb*, 3: 119-120

Bennet, S.G. (1943). *Annual Report of Sudan Veterinary Services*, 29-32.

Bertu, W.J., Ducrotoy, M.J., Muuoz, P.M., Mick, V., Zúñiga-Ripa, A., Bryssinck, W., Kwaga, J.K.P., Kabir, J., Welburn, S.C., Moriyon, I., Ocholi, R.A. (2015) Phenotypic and genotypic characterization of *Brucella* strains isolated from autochthonous livestock reveals the dominance of *B. abortus* biovar 3a in Nigeria. *Vet. Microbiol*, 180 (1-2): 103-108. https://doi.org/10.1016/j.vetmic.2015.08.014

Bricke, B.J. (2002). PCR as a diagnostic tool for brucellosis. *Vet. Microbiol*, 90: 435-446.

Bricker, B.J., Ewalt, D.R. (2005). Evaluation of the HOOF-Print assay for typing *Brucella abortus* strains isolated from cattle in the United States: results with four performance criteria. *BMC Microbiol*, 5:37. doi:10.1186/1471-2180-5-37. Available from: http://www.biomedcentral.com/1471-2180/5/37.

Bricker, B.J., Ewalt, D.R., Halling, S.M. (2003). *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiol*, 3 (15). Available from: http://www.biomedcentral.com/1471-2180/3/1

Bricker, B.J., Ewalt, D.R., MacMillan, A.P., Foster, G., Brew, S. (2000). Molecular characterization of *Brucella* strains isolated from marine mammals. *J. Clin. Microbiol*, 38: 1258-1262.

Bricker, B.J., Halling, S.M. (1994). Differentiation of *Brucella abortus* bv 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv 1 by PCR. *J. Clin. Microbiol*, 32: 2660-2666.

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

Capasso, L. (1999). Brucellosis at Herculaneum (79 AD). *Int. J. Osteoarchaeol*, 9: 277-288.

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

Capasso, L. (2007). Infectious disease and eating habits at Herculaneum (1st century AD, Southern Italy). *Int. J. Osteoarchaeol*, 17:250-257.

Catlin, J.E., Sheehan, E.J (1986). Transmission of bovine brucellosis from dam to calve. *J. Am. Vet. Med. Assoc*, 188: 867-9.

Chain, P.S., Comerci, D.J., Tolmasky, M.E., Larimer, F.W., Malfatti, S.A., Vergez, L.M., Aguero, F., Land, M.L., Ugalde, R.A., Garcia, E. (2005).Whole-genome analyses of speciation events in pathogenic Brucellae. *Infect. Immun*, 73: 8353-8361. doi:10.1128/IAI.73.12.8353-8361.2005

Christopher, G.W., Agan, M.B., Cieslak, T.J. (2005). History of US military contributions to the study of bacterial zoonoses. *Mil Med*, 170 (4 Suppl):39-48.

Cirincione J, Wolfsthal JB, Rajkumar M. Deadly Arsenals: Tracking Weapons of Mass Destruction. Washington, DC: Carnegie Endowment for International Peace, 2002. 465pp. DOI. 10.1080/00396338.2002.9688570 .

Cloeckaert, A., Grayon, M., Grepinet, O. (2000). An IS711 element downstream of the bp26 gene is a specific marker of *Brucella* spp. isolated from marine mammals. *Clin. Diagn. Lab. Immunol*, 7, 835–839.

Cloeckaert, A., Grayon, M., Grepinet, O. (2002). Identification of *Brucella melitensis* vaccine strain Rev.1 by PCR-RFLP based on a mutation in the *rpsL* gene. *Vaccine*, 20: 2546-2550.

Cloeckaert, A., Grayon, M., Grepinet, O., Boumedine, K.S. (2003). Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification tests. *Microbes Infect*, 5: 593-602.

Cloeckaert, A., Verger, J.M., Grayon, M., Grepinet, O. (1995). Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. *Microbiology*, 141: 2111-2121.

Cloeckaert, A., Verger, J.M., Grayon, M., Paquet, J.Y., Garin-Bastuji, B., Foster, G., Godfroid, J. (2001). Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. *Microbes Infect*, 3, 729-738.

Constable, P.D., Hinchcliff, K.W., Done, S.H., Grunberg, W. (2017). Diseases Primarily Affecting the Reproductive System in: Veterinary Medicine. 11th edn. Elsevier, 1758–1829. doi: 10.1016/B978-0-7020-5246-0.00018-8 .

Corbel J (2006). Brucellosis in humans and animals. WHO publication, WHO/CDR/2006.7. ISBN 978 92 4 154713 0.

Corbel, M.J. (1997). Brucellosis: an overview. *Emerg Infect Dis*, 3: 213–221

Corbel, M.J., Banai, M. (2005) Genus I. *Brucella* Meyer and Shaw 1920, 173AL. In Brenner, D.J., Krieg, N.R., Staley, J.T (eds). Bergey's Manual of Systematic Bacteriology, 2: 370-386.

Corbel, M.J., Brinley-Morgan, W.J. (1984). Genus *Brucella* Meyer and Shaw 1920, 173AL. In: Krieg NR, Holt JG (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1. Williams & Wilkins, Baltimore, MD, pp. 377–388.

Corbel, MJ. (1988). International committee on systematic bacteriology subcommittee on the taxonomy of Brucella report of the meeting, 5 September 1986, Manchester. *Eng, Int. J. Syst. Bacteriol*, 38: 450-452.

Crasta, O.R., Folkerts, O., Fei, Z., Mane, S.P., Evans, C., Martino-Catt, S., Bricker, B., Yu, G., Du, L., Sobral, B.W. (2008). Genome Sequence of *Brucella abortus* Vaccine Strain S19 Compared to Virulent Strains Yields Candidate Virulence Genes. *PLoS ONE* 3(5): e2193. http://dx.doi/10.1371/journal.pone.0002193.

Cunningham (1977). A difficult disease called brucellosis. In: Crawford RP, Hidalgo RJ, eds. Bovine Brucellosis, International Symposium, Texas A & M University Press, 687-711.

Cutler, S.J., Whatmore, A.M.,Commander, N.J. (2005). A review: Brucellosis-new aspects of an old disease. *J. A.ppl. Microbiol*, 98: 1270-1281. https://dx.doi/10.1111/j.1365-2672.2005.02622.x .

Dafaalla EN (1962). Incidence of Animal and human brucellosis in the Sudan.

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

De Ley, J., Mannheim, W., Segers, P., Lievens, A., Denijn, M., Vanhoucke, M., Gillis, M. (1987). Ribosomal ribonucleic acid cistron similarities and taxonomic neighbourhood of *Brucella* and CDC group Vd. *Int. J. Syst. Bacteriol*, 37: 35-42.

DelVecchio, V,G,, Kapatralm V., Redkar, R.J., Patra, G., Mujer, C., Los, T., Ivanova, N., Anderson, I., Bhattacharyya, A., Lykidis, A., Reznik, G., Jablonski, L., Larsen, N., D'Souza, M., Bernal, A., Mazur, M., Goltsman, E., Selkov, E., Elzer, P.H., Hagius, S., O'Callaghan, D., Letesson, J.J., Haselkorn, R., Kyrpides, N., Overbeek, R. (2002). The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proc. Natl. Acad. Sci*. U.S.A, 99: 443-448.

Doganay, G.D., Doganay, M. (2013). *Brucella* as a Potential Agent of Bioterrorism. Recent Pat. *Anti-Infective Drug Disc*, 8: 27-33 .

Domenech, J,, Corbel, M.J., Thomas, E.L., Lucet, P.H. (1983). La brucellose bovine en Afrique Centrale: VI. Identification et typage des souches isolates au Tchad et au Cameroun. *Rev. Elev. Med. Vet. Pays Trop*, 36 (10): 19-25.

Elnasri, M. (1960). Brucellosis in Southern Sudan. *Vet. Rec*, 72: 1200-1201 .

Enright, M.C., Spratt, B.G. (1999). Multilocus sequence typing. *Trends Microbiol*, 7: 482-487.

Evans, A.C. (1918). Further Studies on Bacterium Abortus and Related Bacteria: II. A Comparison of Bacterium Abortus with Bacterium Bronchisepticus and with the Organism Which Causes Malta Fever. *J. Inf Dis*, 22(6): 580-593.

Ewalt, D.R., Bricker, B.J. (2000) .Validation of the abbreviated *Brucella* AMOS PCR as a rapid screening method for differentiation of *Brucella abortus* field strains isolates and the vaccine strains, S19 and RB51. *J. clin. microbiol*, 38(8): 3085-3086.

