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Isolation, Identification and Typing of *Brucella* Species as Zoonotic Pathogens by Using Conventional and Molecular Biological Methods

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Mayada Mosad Ahmed Shaaban Gwida

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List of Abbrevation

2MET	2 Mercaptoethanol Test
AFI	Acute Febrile Illness
AFLP	amplified fragment length polymorphism
B. melitensis	*Brucella* melitensis
B. ovis	*Brucella* ovis
B.abortus	*Brucella* abortus
B.canis	*Brucella* canis
B.ceti	*Brucella* ceti
B.inopinata	*Brucella* inopinata
B.microti	*Brucella* microti
B.neotoma	*Brucella* neotoma
B.pinnipedialis	*Brucella* pinnipedialis
B.suis	*Brucella* suis
BAPAT	Buffered acidified Plate Agglutination Test
BCT	Brewer Card Test
CFT	Complement Fixation Test
C_t	Threshold cycle
CT	Coagglutination test
Cu	Copper
CVRL	Central Veterinary Research Laboratory
ELISA	Enzyme Linked Immunosorbant Assay
EU	European Union
FPA	Fluorescent polarization Assay
HGDI	Gaston diversity index (HGDI)
ICFTU/ml	International CFT unit per ml
iELISA	Indirect Enzyme Linked Immunosorbant Assay
MLST	Multilocus sequence typing
MLVA	Multilocus variable number tandem repeats
mp	Millipolarization units
MRT	Milk Ring Test
MSAT	Microserum agglutination Test
PCR	Polymerase Chain Reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphisms
PFGE	pulsed field gel electrophoresis
RAPD-PCR	random amplified polymorphic DNA
RAT	Rivanol Agglutination Test
RBT	Rose Bengal Test
Riv. T	Rivanol Test
S.19	Strain 19
SAT	Serum Agglutination Test
Spp.	Species
TAT	Tube Agglutination Test
TRs	Tandem repeat units
UPGMA	unweighted pair group method using arithmetic averages
Y. enterocolitica	Yesinia enterocolitica
Zn	Zinc

1. Introduction

Brucellosis is one of the most important zoonoses worldwide affecting livestock and humans **(Corbel, 1997).** *Brucellae* are facultative intracellular, Gram-negative coccobacilli that lack capsules, flagellae, and endospores. The genus *Brucella* is composed of nine recognized species, six of which are the "classical" members *(B. abortus, B. suis, B. melitensis, B. canis, B. ovis, and B. neotomae)* **(Cutler *et al.,* 2005).** Recently, the species *B. ceti, B. pinnipedialis,* and *B. microti* have been described **Foster *et al.,* (2007)** and the species *B. inopinata* has been proposed **(Scholz *et al.,* 2009).** *B. melitensis, B. suis and B. abortus* are of major impact by causing significant economic losses to animal owners and by provoking severe human disease. *Brucella* spp. are also a focus of interest as they are categorized as biological agents due to their high contagiousness and their impact on human and animal health. *Brucella suis* was among the earliest agents investigated and developed as a bioterrorism weapon in the United States offensive bioterrorism program in the 1950s. The zoonotic pathogens *B. abortus*, *B. melitensis*, and *B. suis* have been identified as category B bioterrorism agents **(Rotz *et al*., 2002)**. These *Brucella* spp. are also designated as selected agents by the US Government **(CDC, 2005).**

In countries of the Near East region, brucellosis was reported in almost all domestic animals, particularly cattle, camels, sheep and goats. In Egypt, brucellosis has been reported also in buffaloes, equines and swine. Brucellosis has been recognized during the last 20 years to be a serious zoonotic disease in most countries of the region. The disease has been reported in animals in most of the countries in the region, which depend on import of animals, particularly from outside the region for slaughter and breeding. With the intensification of the import of animals and the establishment of big farms in the last few years, the incidence of brucellosis rose sharply in many countries, both in man and animals. A high incidence rate of brucellosis was reported particularly from several modern commercial dairy farms. The incidence of reactors in those newly established farms of cattle reached in some countries more than 30% **(Refai, 2002)**.

A camel is an even-toed ungulate within the genus *Camelus*, bearing distinctive fatty deposits known as humps on its back. There are two recognised species of camels *(Dromedarius and Bactrianus).* The dromedary or Arabian camel has a single hump and the Bactrian camel has two humps. They are native to the dry desert areas of West Asia, Central and East Asia, respectively. Both species are domesticated to provide milk and meat **(Wilson, 1984).**

The world population of camels is about 20 million mainly in arid zones. Of which, 15 million camels live in Africa and 5 million in Asia **(GLIPHA, 2006)**. In 2001, the total camel population was 19 million. Of which, 17 million were

dromedaries and 2 million were Bactrian **(Farah and Fischer 2004)**. In most countries, the camel population increased after a period of decreasing number due to the introduction of modern transport facilities.

Camels are not known to be primary hosts of *Brucella*, but they are susceptible to both *B. abortus* and *B. melitensis* **(Cooper, 1991).** Consequently, the prevalence depends upon the infection rate in primary hosts being in contact with them. Brucellosis may spread from camels to humans, especially via milk. Therefore, the zoonotic risks from camel milk must be considered in view of the traditional African and Arabian preference for raw milk consumption.

Accurate diagnosis is the key to prevent the spread of and to control brucellosis. However, diagnosis of brucellosis is frequently difficult to establish. This is not only because the disease can mimic many infectious and non infectious diseases, but also because the established diagnostic methods are not always sensitive enough. Although serological tests have been used as diagnostic tool for screening of camels brucellosis, they are neither adequately sensitive nor specific due to an insufficient immune status of the host or serological cross reactivity **(Morgan and Mackinnon, 1979; Farina, 1985)**, as well as most tests have been directly transposed, without validation for camels. The most specific diagnostic test is isolation of the causative agent; however, it is time consuming and low sensitive especially in the chronic stage of the disease **(Alton *et al.,* 1988).** Because of these difficulties, the development of new diagnostic tests for direct detection of *Brucella* species is increasingly drawing interest.

Recently, polymerase chain reaction (PCR) has been shown to be a valuable method for detecting DNA from different fastidious and non-cultivable agents **(Brikenmeyer and Mushahwar, 1991).** Although there are several studies on Brucella-DNA detection by PCR from pure culture **(Fekete et al., 1990; Herman and Ridder, 1992)**, only a few studies have been performed in camels with clinical or field samples **(Hamdy and Amin, 2002; Alshaikh *et al*., 2007).** To our knowledge, no available data was present on the application of real-time PCR for detection of *Brucella* organisms in camel sera. Therefore; the present study considered as the first standards for the use of real-time PCR and serological tests in camels.

Brucella species have a high DNA homology of greater than 90% (**Whatmore *et al*., 2006**). Characterization of *Brucella* species and biovar level can be performed by differential microbiological approaches used for phenotyping **(Alton *et al*., 1988)**. A few tools have been introduced to molecular genotyping methods such as polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP), random amplified polymorphic DNA (RAPD)-PCR, amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE) and multilocus

sequence typing (MLST) (**Vizcaíno *et al*., 2000**; **Jensen *et al*., 1999; Whatmore *et al*., 2007**). None of them, however, has proven to be fully satisfactory for epidemiological investigation or for tracing strains back to their origin. The multilocus variable number tandem repeats (VNTR) analysis (MLVA) methods based on the monitoring of variability in the copy numbers of tandem repeat units (TRs) for several loci were introduced to the assessment of the discrimination potential of genotype based typing and epidemiological trace back.

Recently, it is announced that the MLVA typing assay for the *Brucella* species has a good species identification capability and a higher discriminatory power. Thus, it would be proposed as a complement of, or even as a substitute for, the classical biotyping methods (**Le Fleche *et al*., 2006**). Moreover, this assay shows that it could discriminate the *Brucella* isolates originating from restricted geographic sources, indicating its potential as an epidemiological tool **(Kattar *et a*l., 2008)**.

Based on the previously mentioned facts, the present study was delineated to:

1. Gain deeper insight into the prevalence of brucellosis, which remains a zoonotic disease of worldwide public health concern.
2. Assess threats of transmission into the European Union.
3. Identify areas where research is sorely needed to ensure that brucellosis epidemics are avoided in the future.
4. Determine the prevalence of brucellosis among single humped camels in Dubai and its public health significance.
5. Evaluate various serological tests as a diagnostic tool for camel brucellosis.
6. Establish a real-time PCR assay based on *Brucella* genus specific *BCSP31* and species specific insertion sequence *IS711* for rapid diagnosis of camel brucellosis in term of sensitivity and specificity in comparison with other conventional serological tests.
7. Evaluate the polymorphisms of the MLVA-16 loci in a series of *Brucella* isolates.
8. Determine the relatedness of the strain isolated from sheep in Turkey with that isolated from German tourists being infected during a stay in Turkey.

2. Literature

2.1 Epidemiology of Brucellosis

Brucellosis remains an important disease in both human, causing (chronic) febrile illness, and animals in which the main symptom is reproductive failure. A number of characteristics make *Brucella* species attractive targets for weaponization **(Rotz *et al*., 2002)**. In humans, brucellosis caused by *B. melitensis* is by far the most important clinically apparent disease and is usually associated with occupational exposure to infected animals or the consumption of unpasteurized dairy products **(Corbel, 1997)**. Additionally, *B. melitensis* is one of the major causes of abortions in sheep and goats. *B. melitensis* persists in Mediterranean and Middle East countries. Brucellosis is distributed globally and continues to pose a thread despite efforts to eradicate it from domestic animal population **(Mantur *et al*., 2007)**. The organism is secreted in the milk of infected animals. Infection with *Brucella spp*. continues to pose a human health risk also globally despite strides in eradicating the disease from domestic animals.

Animal brucellosis is well established in the Mediterranian Basin and the Middle East and affects both cattle and small ruminants **(Abdel- Ghani *et al*., 1983; Ismaily *et al*., 1988; Aldomy *et al*., 1992; Darwish and Benkirane 2001)**. It is associated with nomadic animal husbandry which is related to developing countries **(Cardoso *et al*., 2006)**. For this reason, a test and slaughter policy is not realistic in the majority of areas where *B. melitensis* is endemic due to lack of financial resources needed for compensation. Thus, international health agencies have proposed that whole flock vaccination should precede any test and slaughter programs until disease prevalence is significantly reduced (**WHO, 1998a**). In Egypt, brucellosis is still remaining one of the major disease problems in spite of attempts that were implemented to control the disease through bilateral projects with agencies or international organization **(Sahin *et al*., 2008)**. Brucellosis has been recorded in Egypt since 1939 **(Ahmed, 1939)**, and the estimated annual economic losses due to brucellosis were about 60 million Egyptian pounds yearly **(AOAD, 1995)**. The annual incidence of brucellosis in people in the Mediterranean and Middle East countries varies from 1 to 78 cases per 100.000 **(OIE, 2000; El-Sherbini *et al*., 2007)**. Although many authors had reported the incidence of brucellosis among animals in Egypt **(Refai, 1994; Abdel Hafeez *et al*., 2001)**, only few reports concerning the role of risk factors and the epidemiologic evaluation of the disease have been reported **(Kaoud, *et al*., 2010)**.

Human brucellosis is transmitted by inhalation, animal contacts, and consumption of unpasteurized dairy products and undercooked meat products. For example, consumption of traditional Arabian / African delicacies such as raw liver

can cause human infection **(Malik, 1997).** In female animals, the bacterium is localized in the tissues of the udder and then excreted via milk. In male animals, orchitis and epididymitis can lead to temporary or permanent infertility **(Corbel, 2006).** *Brucella* spp. can survive for long periods in dust, dung, water, slurry, aborted foetuses, soil, meat and dairy products. As the infectious dose is very low, infections are an occupational risk for farmers, veterinarians, abattoir workers, laboratory personnel, and others who work with animals and consume their products **(Smith and Cutler 2004).** The increase in business and leisure travel to brucellosis-endemic countries has led to importat the disease into non-endemic areas **(Corbel, 2006).** The prevalence of brucellosis in humans depends upon several factors such as nutrition habits, methods of processing milk and milk products, husbandry practices, and environmental hygiene.

The "gold standard" in the diagnosis of brucellosis is bacterial isolation, which requires long cultivation time and is often unsuccessful. Although several PCR assays have been developed, serological tests are still frequently used as diagnostic methods. The most commonly used serological screening tests are the serum agglutination test (SAT), Rose Bengal test (RBT), complement fixation test (CFT), and enzyme-linked immunosorbent assay (ELISA) **(Al Dahkou *et al*., 2003)**. All tests have limitations concerning sensitivity and specificity, especially when testing individual animals. The SAT appears less sensitive and less specific than any other standard test for all animal species compared **(Annex EFSA, 2006).** Thus, the SAT is no longer recommended as an official screening test for brucellosis within the European Union **(Commission Decision, 2008).**

Brucellosis causes more than 500,000 human infections per year worldwide. In the European Union, the highest prevalence of human brucellosis occurs in the countries of the Iberian Peninsula and the Mediterranean littoral or basin region (Portugal, Spain, Southern France, Italy and Greece) **(WHO 1998b).** The disease has a limited geographic distribution, but it remains a major public health problem in the Mediterranean region, western Asia, parts of Africa and Latin America. Human brucellosis was discovered as early as 1895 in the Pasteur Institute d'Algeria **(Refai, 2002)**. Recently, attention has been given to the disease following the serious epidemic reported in 1984 in Ghardaia, where 600 cases were diagnosed. The disease was then reported in other regions. The analysis of data obtained in the years 1988–1990 revealed that the infection rate varied from 0.36 to 0.67 per 100,000. The highest rate was recorded in May and August, which corresponds to the period of parturition and lactation of sheep and goats. Consumption of cheese was considered the main source of infection **(Cherif *et al*., 1986).** In Egypt, **El-Taweel (1999)** reported that the rate of the disease in human is greatly affected by the rate of diseases in animals. The

middle age group from 20 to 40 years had a higher incidence of brucellosis, and the number of infected females was higher than that of males. Direct contact with infected animals is responsible for 67.9% of human patients, while the indirect means were responsible for 27.3 and 4.8% of the patients are due to unknown means. No human deaths were reported from brucellosis in Egypt, although the prevalence of the disease is increasing from 0.5 during 1994 to 1.9 (per 100,000 person) during 1998. Eighteen *B. melitensis* biovar 3 and one *B. melitensis* Rev.1 vaccinal strain were isolated from humans.

Asmaa *et al.* (2005) collected 7154 peripheral blood samples from patient with fever at Assiut Fever Hospital during the period of 2002-2003. A full detailed anamnestic and clinical assessment in the form of a questionnaire was made for each individual to determine the risk factors with specific emphasis to age, sex, residence and occupation. The authors added that the prevalence of brucellosis was ($1.29 \pm 0.004\%$) and ($1.22 \pm 0.002\%$) as detected by agglutination and ELISA, respectively.

Jennings *et al.* (2007) conducted a survey for acute febrile illness (AFI) in Fayoum governorate, Egypt (population 2347249) during two summer periods (2002 and 2003). AFI patients without obvious aetiology were tested for brucellosis by culture and serology and an incidence estimate was 7.1%. 321 patients of 4,490 patients AFI enrolled met the brucellosis case definition. The estimated annual incidence of brucellosis per 100,000 populations for the governorate was 64 and 70 in 2002 and 2003, respectively. The median age of brucellosis patients was 26 years and 70% were male. 53% of the patients were initially diagnosed to have typhoid fever. Close contact with animals and consumption of unpasteurized milk products were associated with brucellosis. The high incidence of brucellosis in Fayoum highlights its public health importance, and the need to implement prevention strategies in humans and animals.

Brucellosis in animals causes tremendous economic losses due to abortion, premature birth, decreased milk production and reduced reproduction rate. Despite the advances made in surveillance and control, the prevalence of brucellosis is increasing in many developing countries due to various sanitary, socioeconomic, and political factors **(Pappas *et al.*, 2006).** Brucellosis in cattle seems to be associated primarily with poor farm hygiene, unrestricted trade and movement of animals, use of local cattle yards and fairs for trading, the practice of returning non-lactating animals to villages for seasonal maintenance and use of semen from infected bulls of unknown health status for artificial insemination.

To review the literature on animal brucellosis seroprevalence, only few published studies on brucellosis in cattle, buffaloes, camels, sheep and goats from

Egypt, Iran, Iraq, Saudai Arabia, Kuwait, Jordan, India, Yemen, Libya; Syria, Algeria and Turkey were found.

The initial aim of surveillance and control programs is the reduction of infection in the animal populations to reduce the effect of the disease on animal health and production, thus minimizing its impact on human health**.** Within the European Union (EU), measures for the eradication of brucellosis are contained in Directive 2003/99/EC of the European Parliament. The epidemiological situation in its neighboring regions is of great importance for the EU due to the potential risk of importation of infected animals or their products **(Melzer *et al*., 2007)**. An effective control of animal brucellosis requires the following elements: (1) surveillance to identify infected animal herds, (2) prevention of transmission to non-infected animal herds, and (3) eradication of the reservoir to eliminate the sources of infection in order to protect vulnerable animals or herds coupled with measures to prevent re-introduction of the disease**.** In areas where a brucellosis-free status has been established or where such a status is assumed from epidemiological data, the risk of importing the disease by means of animal movement must be eliminated. Movement of infected animals must be prohibited and import permissions should be given only to certified brucellosis-free farms or areas. This is also true for national and international transport of animal products, in accordance with the general principles and procedures specified in the International Zoo-Sanitary Code of the OIE **(OIE, 2009)**. This code also describes the testing procedures for animals and quarantine measures. Control programs should take into account incidental spreading of brucellosis by infected but serologically negative animals originating from inadequately certified sources.