Ewalt, D.R., Bricker, B.J. (2003). Identification and differentiation of *Brucella abortus* field and vaccine strains by BaSS-PCR. *Methods Mol. Biol*, 216: 97-108.

FAO, (2005). Country report, Livestock sector Brief, Sudan. Availabe from http://www.fao.org/ag/againfo/resources/en/publications/sector_briefs/lsb_SDN.pdf. Accessed July 2018 .

Farrell, I.D. (1974). The development of a new selective medium for the isolation of *Brucella abortus* from contaminated sources. *Res. Vet. Sci*, 16: 280-286.

Farrell, I.D. (1996). *Brucella* In: Mackie & McCartney's Practical Medical Microbiology, 14th edit. Section B., Bacteria and related organisms. Pp: 473-478. Collee, A.G., Fraster, A.F., Marmion, B.P. and Simmons, A. (eds). Churchill Livingstone (publisher), London.

Federal Ministry of Finance and National Economic (2002). The economic Review, Sudan.

Ferreira, A.C., Chambel, L., Tenreiro, T., Cardoso, R., Flor, L., Dias, I.T., Pacheco, T.,Garin-Bastuji, B., Le Fleche, P., Vergnaud, G., Tenreiro, R., de Sa, M.I. (2012). MLVA16 typing of Portuguese human and animal *Brucella melitensis* and *Brucella abortus* isolates. *PLoS One* 7, e42514 .

Ficht TA, Bearden SW, Sowa BA and Marquis H (1990) Genetic variation at the *omp2* porin locus of the brucellae: species-specific markers. *Molecular Microbiology*., 4(7), 1135-1142.

Forbes, L.B. (1990). *Brucella abortus* infection in 14 farm dogs. J. *Am. Vet. Med. Asso*, 196(6):911-916.

Foster, G., Osterman, B.S., Godfroid, J., Jacques, I., Cloeckaert, A. (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int.ernational *J. Syst. Evol. Microbiol*, 57: 2688-2693. https://dx.doi/10.1099/ijs.0.65269-0.

Foster, J.T., Beckstrom-Sternberg, S.M., Pearson, T., Beckstrom-Sternberg, J.S., Chain, P.S., Roberto, F.F., Hnath, J., Brettin, T., Keim, P. (2009). Whole genome-based phylogeny and divergence of the genus *Brucella. J. Bacteriol*, 191: 2864-2870.

Foster, J.T., Okinaka, R.T., Svensson, R., Shaw, K., De, B.K., Robison, R.A., Probert, W.S., Kenefic, L.J., Brown, W.D., Keim, P. (2008). Real-time PCR assays of

single-nucleotide polymorphisms defining the major *Brucella* clades. *J. Clin. Microbiol*, 46: 296-301.

Garcia-Yoldi, D. (2006). Multiplex PCR Assay for the Identification and Differentiation of all *Brucella* Species and the Vaccine Strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clin. Chem*, 52(4), 779–781. http://x.Doi.10.1373/clinchem.2005.062596..

Garcıa-Yoldi, D., Le Fleche, P., De Miguel, M.J., Munoz, P.M., Blasco, J.M. (2007a). Comparison of Multiple-Locus Variable-Number Tandem-Repeat Analysis with Other PCR-Based Methods for Typing *Brucella suis* Isolates. *J. C. Microbiol*, 45(12): 4070-4072. http://dx.doi/10.1128/JCM.01096-07 .

Garcia-Yoldi, D., Le Fleche, P., Marin, C.M., De Miguel, M.J. (2007b) Assessment of genetic stability of *Brucella melitensis* Rev 1. vaccine strain by multiple-locus variable-number tandem repeat analysis. *Vaccine*, 25: 2858-2862. http://dx.doi/10.1016/j.vaccine.2006.09.063 .

Garin-Bastuji, B. (2011). Pathogens in Milk | Brucella spp. *Encyclopedia of Dairy Sciences*, 31-39. https://dx.doi/10.1016/b978-0-12-374407-4.00389-7.

Garin-Bastuji, B., Blasco, J.M. (2016). Pathogens in Milk - *Brucella* spp. *Reference Module in Food Science*. https://dx.doi/10.1016/b978-0-08-100596-5.00983-5.

Geoffrey, T.F., Tim, E.C., Bruno, B.C., James, T.C., Emilio, E.D., Kevin, F.R. (2002). Time space clustering of human brucellosis, California, 1973-1992. *Emerg. Infect. Dis*, 8(7): 672-678. DOI:10.3201/eid0807.010351.

Godfroid, J., Cloeckaert, A., Liautard, J.P., Kohler, S., Fretin, D., Walravens, K., Garin-Bastuji, B., Letesson, J.J. (2005). From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Vet. Res*, 36:313-326.

Godfroid, J., Scholzc, H.C., Barbierd, T., Nicolasd, C., Wattiaue, P., Fretine, D., Whatmore, A.M., Cloeckaert, A., Blascoh, J.M., Moriyoni, I., Saegermanj, C., Mumak, J.B., AlDahoukl, S., Neubauern, H., Letessond, J.J. (2011). Brucellosis at

the animal/ecosystem/human interface at the beginning of the 21st century. *Prev. vet. Med*, 102:118-131. http://dx.doi.org/10.1016/j.prevetmed.2011.04.007..

Gopaul, K.K., Koylass, M.S., Smith, C.J., Whatmore, A.M. (2008). Rapid identification of *Brucella* isolates to the species level by real time PCR based single nucleotide polymorphism (SNP) analysis. *BMC Microbiol*, 8: 86. Available from: http://www.biomedcentral.com/1471-2180/8/86.

Groussaud P, Shankster SJ, Koylass MS, Whatmore AM (2007). Molecular typing divides marine mammal strains of Brucella into at least threegroups with distinct host preferences. *J Med Microbiol* 56:1512–1518

Guihot, A., Bossi, P., Bricaire, F. (2004). Bioterrorism with brucellosis. *Presse Med* ; 33: 119-22.

Gul, S.T., Khan, A. (2007). Epidemiology and epizootology of brucellosis: A review. *Pakistan vet. J*, 27(3): 145-151 .

Gumaa, M.M., Osman, H.M., Omer, M.M., El Sanousi, E.M., Godfroid, J., Ahmed, A.M. (2014). Seroprevalence of brucellosis in sheep and isolation of *Brucella abortus* biovar 6 in Kassala State , Eastern Sudan. *Rev. sci. tech. Off. int. Epiz*, 33(3): 957-965. doi: http://dx.doi.org/10.20506/rst.33.3.2333 .

Gyuranecz, M., Wernery, U., Kreizinger, Z., Juhasz, J., Felde, O., Nagy, P. (2016). Genotyping of Brucella melitensis strains from dromedary camels (Camelus dromedaries) from the United Arab Emirates with Multiple-locus variable number tandem repeat analysis. *Vet. microbiol*, 186: 8-12. http://dx.doi.org/10.1016/j.vetmic.2016.02.009.

Habiballa, N., Dafaalla, E.A., Omer, E.E. (1977). Studies on Human and Bovine brucellosis in the Sudan: The incidence of brucellosis and the species of *Brucella* organism isolated from cattle in three provinces. *Sud. Med. J*, 15:9-16.

Halling, S.M., Bricker, B.J. (1994). Characterization and occurrence of two repeated palindromic DNA elements of *Brucella* spp.: Bru-RS1 and Bru-RS2. *Mol. Microbiol*, 14: 681-689.

Halling, S.M., Peterson-Burch, B.D., Bricker, B.J., Zuerner, R.L., Qing, Z., Li, L,L,, Kapur, V., Alt, D.P., Olsen, S.C. (2005). Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. *J. Bacteriol*, 187: 2715-2726.

Halling, S.M., Tatum, F.M., Bricker, B.J. (1993). Sequence and characterization of an insertion sequence, IS711, from *Brucella ovis*. *Gene*, 133: 123-127.

Haseeb, M.A. (1950). Undulant Fever in the Sudan, *J. Trop. Med*, 12(53): 241-4.

Hoyer, B.H., McCullough, N.B. (1968a). Polynucleotide homologies of *Brucella* deoxyribonucleic acids. *J. Bacteriol*, 95: 444-448.

Hoyer, B.H., McCullough, N.B. (1968b). Homologies of deoxyribonucleic acids from *Brucella ovis*, canine abortion organisms, and other Brucella. *J. Bacteriol*, 96: 1783-1790.