Vaccination of animals practically can reduce the likelihood of developing disease after exposure, but it does not affect exposure itself. If a pathogen enters an area, both vaccinated and non-vaccinated animals will be similarly exposed. However, the vaccinated animals are less likely to develop disease following exposure. In small ruminants the initial step in brucellosis control is to vaccinate young animals (kept as replacements) with the *B. melitensis* Rev.1 vaccine. This approach is based on the hypothesis that the Rev.1 vaccine offers life-long immunity and that after implementing the vaccination program for 5-7 years, which is the productive life-span of sheep and goats, the whole population will be vaccinated and fully protected against brucellosis. This method is also recommended to minimize post vaccinal diagnostic problems and to prevent abortion **(Blasco, 1997)**.

The *B. melitensis* Rev.1 vaccine for small ruminants has not been fully evaluated for use in cattle. *B. abortus* vaccines do not effectively protect against *B. melitensis* infection, meaning that bovines infected with *B. melitensis* may pose a

serious problem even for vaccinated cattle. Furthermore, in countries like India, penal codes that prohibit slaughter of cows complicate eradication efforts

2.2 Brucellosis in Camels

Camels are the most capable animal species in utilizing marginal areas and in survival and production under harsh environmental conditions. Many pastoral groups and communities throughout the world are depending on camels for their livelihood.

2.3 Dromedaries

2.3.1 Taxonomy and breeds

In zoological taxonomy, camelids are classified in the suborder *Tylopoda* (pad footed animals) that represents with the suborders *Suiformes* (pig-like) and *Ruminantia* (ruminants) and the order *Artiodactyla* (even-toed ungulates). Thus, camelids (family *Camelidae)* as ruminating animals are classified in proximity to ruminants but developed in parallel and are not part of the suborder *Ruminantia*. Some differences as foot anatomy, stomach system and the absence of horns underline this fact **(Schwartz and Dioli, 1992; Fowler, 1998).** The family *Camelidae* is divided into three genera; the old world camels (genus *Camelus*) and the new world camels (genus *Lama* with the species *L. glama, L. guanicoe*, *L. pacos* and genus *Vicugna* with the species *V. vicugna*) **(Wilson and Reeder, 2005).**

Two domesticated species of old world camels are exist, the dromedary or one humped camel *(Camelus dromedarius)* that has its distribution in the hot deserts of Africa and Asia and the Bactrian or two-humped camel (*Camelus bactrianus)* that can be found in the cold deserts and dry steppes of Asia **(Wilson, 1984).**

2.3.2 Economical Importance of Camels

As dromedaries are very drought tolerant, they thrive in arid zones of many countries in the world and provide food, hides and transport. Therefore, there has developed an increasing interest in dromedary in arid countries, where other domesticated animals have difficulties to survive. Camels can graze on low productive pastures on which the production of milk is possible and economically profitable. For this reason, camels may reduce the dependence of pastoralists on other livestock that is usually much more vulnerable to drought than camels **(Farah and Fischer 2004).**

Camel milk is one of the most valuable food resources for nomads in arid regions and can contribute to a better income for pastoralists, as in the last year's milk consumption among the urban population increased **(Farah and Fischer 2004).** Camel milk possesses superior keeping quality to cows' milk due to its high contents of proteins that have inhibitory properties against bacteria. This makes raw camel milk a marketable commodity, even under conditions of high temperatures. Zoonotic

risks from camel milk must be considered in view of the traditional preference for raw milk consumption.

Besides milk, meat is one of the most important products of camels. It compares favorably with other livestock in yield and quality of the carcasses but camels are still not systemically bred for meat production in many regions as camels are considered too valuable for this production type. Usually males and infertile female camels are sold as slaughter animals by pastoralists. Nevertheless, saling these animals for meat production can present an important source of income. There has been an increasing demand of camel meat in people and societies that do not breed camels, thus leading to a higher number of camel abattoirs and butcheries in several countries that mainly slaughter young animals **(Farah and Fischer 2004; Finke, 2005)**.

Another important product is camel wool. It is one of the world's most expensive natural animal fibers. In some countries, camels are kept in the backyards of cities to gain wool, besides milk and meat. An adult camel usually produces 2 - 3 kg per shearing **(Wernery, 2003)**. Camel hides are known for their strength and durability. They are used by camel breeders, but also as fashion accessories **(Wernery, 2003)**. Other products used are dung as fertilizer and source of fuel for pastoralists and bones for production of jewellery or bone-meal for fertilizing purposes.

In spite of its vital importance, studies about camels are very few due to the fact that camel production is in remote, migratory and poor infrastructure condition. Available studies were based on small animal numbers **(Schwartz and Dioli, 1992).** Published information on diseases revealed that camels may be either carrier, susceptible or suffering from a vast array of infectious and parasitic diseases. Some of these diseases such as brucellosis have considerable public health importance. Brucellosis was reported in camels as early as 1931 **(Solonitsuin, 1949)**.

2.4 Economic Importance of Brucellosis

Brucellosis is characterized by abortion, non-viable offspring in female, orchitis and epididymitis in male animals (**Seifert, 1996; Radostits *et al*., 2007**). Abortion is the major feature that is manifested in camels (**Al-Khalaf and El-Khaladi, 1989**). The disease is also associated with infertility and prolonged calving intervals, and has considerable impact on camel production. Epididymitis, chronic inflammation of the joints, tendon sheath and synovial bursa especially at the carpus may also occur in camels (**Abbas and Agab, 2002; Wernery and Kaaden, 2002**). The disease can generally cause significant loss of productivity through late first calving age, long calving interval time, low herd fertility and comparatively low milk

production (**Radostits *et al.,* 2007**). The disease can also have an impact on export and import of animals constraining livestock trade.

Abu damir *et al*. (1989) experimentally infect six camels with two strains of *B. abortus*, four with S 19 and two with a field bovine strain. They observed that none of the infected camels had any inflammatory reaction at the site of inoculation or any clinical signs during the experimental period. However, camels that inoculated with the bovine field strain showed transient, slight clinical signs including a reduced appetite, a reluctance to rise in the morning, slight lameness with hot coronets, bilateral lacrimation and intermittent pyrexia. Furthermore, camels showed very early serological response by RBT, SAT and CFT. They also added that *B. abortus* had a tendency to localize in the lymph node especially those of the head and genital tract.

Afzal and Sakkir (1994) suggested that subclinical brucellosis can pose problems in racing camels by reducing their performance and productivity in the Arabian Peninsula where camel racing is highly popular.

2.5 Public Health Importance of Brucellosis

Brucellosis in humans represents a major public health hazard, which affects social and economic development in various countries. Animal health workers, butchers, farmers, and those who are habitually consume raw milk and come in contact with animals are at high risk for brucellosis **(Chukwu, 1987).** In man, transmission occurs as a result of ingestion of milk, contact via skin abrasion, mucous membranes and inhalation (**Seifert, 1996; Radostits *et al*., 2007**).

Masoumi *et al.* (1992) recorded a higher prevalence among butchers and people who habitually consume raw milk. Camel keepers consume camel milk as well as liver without heat treatment **(Gameel *et al.*, 1993).** There is also a close contact between herdsmen and the animal during watering, grooming, riding, nursing sick ones and delivery assistance **(Abbas *et al.,* 1987).** The isolation of the two major pathogenic *Brucella* species *B. melitensis* and *B. abortus,* from milk and other samples of camel origin **(Gameel *et al.,* 1993; Agab *et al.,* 1994; Hamdy and Amin, 2002)** clearly indicate the potential public health hazards of camel brucellosis **(Straten *et al*., 1997).** The disease in man may be misdiagnosed due to the prevailing malaria infections in dry areas **(Abou-Eisha, 2000; El-Ansary *et al.,* 2001).**

2.6 Epidemiology of Camel Brucellosis

2.6.1 Aetiology

Brucellosis is caused by Gram negative coccobacilli of the genus *Brucella* which are facultative intracellular that can survive within host cells causing a chronic infectious disease that may persist throughout the life of the animal. In addition to the "classical" *Brucella* spp. (*B.abortus*, *B. suis*, *B. melitensis*, *B. canis*, *B. ovis*, and *B. neotomae*) the genus has recently been expanded to include marine isolates, which

have zoonotic potential as well (**Sohn *et al*., 2003; McDonald *et al*., 2006**). Camels can be infected by *B. abortus and B. melitensis*. Different studies showed that *B. abortus* and *B. melitensis* are most frequently isolated from milk, aborted fetus and vaginal swabs of diseased camels (**Radwan *et al.,* 1992; Gameel *et al*., 1993; Agab *et al.*, 1994; Abou-Eisha, 2000; Hamdy and Amin, 2002)** and the spread of brucellosis depends on the *Brucella* species being prevalent in other animals sharing their habitat and on husbandry (**Musa *et al*., 2008).**

2.6.2 Disease transmission

Animal brucellosis can be transmitted by both vertical and horizontal transmission. Horizontal transmission occurs through ingestion of contaminated feed, skin penetration, via conjunctiva, inhalation and udder contamination during milking. Congenital infection during parturition is frequently cleared and only few animals remained infected as adult **(Radostits *et al.,* 2007).** Spread of the disease is due to movement of infected animals to disease free herds. Proximity of infected herds to non-infected herds occurs at water where camels come together. The important epidemiological risk factors are large herd size, poor management, abortion, milking more animals by a single person and herding with other ruminants. Survival of the organisms in the environment may also play a role in epidemiology of the disease. **(Abbas *et al*., 1987; Radwan *et al.,* 1992; Abuo -Eisha, 2000)**.

Small ruminants act as extensive reservoir of *B. melitensis*, which constitutes a threat of infection to large ruminants including camels and man due to prolonged contact. The chance of transmission is higher during parturition and abortion when most of the *Brucella* contamination occurs **(Dafni *et al.,* 1991)**.

2.6.3 Host Factor

Animals of all age groups are susceptible to *Brucella* infection but infection persists commonly in sexually mature animals. The seroprevalence of brucellosis was three to four folds higher among adult camels than young ones **(Yagoub *et al*., 1990).**

Various studies showed an equal distribution of *Brucella* antibodies among males and females **(Waghela *et al*., 1978; Abu Damir *et al*., 1984; Abbas *et al*., 1987; Radwan *et al.,* 1992)**. However, it was mentioned that females are more susceptible to the disease than males **(Agab 1997; Ajogi and Adamu, 1998)**. Female animals have essential epidemiological importance in disseminating the disease via uterine discharge and milk. The role of males in the spread of disease under natural condition is considered to be not important **(Radostits *et al.,* 2007).**

2.6.4 Pathogenesis and Pathology

Following exposure, the organisms penetrate intact mucosal surface. In the alimentary tract the epithelium covering the ileal Payer's patches are the preferred sites of entry. After penetration, the organisms is engulfed by phagocytic cells and

transported to regional lymph nodes **(Walker, 1999)**. Then they proliferate, disseminate haemogenously and localize in the reticuloendothelial and reproductive tract. Various mechanisms are employed by *Brucella* organisms to survive inside the phagocytic cells, inhibiting phagolysosome fusion, blocking bactericidal action of phagocytes and suppressing the myeloperoxidase H_2O_2 halide system **(Frenchick *et al.,* 1985; Harmon *et al.,* 1988; Tizard, 1992; Walker, 1999).** In ruminants, *Brucella* organisms bypass the most effective host defense by targeting embryonic and trophoblastic tissue. In cells of these tissues, the bacteria grow not only in the phagosome but also in the cytoplasm and the rough endoplasmic reticulum (**Anderson and Cheville 1986**). In the absence of effective intracellular microbicidal mechanisms, these tissues permit exuberant bacterial growth, which leads to fetal death and abortion. The presence of erythritol in the placenta may further enhance growth of Brucellae. Products of conception at the time of abortion may contain up to 10^{10} bacteria per gram of tissue (**Anderson *et al*., 1986**). When septic abortion occurs, the intense concentration of bacteria and aerosolization of infected body fluids during parturition often result in infection of other animals and humans.

Only little information is known about the pathological changes in camels. Gross lesion may be found in the predilection sites uterus, udder, testicles, lymph nodes, joint bursa and placenta. Hydrobursitis was often observed in brucellosis positive dromedaries causing swelling of the bursa **(Werney and Kaaden, 2002).** The probable possibilities for the abortion in farm animals may be due to placentitis, direct effect of endotoxins or inflammatory response in fetal tissue **(Walker, 1999).**

2.6.5 Immune Responses

Naturally infected and vaccinated animals can be serological reactors. After infection, the level of immunoglobulin IgM, IgG and IgA will significantly increase in serum **(Radostits *et al.*, 2007).** IgM antibodies, which appear initially after infection and low levels of IgG will cause complement-mediated lysis of *Brucella.* Secretory IgA is tend to be abundant in milk, where as IgG is high in serum **(Walker, 1999).** The O-chain of the smooth lipo-polysaccharide complex of the bacterial cell envelope together with the outer proteins are potent immunogens. On the other hand, the immunogenicity of the non-smooth cell variant is relatively low **(Corbel *et al*., 1980).** The O-chain specific antibodies play a major role in protective immunity, but do not eliminate the bacteria as they are protected by their intracellular niche (**WHO, 1997)**. This highlights the lack of correlation between protection and high antibody level **(Walker, 1999).**

2.6.6 Cellular Immunity

As the *Brucellae* are facultative intracellular organisms characteristic chronic granulomatous lesions develop in infected tissue where macrophage, neutrophils and lymphocytes respond to *Brucella* antigens. Phagocytes play a key role in initiating T-cells by processing and presenting antigens. Sensitized T-cells release cytokines that activate macrophages which in turn combat *Brucella* by reactive oxygen intermediates. Both CD4 and CD8 subsets are involved in cell-mediated protection. Cytokines also play a role in controlling *Brucella* infections **(WHO, 1997).** Neutrophils effectively utilize the myeloperoxidase H_2O_2 halide system in killing *Brucella*. However, the organisms inhibit degranulation and the respiratory oxidative burst and they are able to survive with in the cell **(Riley and Robertson, 1984).** Macrophages readily ingest *Brucella* when opsonized with either complement or specific antibodies. The survival of the organisms in macrophages may result from a failure of phagosome-lysosome fusion and resistance to oxidative killing by producing superoxide dismutase and catalase **(Frenchick *et al.,* 1985; Harmon *et al.,* 1988; Quinn *et al.,* 2002). Tatum *et al.* (1992)** suggested that anti-oxidant Cu-Zn superoxide dismutase plays a role in the survival of *Brucella* in phagocytic cells.

2.7 Prevalence of brucellosis in different countries

Brucellosis, particularly due to *B. abortus*, is considered to be one of the most important zoonotic diseases of camels and other domestic animals in some countries of northern Africa. Camel brucellosis was recorded to be caused by *B. abortus* and *B. melitensis* with a prevalence of 1.9-20% **(Abbas and Agab 2002).** Several published literature regarding the prevalence of camel brucellosis from different countries were summarized in Table 5.

2.7.1 Egypt:

Hamada *et al.* (1963) examined 175 camel sera collected from Giza abattoir, Cairo. All samples were tested by slide agglutination test. The positive samples were confirmed by tube agglutination test. They found that 18 (10.29%) were positive and 2 cases were suspicious.

Ahmed and Nada (1993) revealed that 12 (11.54%) out of 104 mature male camels with genital pathological affection were positive for brucellosis by RBT, Tube Agglutination test (TAT), Buffered Acidified Plate Agglutination Test (BAPAT) and Rivanol test (Riv. T) with obvious chronic epididymo-orchitis.

El-Sawalhy *et al.* (1996) collected 500 serum samples from camels at different abattoirs of Sharkia and Kaloubia governorates; Egypt. The prevalence of brucellosis was 11.6, 14, 7, 4.4, 2.93 and 2.29% by SAT, BAPAT, RBT, Riv. T, 2MET and cELISA, respectively. They isolated *B. abortus* biovar 7 from only one sample (0.2%).

Atwa (1997) investigated the seroprevalence of brucellosis in 1,258 camels imported from Kenya and Giputi at Suez quarantine station and 116 mares from different governorates of Egypt. Using Rose Bengal test, the incidence of brucellosis in imported camels and local camels were 4.05 and 7.75% respectively.

Abou-Eisha (2000) examined 592 sera from apparently healthy camels from North Sinai Province, Egypt by card test and standard tube agglutination test. 6 (1.01%) and 10 (1.7%) samples were positive with STAT and card test, respectively. *B. melitensis* biovar 3 was isolated from the milk of two seropositive mares. The author mentioned that most cases of infected camels were in close contact to or grazing with sheep and goats.