IGAD "Intergovernmental Authority on Development" and Center for Pastoral Areas & Livestock Development "ICPALD", (2013). The Contribution of Livestock to the Sudan Economy. Policy Brief, ICPALD 6/CLE/8/2013

Ismail, A.A.A. (2007). Prevalence of brucellosis in Kuku Dairy, Khartoum state, and susceptibility of isolates to some chemotherapeutic agents. MSc thesis, University of Khartoum, Sudan. Available from https://core.ac.uk/download/pdf/71668488.pdf.

Kattar, M.M., Jaafar, R.F., Araj, G.F., Le Fleche, P., Matar, G.M., Abi Rached, R., Khalife, S., Vergnaud, G. (2008). Evaluation of a multilocus variable-number tandemrepeat analysis scheme for typing human *Brucella* isolates in a region of brucellosis endemicity. *J. Clin. Microbiol*, 46: 3935-3940.

Kay GL, Sergeant MJ, Giuffra V, Bandiera P, Milanese M, Bramanti B, Bianucci R, Pallen MJ (2014). Recovery of a medieval *Brucella melitensis* genome using shotgun metagenomics. *mBio* 5(4):e01337-14. doi:10.1128/mBio.01337-14.

Keid, B., Soares, M., Vieira, R., Megid, J., Salgado, R., Vasconcello, S.A., da Costa, M., Gregori, F., Richtzenhain, L.J. (2007). Diagnosis of canine brucellosis: comparison between serological and microbiological tests and a PCR based on

primers to 16S-23S rDNA Interspacer. *Vet. Res. Commun*, 31: 951-965. http://dx.doi/10.1007/s11259-006-0109-6.

Khames, M., Mick, V., de Miguel, M.J., Girault, G., Conde-Alvarez, R., Khelef, D., Oumounaa, M., Moriyóne, I., Munozd, P.M., Zuniga-Ripa, A. (2017) The characterization of *Brucella* strains isolated from cattle in Algeria reveals the existence of a *B. abortus* lineage distinct from European and Sub-Saharan Africa strains. *Vet. Microbiol*, 211: 124-128. http://dx.doi.org/10.1016/j.vetmic.2017.10.008.

Le Fleche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nockler, K., Neubauer, H., Guilloteau, L.A., Vergnaud, G. (2006). Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiology*, 6: 9 http://dx.doi:10.1186/1471-2180.

Lopez-Goni, I., Garcia-Yoldi, D., Marin, C.M., De Miguel, M.J., Munoz, P.M., Blasco, J.M., Jacques, I., Grayon, M., Cloeckaert, A., Ferreira, A.C., Cardoso, R., Correa de Sa, M.I., Walravens, K., Albert, D., Garin-Bastuji, B. (2008). Evaluation a Multiplex PCR Assay (Bruce-ladder) for molecular typing of all *Brucella* species including the vaccine strains. *J. Clin. Microbiol*, 46(10):3484-3487. http://dx.doi:10.1128/JCM.00837-08.

Mangen, M.J., Otte, J., Pfeiffer, D., Chilonda, P., (2002). Bovine brucellosis in sub-Saharan Africa: estimation of sero-prevalence and impact on meat and milk offtake potential. FAO Livestock Information and Policy Branch, AGAL, Livestock Policy Paper no. 8. Rome, Italie: 39-41.

Margos, G., Gatewood, A.G., Aanensen, D.M., Hanincova, K., Terekhova, D., Vollmer, S.A., Cornet, M., Piesman, J., Donaghy, M., Bormane, A., Hurn, M.A., Feil, E.J., Fish, D., Casjens, S., Wormser, G.P., Schwartz, I., Kurtenbach, K. (2008). Multiple Locus Sequence Typing "MLST" of housekeeping genes captures geographic population structure and suggests a European origin of Borrelia burgdorferi. *Proc. Natl. Acad. Sci*. U.S.A, 105: 8730-8735.

Marianelli, C., Graziani, C., Santangelo, C., Xibilia, M.T., Imbriani, A., Amato, R., Neri, D., Cuccia, M., Rinnone, S., Di Marco, V., Ciuchini, F. (2007). Molecular epidemiological and antibiotic susceptibility characterization of *Brucella* isolates from humans in Sicily, Italy. *J. Clin. Microbiol*, 45: 2923-2928 .

Marianelli, C., Petrucca, A., Pasquali, P., Ciuchini, F., Papadopoulou, S., Cipriani, P. (2008). Use of MLVA-16 typing to trace the source of a laboratory-acquired *Bruella* infection. *J. Hosp. Infect*, 68: 274-276.

Marin, C.M., Jimenez de Bagues, M.P., Barberan, M., Blasco, J.M. (1996). Comparison of two selective media for the isolation of *Brucella* melitensis from naturally infected sheep and goats. *Vet. Rec*, 138: 409-411.

McCaughey WJ, and Purcell DA (1973). Brucellosis in bulls. *Vet Rec*; 93: 336-7 .

McDermott, J.J., Arimi, S.M. (2002) Brucellosis in sub-Saharan Africa: epidemiology, control and impact. *Vet. Microbiol*, 90: 111-134.

McDermott, J.J., Deng, K.A., Jayatileka, T.N., EL Jack, M.A. (1987) A Cross-sectional Cattle Disease Study in Kongo Rural Council, Southern Sudan. II. Brucellosis in Cows: Associated Factors, Impact on Production and Disease Control Considerations. *Prev. Vet. Med*, 5: 125-132.

McDermott, J.J., Grace, D., Zinsstag, J. (2013). Economics of brucellosis impact and control in low-income countries. *Rev. sci. tech. Off. int. Epiz*, 32(1): 249-261.

McDonald, W.L., Jamaludin, R., Mackereth, G., Hansen, M., Humphrey, S., Short, P., Taylor, T., Swingler, J., Dawson, C.E., Whatmore, A.M., Stubberfield, E., Perrett, L.L., Simmons, G. (2006). Characterization of a *Brucella* sp. strain as a marine mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand. *J. Clin. Microbiol*, 44: 4363-4370.

Mohd, M.G. (1989). Brucellosis in the Gezira area, Central Sudan. *J Trop Med Hyg*, 92(2):86-8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/2496237?report=abstract&format=text

Moreno, E., Cloeckaert, A., Moriyon, I. (2002). *Brucella* evolution and taxonomy. *Vet. Microbiol*, 90: 209-227.

Mukherjee, F., Jain, J., Grillo, M.J., Blasco, J.M., Nair, M. (2005). Evaluation of Brucella abortus S19 vaccine strains by bacteriological tests, molecular analysis of ery loci and virulence in BALB/c mice. *Biologicals*, 33: 153-160.

Musa, M.T., Eisa, M.Z.M., El sanousi, E.M., Abdel Wahab, M.B., Perrett, L.L. (2008). Brucellosis in Camels (*Camelus dromedarius*) in Darfur, Western Sudan. *J. comp. Path*, 138: 151-155. http://dx.doi/10.1016/j.jcpa.2007.10.005 .

Musa, M.T., Jahans, K.L. (1990). The Isolation of Brucella melitensis Biovar 3 from a Testicular Hygroma of a Ram in a Nomadic Flock of Sheep and Goats in Western Sudan. *J. Comp. Path*, 103: 468-470.

Musa, M.T., Jahans, K.L., Fadlalla, M.E. (1990). *Brucella* biovars isolated from nomadic cattle in the South Darfur provinces, Western Sudan. *J. Comp. Path*, 102: 49-54.

Musa, M.T., Shigidi, M.T.A. (2001). Brucellosis in Camels in Intensive Animal Breeding Areas of Sudan "Implications in Abortion and Early-Life Infections". *Revue Élev. Méd. vét. Pays trop*, 54 (1): 11-15 .

Mustafa, A.A.A., Hassan, H.S. (2010). Human Brucellosis in Khartoum State: A Commonly Underdiagnosed Disease. Sudan J Med Sci, 5(3): 213-220. Available from: http://www.ajol.info/index.php/sjms/article/view/62010. http://dx.doi.org/10.4314/sjms.v5i3.62010.

Nicoletti, P. (1981). Prevalence and persistence of *Brucella abortus* strain 19 infections and prevalence of other biotypes in vaccinated adult dairy cattle. *J. Am. Vet. Med. Assoc*, 15: 178 (2): 143-5.

Nicoletti, P. (2002). A short history of brucellosis. *Vet Microbiol*, 90:5-9 .