Abdel Moghney (2004) examined 766 camel serum samples. These samples were collected from 679 camels taken at abattoirs before slaughtering and 87 samples from camels in contact with farm animals at Behira province, Egypt. Two screening tests RBT and BAPAT were performed before slaughtering. The prevalence was 8.68 and 9.42%, respectively. Other confirmatory tests were used where the percentage (%) of positive samples were 9.57, 8.10, 8.89, and 9.13 in TAT, 2MET, Riv.T and ELISA, respectively. Concerning the camels contact with farm animals the seroprevalence was 9.19, 10.3, 12.6, 8.04, 8.04 and 10.3% for RBT, BAPT, TAT, 2MET, Riv.T and ELISA, respectively.

Al-Gaabary and Mourad (2004) investigated the incidence of camel brucellosis at Assiut governorate, Egypt. 430 serum samples (312 from camels before slaughtering and 118 samples from camels in contact with farm animals) were analysed using RBT, Tube Agglutination Test (TAT), 2MET and Riv. T, which yielded 7.67, 8.84, 6.97 and 6.75% positive results, respectively. The high prevalence of brucellosis indicates the importance of this disease in camels.

Ali *et al.* (2005) examined 300 camel sera using BAPAT and RBT. Positive reactors were confirmed by TAT and Riv. T. Out of the 300 sera tested, 7 (2.33%) were detected to be positive. The prevalence in Assiut governorate was 3.04%. They explained that brucellosis in camels represents a serious public health risk.

EL-Boshy *et al.* (2009) examined 340 dromedary camels from Nobaria city using agglutination and complement fixation tests. 25 (7.35%) were positive by both tests; 14 (4.12%) for *B. abortus* and 11 (3.23%) for *B. melitensis.* They mentioned that *B. abortus* provoked more clinicopathological changes than *B. melitensis*.

2.7.2 Australia:

The population of feral camels in central Australia is estimated to be at least 270,000 and the domesticated camel population less than 10,000 which are used e.g. in tourist enterprises throughout Australia **(Brown, 2004).**

Many camel herds in central Australia coexist on pastoral properties stocked with cattle. Since 1978, 1,693 camels from Central Australia had been tested using SAT, RBT and CFT, and all were brucellosis negative. Two camels were positive by SAT as part of an export consignment during 1995. Three months later they were negative to the SAT test. During Eradication Campaign (BTEC, 1974-1997), no brucellosis outbreak was recorded. Australia has been declared free from *B. abortus* in 1989; also *B. melitensis* infection has not been reported in Australia **(Brown, 2004).**

2.7.3 Sudan:

In eastern Sudan, camel brucellosis was firstly reported by **Mustafa and Nur (1968)** in Gash and Tocker where the prevalence was ranged from 0.1 to 5.5%.

In Kassala and Butana, **Mustafa and El Karim (1971)** examined 310 camels and reported that the prevalence was 1.75 and 5.7%, respectively.

Abu-Damir *et al*., (1984) stated that the prevalence of *B. abortus* antibodies was 4.9% in 740 camel serum samples tested by RBT, SAT and CFT.

Bitter (1986) examined 948 camels from different herds in eastern Sudan and reported a prevalence of 16.5- 32.3%.

Abbas *et al.* (1987) investigated 238 camel serum samples by slide agglutination test. Antibodies against *B. abortus* were detected in 8 (3%). They attributed the low prevalence of brucellosis in Sudan to the fact that camels were raised on extensive ranges without overcrowding.

Yagoub *et al.* (1990) collected 1,502 serum samples from one humped camels *(Camelus dromedaries).* The prevalence rate of *B. abortus* tested by RBT was 6.54, 5.79, 9.32, 5.03 and 8.06%, respectively from 1985 to 1989.

Agab *et al.* (1994) examined 38 serum samples by RBT. They found that 32 (84.2%) were positive for *Brucella* and they isolated *B. abortus* biovar 3 from 3 samples.

Musa (1995) examined 416 camels from seven herds in western Sudan. The prevalence was 7.9, 9.32, 5.03 and 8.06 %, respectively from 1985 to 1989. The author suggested that camels are the second most affected animal species besides cattle.

EL-Ansary *et al*., (2001) randomly collected 64 camel sera from 5 herds. All sera were screened for *Brucella* antibodies by the slide agglutination test. Seroprevelance for brucellosis was 0%.

Musa and Shigidi (2001) examined 3,303 camel sera in Nyala abattoir, Sudan. Of which 3,274 camels were examined by conventional serological tests as RBT, SAT and CFT. 256 (7.82%) were positive. The remaining 29 sera were examined by RBT and competitive ELISA (cELISA). Four (13.8%) out of the 29 sera

samples examined by cELISA were positive, while only 3 (10.3%) were positive by RBT.

Yagoub (2005) examined 756 camel serum samples. Only 12 (1.6%) showed high agglutination titres**.** On the other hand *Brucella* was not isolate from the herd.

Omer *et al.* (2007) estimated the prevalence of brucellosis in camels in Kassala area during 2004 to 2006. The serum samples were collected from 14,372 camels. All samples were investigated using RBT. The percentage of the positive sera during 2004, 2005 and 2006 was found to be 12.3, 15.5 and 30.5% (mean 19.4%), respectively.

Musa *et al.* (2008) examined 83 samples obtained from a field outbreak of brucellosis (21 camels mixed with cattle, sheep and goats and 62 apparently healthy camels from the abattoir in Darfur). Out of 21 camels, 5 (23.8%) were serologically positive and only three camels exhibited clinical signs of brucellosis. From the abattoir samples, 6 (9.7%) were serologically positive for brucellosis.

2.7.4 Kenya:

Waghela *et al.* (1978) collected 172 serums samples from camels from the north eastern province of Kenya. Three serological tests RBT, SAT and CFT were performed on all sera. 11 samples (6.39%) reacted with RBT and SAT; while 21 (12.2%) with CFT. They suggested that CFT is more specific than either the agglutination or allergic test for the diagnosis of brucellosis.

Paling *et al.* (1988) collected 300 sera from 102 camels, sera were serologically tested for *Brucella* spp. by RBT, SAT and CFT. 8 (8%) reacted positive with one or more tests. *Brucella* spp. was not isolated from serologically positive animals. They mentioned that control of camel brucellosis by elimination of reactors and vaccination of young animals is possible.

2.7.5 Iran:

Zowghi and Ebadi (1988) collected 953 serum samples and 3,500 lymph nodes from 300 camels from slaughterhouses in Iran. They found that 77 camels (8%) were serologically positive by RBT and SAT while *Brucella* was isolated from 3 out of 300 camels. The bacteria were biotyped as *B. melitensis* biotype 1 (1 case) and *B. melitensis* biotype 3 (2 cases).

Khadjeh *et al.* (1999) collected 258 serum samples from one humped camels from different parts of Boushehr, Iran. All serum samples were examined by RBT, SAT and 2 mercaptoethanol brucella agglutination test (2MET). Only 5 cases (1.93%) were positive. All positive camels were females in the age group of 5-7 years and had a past history of abortion. The lymph nodes of all serologically positive camels were cultured. *B. melitensis* biotype 1 was isolated from two camels (0.77%).

Ahmed and Nemate (2007) examined 1,123 camels by RBT, 2MET and Milk Ring Test (MRT). The RBT was used for all serum samples and both, MRT and 2MET were used to confirm RBT. Positive test results were recorded in 118 (10.5%) camels for RBT, in 96 (8.54%) camels for MRT and in 89 (7.92%) camels for 2MET. 69% of the positive camels were adults older than 4 years and the remaining 31% were younger. In infected herds, abortion rates associated with the disease ranged from 10 to 39% depending on the location. Other conditions caused by the disease were retention of placenta, fetal death and mummification, delayed maturity and infertility.

Ebrahimi *et al*. (2007) examined serologically 18 female and 135 male serum samples using rose Bengal (RBT), tube agglutination (TAT) and mercaptoethanol (MET) tests in 2006. It was shown that RBT, TAT and MET recorded 1.3, 3.9 and 2.6% reactors, respectively.

2.7.6 Libya:

Gameel *et al.* (1993) tested 967 camel serum samples of both sexes for antibodies using RBT, SAT and CFT. They found that 4.1% were positive in all 3 tests used. Also they collected 124 samples for cultural examination, only nine isolates were obtained from these samples, five isolates were from milk samples, three from aborted foetuses and one from a vaginal swab. All isolates were identified as *B. melitensis* biovar 1.

Azawi *et al.* (2001) examined 520 serum samples from camels of both sexes and from different localities for *Brucella* antibodies by RBT, SAT, iELISA and cELISA. They found that seropositivity varied from 7 (1.4%) by RBT, 6 (1.2%) by SAT 16 (3.0) % by cELISA and 18 (3.5%) by iELISA. They suggested that ELISA technique is valid for brucellosis serodiagnosis in camels and could be applied in eradication and control of brucellosis in camels.

2.7.7 Saudi Arabia:

Kiel and Khan (1989) suggested that the epidemiology of brucellosis in camels in Saudi Arabia was complicated by consumption of raw camel milk, by importation of live animals with higher prevalence of brucellosis than it is in the local animal population and by the uncontrolled movement of animals and humans across national borders. These factors are relevant in most countries where camels are kept.

Radwan *et al.* (1992) examined 2,630 apparently healthy adult camels *(Camelus dromedarius)* by RBT and "Standard United States of America *Brucella* plate agglutination test". 212 (8%) samples were found to be positive. The authors isolated *B. melitensis* biovars 1 and 2 out of 26 milk samples from 100 seropositive milking camels.

Radwan et al. (1995) examined 2,536 serum samples by the RBT and "Standard United States of American buffered *Brucella* plate agglutination test". *Brucella* prevalence was 8%. They mentioned that *B. melitensis* biovars 1, 2 and 3 were bacteriologically isolated from 41 (34%) milk samples of 120 seropositive milking camels.

Hegazy *et al.* (2004) examined serologically 98 serum samples collected from female camels at AL-Ahsa slaughterhouse. 7.14% were positive by RBT and SAT. No clinical or gross changes were detected in the mammary glands of these animals despite the fact that *B. melitensis* was isolated from highly positive animals.

Alshaikh *et al.* (2007) collected 859 serum samples from housed and free ranged camels. All samples were tested by RBT, STA, cELISA and CFT. They found that 16 samples (1.86%) were positive by RBT, 27 (3.14%) by STA, 26 (3.03%) by cELISA and 34 (3.96%) by CFT, also they were examined the sera positive in CFT by PCR and they mentioned that all were *B. abotus.*

2.7.8 Nigeria:

Okoh (1979) collected 232 serum samples from adult one humped camels at an abattoir in Kano city. All serum samples were tested by RBT and SAT. The author found that the prevalence of antibodies to *B. abortus* was 1%.

Kudi *et al.* (1997) investigated 480 serum samples from adult camels (*Camelus dromedarius*) of both sexes at Kano abattoir, northern Nigeria. The animals had been mixed with sheep and goats. They found that *B. abortus* seoprevalence was 7.5% by using the MSAT.

Junaidu *et al.* (2006) screened 329 blood samples collected over a period of 52 weeks. 37 (11.42%) were found to be positive by using RBT, SAT and cELISA. 19 (10.10%) out of 188 females and 18 (12.78%) out of 141 males were positive. The highest prevalence of 12.4% was recorded in the age range from 5.5 -10 years. Dry season was connected to the highest prevalence of 15.07%.

2.7.9 United Arab Emirates:

Wernery and Wernery (1990) recorded a prevalence of 2% in breeding camels and 6.6% in racing camels. Meanwhile, **Afzal and Sakkir (1994)** mentioned that the incidence of camel brucellosis among racing camels in Abu Dhabi was 1.5%. The authors added that 6 out of 392 camels were positive by SAT.

Moustafa *et al.* (1998) studied the prevalence of camel brucellosis in the eastern region of Abu Dhabi Emirate (Al Ain) from 1991 to 1996. The numbers of monitored sera were 1,794, 11,323, 1,900, 1,433, 3,145 and 7,899 in the years 1991, 1992, 1993, 1994, 1995 and 1996, respectively. All serum samples were screened using RBT. The positive samples were confirmed by SAT and CFT. The

seroprevalence in 1990/1991 was as high as 5.8% then declined gradually in the following years to 0.1% in 1994 to 1996.

2.7.10 Somalia:

Ghanem *et al.* (2009) randomly collected 1,246 serum samples. All samples were examined using RBT and iELISA. 49 camels (3.9%) were positive by RBT while 39 (3.1%) were positive by iELISA. They stated that iELISA detects lower antibody titers than RBT due to a high sensitivity in detection of IgM and IgG thus being more specific than RBT.

2.7.11 Eritrea:

Omer *et al.* (2000) screened samples from 98 camels by RBT and CFT. They added that the prevalence rate of *Brucella* infection in camels was 3.1%.

2.7.12 Jordan:

Al-Majali *et al.* (2008) randomly collected 412 camel sera. All sera were initially screened for presence of antibodies against *Brucella* using RBT. All positive and inconclusive serum samples were tested using CFT. They found that 47 (11.4%) were positive by RBT, and from those positive samples, 39 (9.5%) were also tested positive by CFT. Therefore, the true seroprevalence of camel brucellosis was 12.1%. *B. melitensis* biotype 3 was isolated from 4 aborted camel foetuses.

Dawood (2008) randomly collected 640 camel sera. Of which 91 (14.2%) were positive by RBT and 79 (12.3%) by CFT. Out of 26 milk samples, two (7.7%) were positive for *B. melitensis* biotype 3. Therefore, he mentioned that the true seroprevalence of camel brucellosis in the southern province of Jordan was 15.8%. The author added the high prevalence of camel brucellosis in this region to the high prevalence of brucellosis in small ruminants and lack of adequate *Brucella* control programs in sheep and goat as well as the high number of uncontrolled animal movements even across borders.

2.7.13 Kuwait:

Al-Khalaf and El-Khaladi (1989) investigated the presence of *Brucella* antibodies in serum and milk of camels by applying three serological tests, namely RBT, STAT and CFT, and additionally the MRT for milk. The prevalence rate was 14.8% from serum by CFT and RBT and 10.8% by the STAT. For milk the prevalence was 8.0%. They were unable to isolate *Brucella* organisms from the sediment and the cream of milk. However, *B. abortus* was isolated from two aborted fetuses.

2.8 Diagnosis of brucellosis:

Reliable and sensitive diagnostic tools play a crucial role in the control of brucellosis in livestock, wildlife and humans. Although blood and tissue culture remains the "gold standard" for diagnosis, culture has low sensitivity, is time

consuming, and is a risk for the laboratory personnel (**Bricker, 2002, Navarro *et al.* 2004).**

Serology is a standard technology for the epidemiological surveillance of brucellosis. However, cross-reactions between *Brucella* species and other Gram-negative bacteria such as *Yersinia enterocolitica* O: 9, *Francisella tularensis*, *Escherichia coli* O: 157, *Salmonella urbana* group N, *Vibrio cholerae* and *Stenotrophomonas maltophilia* are a major problem (**Muñoz *et al.,* 2005**).

False-positive serological results caused by *Y. enterocolitica* O: 9 may affect up to 15% of the cattle herds in regions free from brucellosis, generating considerable additional costs for surveillance programs (**Muñoz *et al.,* 2005**).

False-negative results have also been observed in serological diagnosis of brucellosis. They occur mostly due to the fact that the antibody response is dependent upon the stage of infection during sample collection (**Carpenter 1975).**

Leal-Klevezas *et al.* (2000) stated that detectable amounts of antibodies are not recorded in the first 12–16 days after artificial inoculation of goats with *Brucella abortus.* On the other hand, when the disease becomes chronic, the antibody titre could fall to undetectable level **(Tittarelli *et al.,* 2007)** which is especially the case in intracellular organisms like *Brucella* spp. Latent infection without seroconversion complicates this problem particularly in pre-pubertal animals.

It is important to note that the slide-agglutination test has been shown repeatedly to have poor diagnostic sensitivity when compared to other conventional tests **(Alton *et al.,* 1988).** The buffered-plate agglutination test (BPAT), the buffered acidified plate antigen test (BAPAT) and the card or Rose Bengal test (RBT) are comparable in sensitivity and specificity and have greater analytical sensitivity especially in the detection of IgG1. Although the three tests differ in diagnostic performance, it is generally agreed that they exhibit greater sensitivity and specificity than the SAT **(Neilsen *et al.,* 1984; Wright and Nielsen, 1990).**

In the Riv.T and 2-mercaptoethanol test (2-ME), the agglutinating activity of IgM has to be removed thus improving the diagnostic specificity. The promotion of the IgG1 reactivity has led to improved diagnostic specificity **(Nielsen *et al.,* 1984).**

The diagnostic sensitivity of the complement-fixation test (CFT) is slightly lower than that of the buffered agglutination tests, but its specificity is the highest of any of the classical tests. Consequently, CFT has often been considered to be the confirmatory test for brucellosis **(Wright and Nielsen, 1990).**

Molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. A number of genus or species-specific conventional PCR assays using primers derived from different gene sequences from the *Brucella* genome, such as 16S rRNA gene, the 16S-23S gene intergenic spacer region, *omp2* and *bcsp31* have

been established. These assays were adapted for *Brucella* detection in different clinical specimens. In the majority of studies, conventional PCR proved to be a good means to detect *Brucella* DNA from clinical specimens **(Leal-Klevezas *et al.* 1995).** The introduction of real-time PCR offers improved sensitivity, specificity and speed of performance compared with conventional PCR. Several real-time PCR assays using different detection chemistries have already been established. Moreover, some of them were evaluated with various clinical samples of human and animal origins. Most of the authors confirmed that real-time PCR was a very sensitive method for clinical samples **(Debeaumont *et al.* 2005; Queipo-Ortuño *et al.* 2005; Queipo-Ortuño *et al.* 2006).** Nevertheless, **O'Leary *et al.* (2006)** found that there was no advantage in using real-time PCR on blood, milk and lymph node samples of naturally infected cows when compared to standard serological and bacteriological methods.