Ntirandekura J-B, Matemba LE, Ngowi HA, Kimera SI, Karimuribo ED (2018). Knowledge, perceptions and practices regarding brucellosis in pastoral communities of Kagera Region in Tanzania. *Journal of Advanced Veterinary and Animal.Research*. 5(3):243-253.

Ocampo-Sosa, A.A., Aguero-Balbin, J., Garcia-Lobo, J.M. (2005). Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. *Vet. Microbiol*, 110: 41-51

Oguz, K., Irfan, S., Derya, K., Idris, S. (2004). Oxfloxacin plus Rifampicin versus Doxycycline plus Rifampicin in the treatment of brucellosis: A randomized clinical trial. *BMC infectious diseases*, 4:18. available at: www.biomedcentral.com/1471-2334/4/18.

OIE "World Organization for Animal Health" (2018). Chapter 2.4.3 Bovine brucellosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals, 355-398. Available from www.oie.int.

Omer MM, Musa MT, Bakhiet MR, Perrett L (2010). Brucellosis in camels, cattle and humans: associations and evaluation of serological tests used for diagnosis of the disease in certain nomadic localities in Sudan. *Rev. sci. tech. Off. int. Epiz*., 29 (3): 663-669

Omran, E.O.M., Musa, M.T. (2015). Prevalence of Brucellosis in Different Animal Species and Man and Isolation of Brucella abortus biovars 1 and 6 from Cattle in Sennar *State. Sud. J. Vet. Res*, 30: 25-28. www.sudanjvr.net.

Osman, A.E.F., Hassan, A.N., Ali, A.E., Abdoel, T.H., Smits, H.L. (2015) *Brucella melitensis* biovar 1 and *Brucella abortus* S19 Vaccine Strain infections in Milkers Working at Cattle Farms in the Khartoum Area, Sudan. *PLoS ONE*, 10 (5): e0123374. http:www//dx.doi:10.1371/journal.pone.0123374..

Ouahrani, S., Michaux, S., Sri Widada, J., Bourg, G., Tournebize, R., Ramuz, M., Liautard, J.P. (1993). Identification and sequence analysis of IS6501, an insertion sequence in *Brucella* spp.: relationship between genomic structure and the number of IS6501 copies. *J. Gen. Microbiol*, 139: 3265-3273 .

Pappas G, Akritidis N, Tsianos E (2005). Effective treatment in the management of brucellosis. *Expert Opinion. Pharmacotber*., 6: 201-209. DOI. 10.1517/14656566.6.2.201. .

Pappas, G., Panagopoulou, P., Christou, L., Akritidis, N. (2006a). Category B Potential Bioterrorism Agents: Bacteria, Viruses, Toxins, and Foodborne and Waterborne Pathogens. *Infect. Dis. Clin. N. Am*, 20: 395-421.

Pappas, G., Panagopoulou, P., Christou, L., Akritidis, N. (2006b). *Brucella* as a biological weapon. Cell. Mol. Life. Sci., 63: 2229-2236. http://dx.doi/10.1007/s00018-006-6311-4 .

Pappas, G., Papadimitriou, P. (2007). Challenges in Brucella bacteraemia. *Int. J. Antim. Agents*, 30: 29-31. http://dx.doi/10.1016/j.ijantimicag.2007.06.011.

Paulsen, I.T., Seshadri, R., Nelson, K.E., Eisen, J.A., Heidelberg, J.F., Read, T.D., Dodson, R.J., Umayam, L., Brinkac, L.M., Beanan, M.J., Daugherty, S.C., Deboy, R.T., Durkin, A.S., Kolonay, J.F., Madupu, R., Nelson, W.C., Ayodeji, B., Kraul, M., Shetty, J., Malek, J., Van Aken, S.E., Riedmuller, S., Tettelin, H., Gill, S.R., White, O., Salzberg, S.L., Hoover, D.L., Lindler, L.E., Halling, S.M., Boyle, S.M., Fraser, C.M. (2002). The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc. Natl. Acad. Sci.* U.S.A, 99: 13148–13153.

Philippon, A., Renoux, G., Plommet, M. (1970). Experimental Bovine brucellosis. III. Vaginal excretion of *Brucella abortus* before and after calving. *Ann. Rech. Vet*, 1: 215-24.

Philipsson, J. (2000). Dairy cattle breeding in less developed countries (case study) in: workshop on Developing breeding strategies for lower input animal production environments, Bella, Italy 22-25 September 1999. The International Committee for Animal Recording (ICAR) Technical Series - No 3. Developing Breeding Strategies for Lower Input Animal Production Environments. Galal, S., Boyazoglu, J. and Hammond, K. (eds). Publisher: ICAR, Villa del Ragno, Via Nomentana 134, 00162 Rome, Italy. ISSN: 1563-2504

Plommet, M. (1991). Minimal requirements for growth of *Brucella suis* and other *Brucella* species. *Zentralbl. Bakteriol*, 275: 436-450.

Radwan, A.I., Bekairi, S.I., al-Bokmy, A.M., Prasad, P.V., Mohamed, O.M., Hussain, T.T. (1993). Successful therapeutic regimens for treating *Brucella melitensis* and *Brucella abortus* infections in cows. *Rev. Sci. Tech*., 64: 909-922.

Ray, W.C. (1979). Brucellosis (due to *Brucella abortus* and *B. suis*). In: Steele J. Handbook of series in zoonoses., section A: Bacterial, Rickettial and Mycotic diseases. Roca Raton, Florida, USA: CRC press, Inc, 99-127 .

Robinson, A. (2003). FAO Guidelines for coordinated human and animal brucellosis surveillance, Animal Production and Health Division, FAO Agriculture Department, Room. paper 156; ISSN: 0254-6019 .

Roth, F., Zinsstag, J., Orkhon, D., Chimed-Ochir, G., Hutton, G., Cosivi, O., Carrin, G., Otte, J. (2003). Human health benefits from livestock vaccination for brucellosis: Case study. *Bull. WHO*, 81(12): 867-876.

Sangari, F.J., Aguero, J. (1994). Identification of Brucella abortus B19 vaccine strain by the detection of DNA polymorphism at the ery locus. *Vaccine*, 12: 435-438. http://dx.doi/10.1016/0264-410X(94)90121-X.

Sangari, F.J., Garcia-Lobo, J.M., Aguero, J. (1994). The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. *FEMS Microbiol. Lett*, 121:337-342.

Sangari, F.J., Grillo, M.J., Jimenez De Bagues, M.P., Gonzalez-Carrero, M.I., Garcia-Lobo, J.M., Blasco, J.M., Aguero, J. (1998). The defect in the metabolism of erythritol of the *Brucella abortus* B19 vaccine strain is unrelated with its attenuated virulence in mice. *Vaccine*, 16 (17): 1640-1645.

Scholz HC, Hubalek Z, Sedlacek I, Vergnaud G, Tomaso H, Al Dahouk S, Melzer F, Kampfer P, Neubauer H, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Falsen E, Bahn P, Gollner C, Pfeffer M, Huber B, Busse H and Nockler K (2008). *Brucella microti* sp. nov., isolated from the common vole Microtus arvalis. International Journal of Systematic and Evolutionary Microbiology, 58, 375–382. DOI 10.1099/ijs.0.65356-0 .

Scholz HC, Nockler K, Gollner C, Bahn P,Vergnaud G, Tomaso H, Al Dahouk S, Kampfer P, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Pfeffer M, Huber B, Busse H and Barun Kumar De (2010). *Brucella inopinata* sp. nov., isolated from a breast implant infection. *International Journal of Systematic and Evolutionary Microbiology*, 60, 801–808. DOI 10.1099/ijs.0.011148-0 .

Scott, J.C., Koylass, M.S., Stubberfield, M.R., Whatmore, A.M. (2007). Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. *Appl. Environ. Microbiol*., 73: 7331-7337.

Shepherd, A.A., Simpson, H.H,. Davidson, R. M. (1979). An Economic Evaluation of the New Zealand Bovine Brucellosis Eradication scheme. 2nd Int. Symp. *Vet. Epid. And. Econ*. 443-447.

Smits, H..L, Espinosa, B., Castillo, R., Hall, E., Guillen, A., Zevaleta, M., Gilman, R.H., Melendez, P., Guerra, C., Draeger, A., Broglia, A., Nockler, K. (2009). MLVA genotyping of human *Brucella* isolates from Peru. *Trans. R. Soc. Trop. Med. Hyg*, 103: 399-402.

Sohn, A.H., Probert, W.S., Glaser, C.A., Gupta, N., Bollen, A.W., Wong, J.D., Grace, E.M., McDonald, W.C. (2003). Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerg. Infect. Dis*, 9:485-488.

Sriranganathan, N., Seleem, M.N., Olsen, S.C., Samartino, L.E., Whatmore, A.M., Bricker B, O'Callaghan D, Halling SM, Crasta OR, Wattam AR, Purkayastha A, Sobral BW, Snyder EE, Williams KP, Yu GX, Ficht TA, RoopII RM, de Figueiredo P, Boyle SM, He Y, Tsoliss RM et ak. (2009). Vishvanath N and Chittaranjan K (eds). Chapter: *Brucella* in: Genome mapping and Genomics in Animal-Associated Microbes. Springer Berlin Heidelberg (publisher). Pp: 1-64. Doi: 10.1007/978-3-540-74042-1 .

Tesfaye G, Tsegaye W, Chanie M, Abinet F. Seroprevalence and associated risk factors of bovine brucellosis in Addis Ababa dairy farms. *Trop Anim Health Prod*. 2011; 43:1001±1005. doi: 10.1007/s11250-011-9798-0 PMID: 21331496

Thomas, E.L., Bracewell, C.D., Corbel, M.J. (1981). Characterisation of Brucella abortus strain 19 cultures isolated from vaccinated cattle. *Vet Rec*. ,108: 90-93. pmid:6789543.

Valdezate, S., Cervera, I., Hernandez, P., Navarro, A., Saez, Nieto, J.A. (2007). Characterisation of human outbreaks of brucellosis and sporadic cases by the use of hyper-variable octameric oligonucleotide fingerprint (HOOF) variable number tandem repeats. *Clin. Microbiol. Infect*, 13: 887-892.

van Belkum, A., Scherer, S., van Alphen, L., Verbrugh, H. (1998). Short-sequence DNA repeats in prokaryotic genomes. *Microbiol. Mol. Biol. Rev*, 62:275-93.

Vemulapalli, R., McQuiston, J.R., Schurig, G.G., Sriranganathan, N., Halling, S.M., Boyle, S.M. (1999). Identification of an IS711 element interrupting the wboA gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. *Clin. Diagn. Lab. Immunol*, 6: 760-764.

Verger, J.M., Grimont, F., Grimont, P.A., Grayon, M. (1987). Taxonomy of the genus Brucella. Ann. Inst. Pasteur Microbiol, 138: 235-238.

Verger, J.M., Grimont, F., Grimont, P.A.D., Grayon, M. (1985). Brucella, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol, 35: 292-295.

WAHID, (2017). World Animal Health Information Database "WAHID" Interface version of the World Organization for Animal Health 'OIE". Available from: https://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Animalpopulation.

Wattam, A.R., Williams, K.P,, Snyder, E.E., Almeida, N.F. Jr., Shukla, M., Dickerman, A.W., Crasta, O.R., Kenyon, R., Lu, J., Shallom, J.M., Yoo, H., Ficht, T.A., Tsolis, R.M., Munk, C., Tapia, R., Han, C.S., Detter, J.C., Bruce, D., Brettin, T.S., Sobral, B.W., Boyle, S.M., Setubal, J.C. (2009). Analysis of ten *Brucella* genomes reveals evidence for horizontal gene transfer despite a preferred intracellular lifestyle. *J. Bacteriol*, 191:3569-79.

Whatmore, A.M. (2009) Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infect. Genet. Evol*, 9:1168-1184 .

Whatmore, A.M., Davison, N., Cloeckaert, A., Al Dahouk, S., Zygmunt, M.S., Brew, S.D., Perrett, L.L., Koylass, M.S., Vergnaud, G., Quance, C., Scholz, H.C., Dick, Jr. EJ., Hubbard, G., Schlabritz-Loutsevitch, N.E. (2014). *Brucella papionis* sp. nov., isolated from baboons (*Papio* spp.), *Int. J. Syst. Evol. Microbiol*, 64: 4120-4128. http://dx.doi:10.1099/ijs.0.065482-0 .

Whatmore, A.M., Dawson, C,E., Groussaud, P., Koylass, M.S., King, A.C., Shankster, S.J., Sohn, A.H., Probert, W.S., McDonald, W.L. (2008). Marine mammal *Brucella* genotype associated with zoonotic infection. *Emerg. Infect. Dis*, 14: 517-518.

Whatmore, A.M., Koylass, M.S., Muchowski, J., Edwards-Smallbone, J., Gopauland, K.K., Perrett, L.L. (2016). Extended Multilocus Sequence Analysis to Describe the Global Population Structure of the Genus *Brucella*: Phylogeography and Relationship to Biovars. Front. Microbiol, 7:2049. http://dx.doi:10.3389/fmicb.2016.02049. Available from: http://www.biomedcentral.com/1471-2180/7/34.

Whatmore, A.M., Perrett, L.L., MacMillan, A.P. (2007). Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiology*, 7:34 http://dx.doi:10.1186/1471-2180-7-34 .

Whatmore, A.M., Shankster, S.J., Perrett, L.L., Murphy, T.J., Brew, S.D., Thirlwall, R.E., Cutler, S.J., MacMillan, A.P. (2006). Identification and Characterization of Variable-Number Tandem-Repeat Markers for Typing of *Brucella* spp. *J. Clin. Microbiol*, 44(6): 1982-1993.

Wikipedia 2018. Accessed July 2018. https://en.wikipedia.org/wiki/States_of_Sudan

Wilson, R.T. (2018). Livestock in the Republic of the Sudan: Policies, production, problems and possibilities. *Anim Husb Dairy Vet Sci*, 2: http://dx.DOI/10.15761/AHDVS.1000142..

WITs, (2018). “World Integrated Trade Solutions”. Available from: https://wits.worldbank.org/CountryProfile/en/SUD. Accessed 21st September 2018.

Worldometers (2018). Sudan population. Accessed on Wednesday, 18th July, 2018. http://www.worldometers.info/world-population/sudan-population. /

Wyatt, H.V. (2005). How Themistocles Zammit found Malta Fever (brucellosis) to be transmitted by the milk of goat. *Y R Soc Med*, 95:451-454.

Zinsstag, J., Schelling, E., Roth, F., Bonfoh, B., de Savigny, D., Tanner, M. (2007). Human Benefits of Animal Interventions for Zoonosis Control. *Emerg. Infec. Dis*, 13(4): 527-531. Availabe from www.cdc.gov/eid

Zinsstag, J., Schelling, E., Wyss, K., Mahamat, M.B. (2005). Potential of cooperation between human and animal health to strengthen health systems. *Lancet*, 366: 2142-45.