Real-time PCR assay makes post amplification manipulations unnecessary. Sample processing can be automated minimizing the risk of carry-over contamination. Additionally, real-time PCR allows detection and quantification by online monitoring the accumulation of PCR amplification product during cycling thus getting first results before the procedure is ended. A fluorescence signal can be measured during the PCR process which is obtained by different approaches e.g. relying on the cleavage of fluorogenic probes by double-stranded DNA intercalating dye (SYBR Green I), by enzymatically released fluorophores (5'exonuclease assay) or by fluorescence resonance energy transfer (hybridization probes).

Redkar *et al.* (2001) developed a real-time PCR assay specific for *B. abortus, B. melitensis and B. suis.* The assay used an upstream primer that was derived from the 3' end of the genetic element IS711, whereas the downstream primers and probes were designed from signature sequences specific for a species or a biovar. The assays were tested on typed strains as well as field isolates and were found to be specific for all known biovars of *B. abortus, B. melitensis* and biovar 1 of *B. suis*.

Colmenero *et al.* (2003) used the Light Cycler detection system and SYBR Green I to develop a rapid diagnostic tool for human brucellosis. This quantitative real-time PCR assay detects a 223 bp target sequence in a gene, which is highly conserved at genus level encoding an immunogenic 31 kDa protein of the external membrane of *B. abortus.* They examined serum samples of 60 patients suffering from active brucellosis and the assay had a sensitivity of 91.9% and a specificity of 96.4%.

2.9 Comparative studies on antibody detection, cultural and molecular methods.

Various research workers have tried to evaluate PCR techniques for diagnosis of brucellosis in different animal species comparison with "conventional" techniques

like cultural isolation and serological methods. Unfortunately, little information was available in camel.

Gallien *et al.* (1998) used PCR to detect *Brucella* species from the uterus, udder, spleen, lymph nodes, kidney and liver of 3 cows, which had been naturally infected in an outbreak. They compared their results with the result of bacteriological investigations. All 18 samples reacted positive in the PCR, but 5 samples had only weak electrophoretic bands.

Chan *et al.* (1998) compared PCR with conventional methods by collecting semen samples from 185 bulls from serologically negative herds on Cheju Island in Korea. They found that 5 bulls were positive by cultural and PCR methods whereas one was positive and 5 were suspicious by the semen plasma agglutination test. Finally the results of comparative testing suggested that the PCR was more sensitive than the agglutination test on semen from bulls.

Guarino *et al.* (2000) mentioned that PCR can be complementary to classical serological tests in living buffaloes especially in the initial phase when the immune response of the animal is not detectable.

Leal-Klevezas *et al.* (2000) compared PCR sensitivity against some commonly used serological and bacteriological techniques on 23 milk and blood samples from 300 clinically healthy goats. The results showed that 86% of the blood samples were positive on the PCR test, while 60% were positive on the serological test. *Brucella* was isolated only from one blood culture. 64% of the milk samples were positive using PCR, but failed to yield bacteria in the culture. This study demonstrated the higher sensitivity of PCR when compared to RBT and blood culture.

Al-Attas *et al.* (2000) collected 17 blood samples from patients with brucellosis, only 14 were obtained before treatment started. The samples were tested by serology, blood culture and PCR. They found that 7 (57%) were positive by culture while all samples showed high titer by standard tube agglutination test (STA). PCR was positive in the 14 pretreatment samples reflecting 100% sensitivity. They suggested that both the sensitivity and specificity of PCR were significantly higher than that of culture and serology.

Amin *et al.* (2001) compared traditional culture with PCR on 120 serologically positive bull and ram semen samples. *B. melitensis* was detected in 12 out of 120 (10%) samples by PCR, while only 7 samples (5.8%) were positive by isolation. They suggested that PCR is a good supplementary test for the detection of *Brucella* in semen of infected animals.

Leyla *et al.* (2003) evaluated the detection of *Brucella* DNA directly from the stomach contents of aborted sheep fetuses. Out of 39 positive cultures, 38 were *B. melitensis* detected by PCR. Sensitivity and specificity of the PCR were as 97.4 and

100%, respectively. The results indicated that this PCR procedure had a potential for use in routine diagnosis of sheep brucellosis.

Manterola *et al.* (2003) concluded that PCR had sensitivity similar to that of semen culture and could be used as a complementary test for the direct diagnosis of *B. ovis* in semen samples of rams.

Nimri (2003) mentioned that PCR can be applied together with serology for the diagnosis of brucellosis in suspected and relapsed human cases regardless of the duration or type of disease without relying on blood culture. This is especially true in chronic cases.

The sensitivity and specificity values of assays used for the detection of *B. abortus* were examined by **Gall and Nielsen, (2004)**. They found that the mean sensitivity of culture, RBT, STAT, PCR and iELISA were found to be 46.1, 81.2, 75.9, 82.0 and 96.0 %, respectively, while mean specificity was found to be 100.0, 86.3, 95.7, 98.6 and 93.8 %, respectively. Furthermore, the buffered antigen plate agglutination test (BPAT) had better accuracy than the other conventional tests including RBT and CFT. In addition, the primary binding assays, including the fluorescence polarization assay, the iELISA and the cELISA, were overall more accurate than the conventional tests, except BPAT.

Lavaroni *et al.* (2004) mentioned that bovine brucellosis was diagnosed using PCR in blood, iELISA, CFT and cELISA in serum. Serological tests showed 100% sensitivity when compared to PCR. The specificity of CFT, cELISA and iELISA was 100, 99 and 95%, respectively. They dedicated that PCR could be useful to identify *Brucella* biotypes and to complement serological tests.

Scarcelli *et al.* (2004) analyzed samples of abomasal content and organs of 67 aborted bovine fetuses by means of bacteriological methods and by multiplex PCR for *Brucella* and *Leptospira*. Out of 67 samples, 34 (50.7 %) were positive for *Brucelle* by multiplex PCR; however, *Brucella* was isolated only from 26 samples (38.8 %), which showed 88 % agreement rate between the two methods. PCR was found to be more sensitive than culture in bovine brucellosis cases.

Gupta *et al.* (2006) examined 54 milk samples from goats with a history of abortions by PCR. Of 54 goats, 32(59%) were serologically positive. The PCR assay used amplified a 720 bp sequence of the *omp31*. 48 samples (88.8 %) out of 54 milk samples were positive by PCR including the 32 samples positive by serology. They also mentioned that PCR had high sensitivity and specificity.

Khosravi *et al.* (2006) examined 30 blood samples from human patients with brucellosis using culture and PCR. Only 8 (26.6 %) were positive by routine blood culture, while 28 (93.3%) were positive by PCR. They considered PCR to be a very useful tool for the diagnosis of brucellosis when compared to conventional culture.

Sanjiv and Puran (2006) mentioned that PCR is sensitive and capable of demonstrating the presence of *Brucella* agent. Unlike serological assays, PCR can be used as a routine diagnostic assay for the diagnosis of brucellosis in small ruminants.

Amani *et al.* (2007) stated that the sensitivity of PCR were 100 % in culture positive human cases and suggested that PCR could replace blood culture as the gold standard for the diagnosis of acute brucellosis.

Kanani (2007) compared serology, culture and PCR for their diagnostic use in serum and semen samples of 101 bulls. Out of 101, 6 (5.94%), 10 (9.90%) and 10 (9.90%) were positive by RBT, STAT and ELISA, respectively. 8 (7.92%) were found to be positive by culture. Among the three PCR assays used, 19 (18.81%), 2 (1.98%) and 5 (4.95%) were positive by B4/B5, JPF/JPR and F4/R2 PCR, respectively. The author suggested that PCR is more sensitive when compared to the other methods.

Iihan *et al.* (2008a) compared culture and PCR for detection of *Brucella* from blood and lymphoid tissue obtained from 162 slaughtered sheep. They found that serum samples examined by RBT and SAT had a sensitivity of 31.4 % and 27.7 %, respectively. *B. melitensis* was isolated from 1.2 % of blood and 17.2 % of lymphoid tissue samples. *B. melitensis* DNA was detected in 27.7 % samples by "blood PCR" and in 29.0 % by "tissue PCR". They focused on the importance of using more than one diagnostic technique for the detection of animals being positive for brucellosis.