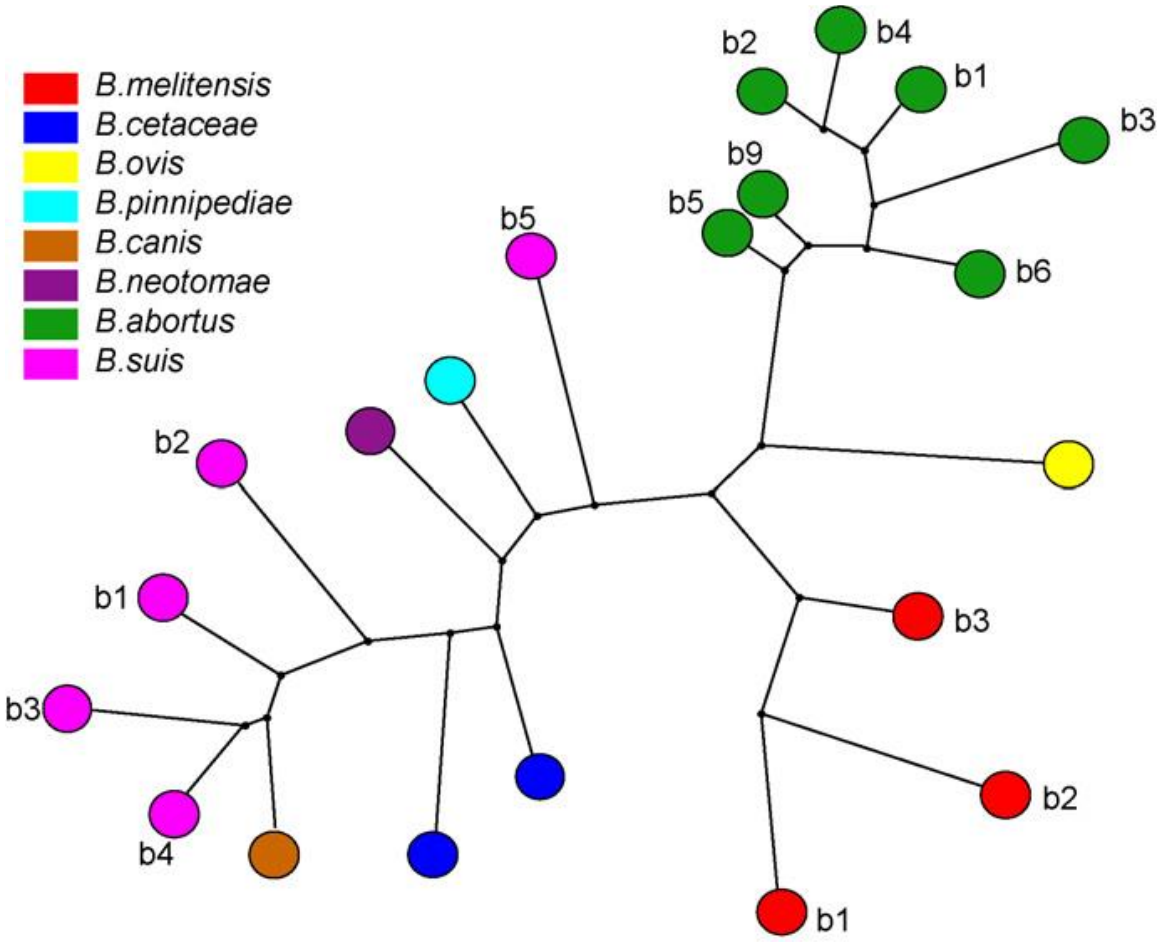
Appendices

Appendix 1. Differential characteristics of *Brucella* spp. and biovars (bv)

Species	bv	Host	Growth characteristics					Agglutination			Growth on dye		Phage lysis				
			Morph-ology	CO_2	H_2O	Oxidase	Urease	A	M	R	Th	BF	Tb RTD	104 ^{Tb} RTD	Wb RTD	Is2 RTD	R/C RTD
B. melitensis	1		S	-	-	+	+	-	+	-	+	+	-	-	-	+	-
	2	Sheep	S	-	-	+	+	+	-	-	+	+	-	-	-	+	-
	3	goats	S	-	-	+	+	+	+	-	+	+	-	-	-	+	-
B. abortus	1		S	(+)	+	+	(+)	+	-	-	+	+	+	+	+	+	-
	2		S	(+)	+	+	+	+	-	-	+	+	+	+	+	+	-
	3		S	(+)	+	(+)	+	+	-	-	+	+	+	+	+	+	-
	4	Cattle	S	(+)	+	+	+	-	+	-	+	+	+	+	+	+	-
	59		S	-	-	+	+	-	+	-	+	+	+	+	+	+	-
	6		S	-	-	+	+	+	-	-	+	+	+	+	+	+	-
	7		S	-	+/-	+	+	+	+	-	+	+	+	+	+	+	-
	9		S	+/-	+	+	+	-	+	-	+	+	+	+	+	+	-
B. suis	1		S	-	+	+	+	+	-	-	+	+	-	+	+	+	-
	2	Swine,	S	-	-	+	+	+	-	-	+	+	-	+	+	+	-
	3	hares,	S	-	-	+	+	+	-	-	+	+	-	+	+	+	-
	4	rodents	S	-	-	+	+	+	+	-	+	+	-	+	+	+	-
	5		S	-	-	+	+	-	+	-	+	+	-	+	+	+	-
B. neotomae		rodents	S	-	+	-	+	+	-	-	-	-	+/-	+	+	+	-
B. ovis		sheep	R	+	-	-	-	-	-	+	+	(+)	-	-	-	-	+
B. canis		dogs	R	-	-	+	+	-	-	+	+	(-)	-	-	-	-	+
B. ceti		whales	S	(-)	-	+/-	+	+	(-)	-	(+)	(+)	-		+	+/-	-
B. pinnipedialis		Seals	S	(+)	-	+	+	(+)	(-)	-	+	(+)	-		+	+/-	-
B. microti		Voles	S	-	-	+	+	-	+	-	+	+	-	+	+	+/-	-
B. inopinata		Unknown	S	-	+	+	+	-	+		+	+	-	+/-	-	-	-
B. papionis		Baboons															
B. vulpis																	

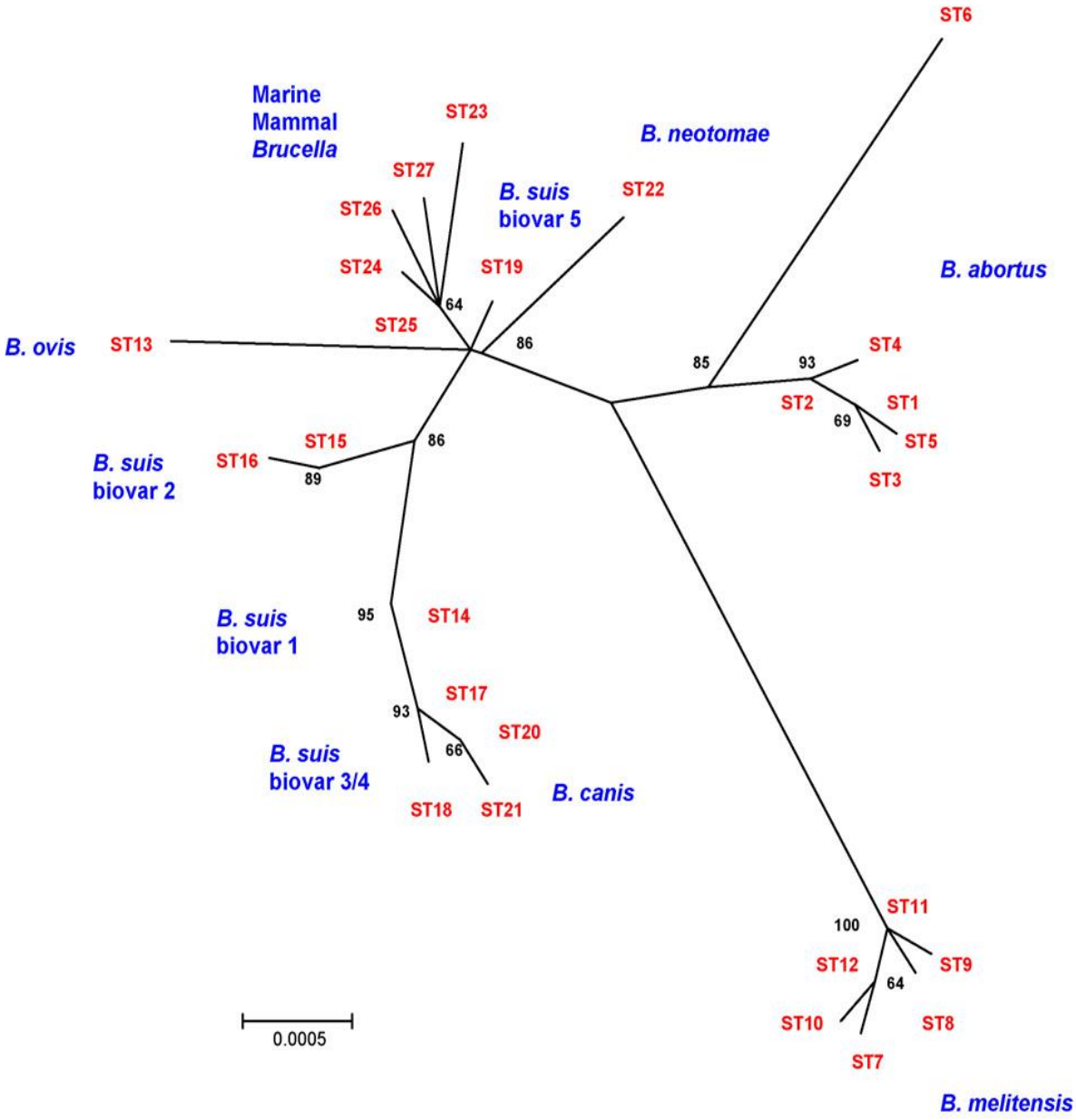
A, M and R: Brucella specific antisera; BF: Basic fuchsin dye (at 20 µg, w/v); TH: Thionin dye (at 20 and 40 µg, w/v); (Wb: Weybridge; Tb: Tibilisi; BK2: Berkingy 2; Fi: Frinzi; Iz: Izantar; R/C) Brucella specific bacteriophage; TRD: routine test dilution; S: smooth colony morphology; R: rough colony morphology; (+): most strains positive; (-): most strains negative, (+/-); positive or negative. (table adapted from Garin-Bastuji et al., 2016).

Appendix 2. Maximum parsimony analysis of Brucella reference strains.



Different colours as indicated represent the different species and biovars (B) are mentioned where relevant. Reproduced from Le Fleche et al. (2006). Note that the names of the Brucella species isolated from marine mammals were modified on formal publication subsequent to the original publication of this figure.

Appendix 3. Unrooted phylogenetic tree of the relationships etween Brucella spp.



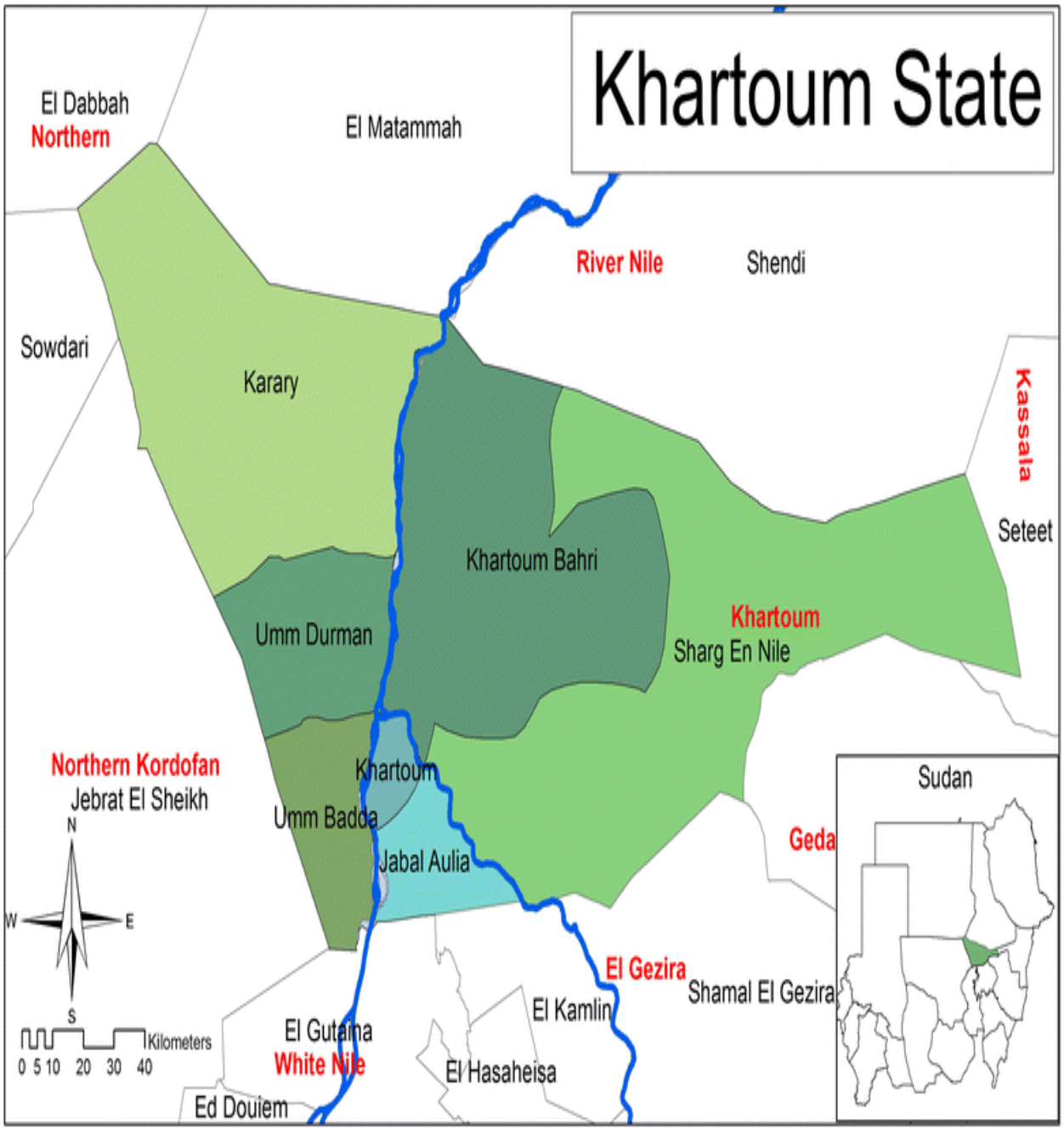
This tree was constructed with the concatenated sequence data of the nine loci (4396 bp) using the neighbour joining approach. The Jukes-Cantor model, which is based on the assumption that all nucleotide substitutions are equally likely, was used to determine genetic distances. The percentage bootstrap confidence levels of internal branches were calculated from 1000 re-samplings of the original data. Reproduced from Whatmore et al., (2007).

Appendix 4. Primers used for PCR assays.

PCR test	Primer's (Oligonucleotide) sequence	
ITS PCR primers pair [a]		
ITS66	ACA TAG ATC GCA GGC CAG TCA	
ITS279	AGA TAC CGA CGC AAA CGC TAC	
AMOS PCR primers pairs [b]		
BAspes	GACGAACGGAATTTTTCCAATCCC	
BMspes	AAATCGCGTCCTTGCTGGTCTGA	
BOspes	CGGGTTCTGGCACCATCGTCG	
BSspes	GCGCGGTTTTCTGAAGGTTCAGG	
IS711spes	TGCCGATCACTTAAGGGCCTTCAT	
Bruce-Ladder PCR primers pairs [c]		
BMEI0998 F	ATCCTATTGCCCCGATAAGG	
BMEI0997 R	GCTTCGCATTTTCACTGTAGC	
BMEI0535 F	F GCGCATTCTTCGGTTATGAA	
BMEI0536 R	CGCAGGCGAAAACAGCTATAA	
BMEII0843 F	TTTACACAGGCAATCCAGCA	
BMEII0844 R	GCGTCCAGTTGTTGTTGATG	
BMEI1436 F	ACGCAGACGACCTTCGGTAT	
BMEI1435 R	TTTATCCATCGCCCTGTCAC	
BMEII0428 F	GCCGCTATTATGTGGACTGG	
BMEII0428 R	AATGACTTCACGGTCGTTCG	
BR0953 F	GGAACACTACGCCACCTTGT	
BR0953 R	GATGGAGCAAACGCTGAAG	
BMEI0752 F	CAGGCAAACCCTCAGAAGC	
BMEI0752 R	GATGTGGTAACGCACACCAA	
BMEII0987 F	CGCAGACAGTGACCATCAAA	
BMEII0987 R	GTATTCAGCCCCCGTTACCT	
MLVA-16 primers pairs [d, e]		
VNTR locus		
bruce06_134bp_L	ATGGGATGTGGTAGGGTAATCG	
bruce06_134bp_R	GCGTGACAATCGACTTTTTGTC	
bruce08_18bp_L	ATTATTCGCAGGCTCGTGATTC	
bruce08_18bp_R	ACAGAAGGTTTTCCAGCTCGTC	
bruce11_63bp_L	CTGTTGATCTGACCTTGCAACC	
bruce11_63bp_R	CCAGACAACAACCTACGTCCTG	

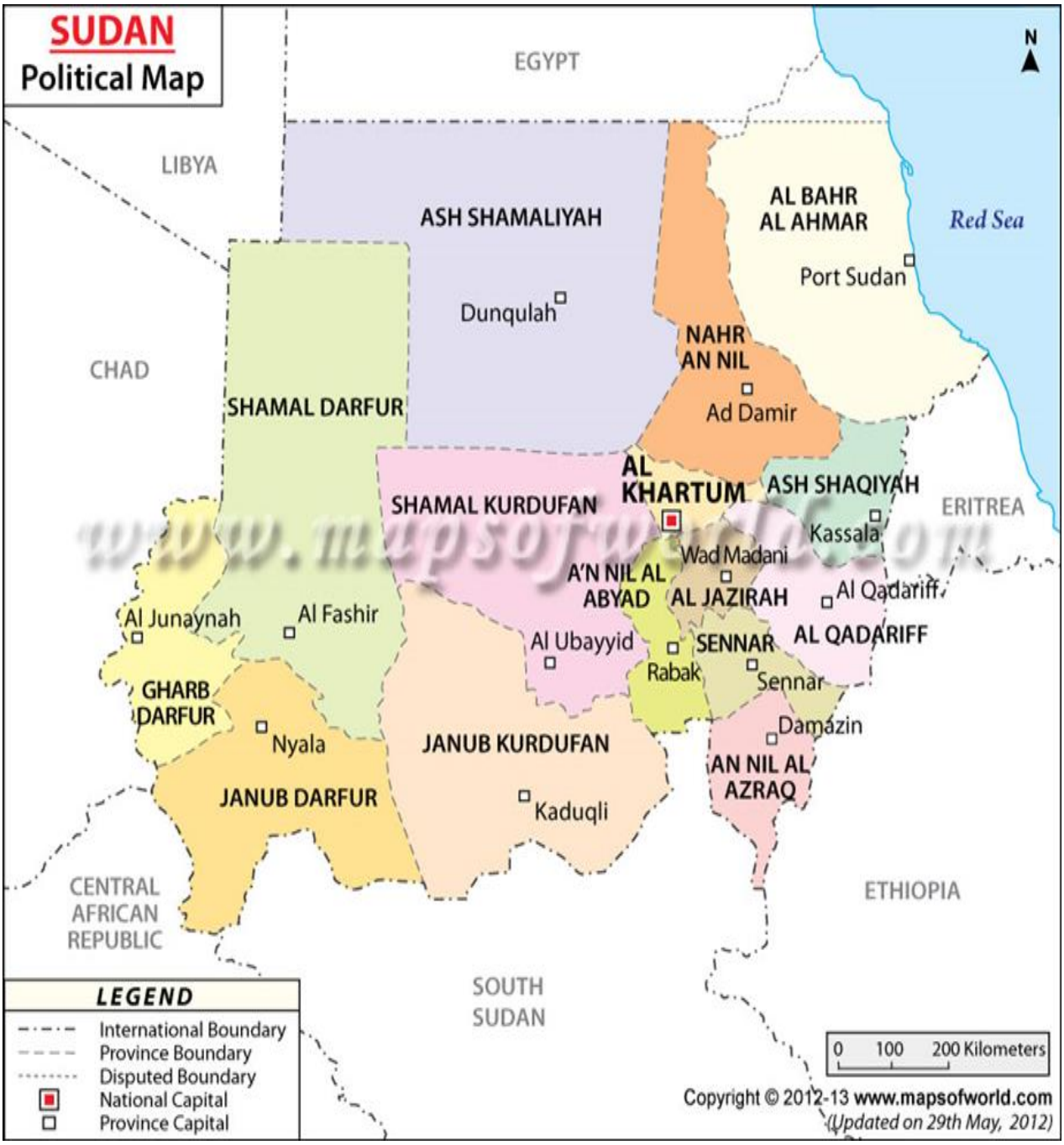
bruce12_15bp_L	CGGTAAATCAATTGTCCCATGA	
bruce12_15bp_R	GCCCAAGTTCAACAGGAGTTTC	
bruce42_125bp_L	CATCGCCTCAACTATACCGTCA	
bruce42_125bp_R	ACCGCAAAATTTACGCATCG	
Bruce43_24bp_L	TCTCAAGCCCGATATGGAGAAT	
Bruce43_24bp_R	TATTTTCCGCCTGCCCATAAAC	
Bruce45_18bp_L	ATCCTTGCCTCTCCCTACCAG	
Bruce45_18bp_R	CGGGTAAATATCAATGGCTTGG	
Bruce55_40bp_L	TCAGGCTGTTTCGTCATGTCTT	
Bruce55_40bp_R	AATCTGGCGTTCGAGTTGTTCT	
bruce04_8bp_L	CTGACGAAGGGAAGGCAATAAG	
bruce04_8bp_R	CGATCTGGAGATTATCGGGAAG	
bruce07_8bp_L	GCTGACGGGGAAGAACATCTAT	
bruce07_8bp_R	ACCCTTTTTCAGTCAAGGCAAA	
bruce09_8bp_L	GCGGATTCGTTCTTCAGTTATC	
bruce09_8bp_R	GGGAGTATGTTTTGGTTGTACATAG	
bruce16_8bp_L	ACGGGAGTTTTTGTTGCTCAAT	
bruce16_8bp_R	GGCCATGTTTCCGTTGATTTAT	
bruce18_8bp_L	TATGTTAGGGCAATAGGGCAGT	
bruce19_6bp_L	GACGACCCGGACCATGTCT	
bruce19_6bp_R	ACTTTACCGTAACGTCGTGGAT	
bruce18_8bp_R	GATGGTTGAGAGCATTGTGAAG	
bruce21_8bp_L	CTCATGCGCAACCAAAACA	
bruce21_8bp_R	GATCTCGTGGTCGATAATCTCATT	
bruce30_8bp_L	TGACCGCAAAACCATATCCTTC	
bruce30_8bp_R	TATGTGCAGAGCTTCATGTTCG	

Appendix 5. Admistrative map of Khartoum State



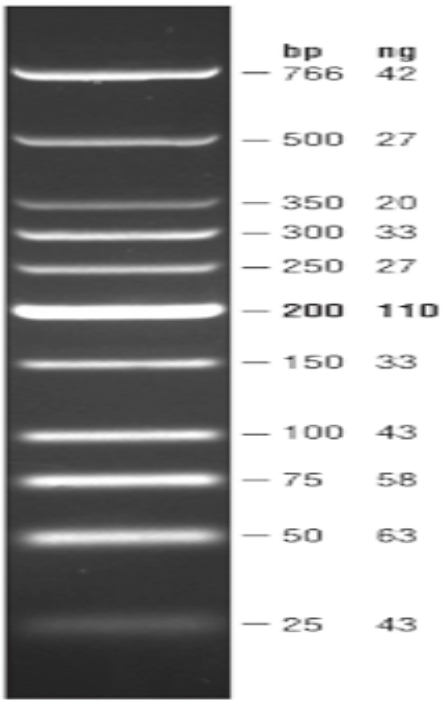
Administrative map of Khartoum State.
Available from, Source:
http://media.springernature.com/full/springer-static/image/art%3A10.1186%2Fs12985-017-0816-3/MediaObjects/12985_2017_816_Fig1_HTML.gif .

Appendix 6. Political map of Sudan

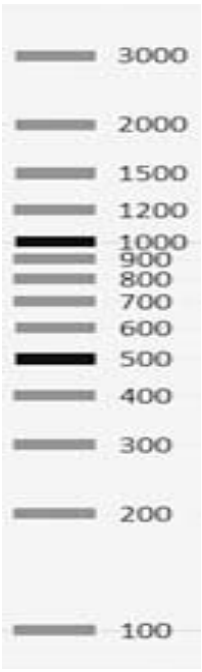


Available from: **www.mapsofworld.com**

Appendix 7. molecular weight ladders



A



B

A= Low molecular weight DNA marker visualized by ethidium bromide staining on a 3% TBE agarose gel. Mass values are for 0.5 µg/lane.

B: Invitrogen GeneRuler 100 bp plus DNA Ladder.

Appendix 8. Thayer Martin medium preparation.

The following culture media had been incorporated in the isolation and identification procedures for *brucellae*.

Thayer Martin medium: -

Thayer martin medium contained haemoglobin, which provided the X factor (hemin), and GC enrichment, which provided the V factor, vitamins, amino acids, coenzymes and dextrose. The vancomycin in the formulation provided improve inhibition of gram-positive cocci. The addition of nystatin had proved to be effective in the suppression of *Candida albicans.* The medium also contained colistin as a selective agent to inhibit most gram-negative organisms, including *Pseudomonas species*.

Thayer Martin medium was made according to NAMRU-3, (2004). The following formula was used:

GC medium	36gm
distilled water	1000ml
Haemoglobin Agar 2%	10gm
Iso-vitalex supplement	10ml
Vancomycin, Colistin and Nystatin (V.C.N) supplement	20ml

Preparation of the medium:

36gm GC media was suspended in 500ml-distilled water.

The Agar was heated with constant stirring until dissolved completely.

Autoclaved at 121 ^{0}C for 15 minutes.

10 gm of haemoglobin powder was added in 500 ml distilled water, heated with constant stirring until the haemoglobin dissolved completely.

Autoclaved at 121^{0}C for 15 minutes.

Both GC media and haemoglobin were mixed into one flask immediately.

The mixture was left to cool to 45-50^{0}C and 10ml of Iso-Vitalex and 20 ml V.C.N supplement was added Stored at 4^{0}C until used.