B. melitensis DNA was detected by **Iihan *et al.* (2008b)** in 24 (23.5%) out of 102 ovine milk samples by PCR, while only 8 (7.8%) samples were positive by culture. The PCR assay was considered to be a very useful tool for the rapid diagnosis of *B. melitensis* in sheep milk.

Kazemi *et al.* (2008) examined 104 blood samples from suspicious human patients by serology, culture and PCR. Of which, 73 were positive by PCR, 15 by culture and 84 by serology. They suggested that PCR is more sensitive and specific than culture and serology for the diagnosis in samples from peripheral blood.

Out of 235 samples from wild boars, 27 (11.5%) were positive by *Brucella* genus specific *BCSP31* PCR. Bacteriological examination revealed that Brucellae were isolated only from 25 cases. The positive results in PCR can be caused by the presence of bacterial fragments (DNA) without any live bacteria in the sample (**Weiner *et al.*, 2009).**

Prevention of brucellosis in humans ultimately depends on control of the disease in the animal hosts. Efforts to control brucellosis are justified economically and in terms of public health. The economic aspects include losses due to clinical disease in humans and animals and other losses associated with agriculture markets for animals and animal products. The recommended forms of control are "test and

slaughter" and vaccination **(WHO, 1997).** The "test and slaughter" policy is recommended when the disease is confirmed serologically and bacteriologically. In such cases the entire herd should be regarded as infected.

2.10 Cross-border molecular tracing of brucellosis in Europe

Brucellosis is prevalent in some middle-eastern countries such as Iran, Iraq, Saudi Arabia, Egypt, Syria **(Refai, 2002)** and some south-eastern European countries such as Greece, Italy and Spain. Turkey has borders with several of these countries. Therefore, it lies within the risky area between Middle East and Europe. Turkey is also a major sheep producing country with a breeding population of approximately 20 million head **(GRAIN, 2010)**. Since Turkey has a border with the European Union (EU) and abridge with Asia, the potential for animal movement and thus disease spread across countries in this region is of particular concern **(Yilmaz, *et al*., 2002).** Therefore, it is very important to have a strain typing epidemiological tool for source trace back in outbreaks of infection. Human brucellosis has serious public health consequences in endemic areas (**Corbel, 2006).** In Turkey, brucellosis is common, especially in the Middle, East and Southeast Anatolia regions. In the last decade, the officially annual report showed incidence rates in Turkey ranged from 15 to 25/100,000 population summing up more than 10,000 human cases per year **(Demirel *et al*., 2009; http://www.oie.int/wahis/public.php).** However, only about one third (31.8 %) of the serologically diagnosed brucellosis cases are notified to Provincial Health Directorates leading to significant underreporting of the disease **(Durusoy and Karababa, 2010)**. Spatial analysis of human brucellosis cases notified to the Turkish Ministry of Health from 1996 through 2006 identified Southeastern Anatolia as high-risk region with an ongoing uptrend of local incidence rates **(Demirel *et al*., 2009).** A major disease cluster was reported from Van province which is located within Southeastern Anatolia sharing a border with Iran. Van is characterized by the highest stock density of the country **(Demirel *et al*., 2009)**. Various prevalence rates of brucellosis have been reported for human population from different parts of Turkey (Table 6). The seroprevalence of brucellosis was found to be 6.2% among farmers in East Anatolia by **Sonmez *et al*. (1997)**. The seroprevalence of brucellosis for humans living in the rural area in Middle Anatolia was 4.8% **Cetinkaya *et al*. (2005)**. However, in another study performed in the same region, the seroprevalence of brucellosis in farmers was 3.2% **(Apan *et al*., 2007).** The Seroprevalences of 2.9 to 8.5% were reported by **Kose *et al*. (2006)** for rural and suburban communities in West Anatolia**.** In the province of Erzurum neighbouring Kars in East Turkey, a cross-sectional study on human brucellosis in 2005 revealed a seroprevalence of 5.4% **(Vancelik *et al*., 2008).**

Otlu *et al*. (2008) collected serum samples from 246 farmers and 28 veterinarians from Kars district of Turkey between 2004 and 2006. Out of farmer samples, 44 (17.9%) were found to be positive by ELISA. Of the 28 sera from veterinarians, 13 (46.42%) were positive by RBT, SAT and ELISA. *Brucella melitensis* is also responsible for most of the losses attributed to brucellosis in sheep in Turkey. Studies carried out in different regions of Turkey have shown that *B. melitensis* is responsible for approximately 20 percent of abortion cases in sheep (**Cetinkaya *et al*., 1999**). The prevalence of brucellosis was found to be 37.1% among sheep population in the country **(Sahin *et al*., 2004)**.

Brucellosis in sheep has to be considered a nation wide problem (Table 7). **Bercovich *et al*. (1998)** examined two flocks of fat tailed sheep, in the Konya province of Turkey, from which *B. melitensis* was isolated. Group A consisted of 55 aborted sheep vaccinated with Rev 1 vaccine, whereas group B consisted of 77 aborted not vaccinated sheep. Sera from those animals were tested with SAT, CFT, RBT and ELISA. 37 (67.2%) out of 55 sheep in flock A and 48 (62.3%) out of 77 sheep in the flock B were positive with all the tests. A serological study in the Region of Central Turkey between 1993 and1997 showed that 15.6% of aborting sheep were positive for brucellosis **(Karaman and Küçükayan, 2000).**

Otlus *et al*. (2008) collected 167 serum samples from aborting sheep from nine different villages, the serum samples were tested by a serum agglutination test. 71 (40.11%) serum samples out of 167 found to be positive for *Brucella melitensis.*

Celebi and Atabay (2009) collected 400 serum samples from 16 different flocks of sheep having a history of abortion in Kars in Turkey. The samples were examined for the presence of antibodies against *Brucella* using Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), Rivanol Agglutination Test (RAT) and Complement Fixation Test (CFT). All animals were unvaccinated against *Brucella*. Of the tested serum samples, 147 (36.7%), 142 (35.5%), 139 (34.75%) and 135 (33.75%) were positive by SAT, RAT, RBPT and CFT, respectively.

Erdenlig and Sen (2000) mentioned that out of 78 *B. melitensis* strains from different regions of Turkey, 88.5% were of biovar 3. Additionally, out of 39 *B. melitensis* strains isolated from sheep in Central Anatolia in Turkey, 94.8% were biovar 3 **(Güler *et al*., 2003)**.

Lihan *et al*. (2007) isolated 26 (19.2%) *B. melitensis* strains from the stomach contents of 135 aborted sheep fetuses in the region of Van in East Anatolia, Turkey. They were all identified as *B. melitensis* biovar 3 by using standard classification test. *B. melitensis* was detected by PCR in 29 (21.4%) stomach content.

Lihan *et al.* (2008a) compared culture and PCR for detection of *Brucella* from blood and lymphoid tissue obtained from 162 slaughtered sheep *B. melitensis*

was isolated from 1.2 % of blood and 17.2 % of lymphoid tissue samples. *B. melitensis* DNA was detected in 27.7 % samples by "blood PCR" and in 29.0 % by "tissue PCR". They found that serum samples examined by RBT and SAT had a sensitivity of 31.4 % and 27.7 %, respectively.

Lihan *et al*. (2008b) collected 102 sheep milk samples from 92 different sheep flocks after abortion. The samples were examined by culture, PCR and MRT. A total number of 8 (7.8%), 24 (23.5%) and 28 (27.4%) *Brucella* positive samples were detected only by culture, PCR and MRT, respectively. The isolated *Brucella* strains were determined as *B. melitensis* biovar 3.

Büyükcangaz *et al*. (2009) investigated 55 abortion cases from sheep during the birth seasons of 2004 and 2005 in North Western Turkey. From the 21 *B. melitensis* strains isolated, 14 were identified as *B. melitensis* biotype 3 (66.6%), 6 were *B. melitensis* biotype 1 (28.5%), and 1 was *B. melitensis* biotype 2 (4.7%).

In contrast to Turkey, Germany has been "officially free from ovine/caprine and bovine brucellosis" since 2000. Nevertheless, about 30 human case being annually reported and the mean annual incidence of human brucellosis in Germany ranges between 0.02 and 0.04/100,000 population (Robert Koch-Institut: SurvStat, http://www3.rki.de/SurvStat, date of query: 1st September, 2010). The incidence rate is known to be significantly higher in the Turkish segment of the German population with 0.3/100,000 population **(Al Dahouk et al., 2007a).** Turkish immigrants are mainly at risk to contract *Brucella* infection while visiting their homeland. Because of its geographic position at the gateway between the Middle East and Europe, Turkey and especially South-eastern Anatolia may play a key role in the re-emergence of brucellosis in the European Union **(Gwida *et al*., 2010)**. To assess the general impact of this interface, the genetic fingerprints of *B. melitensis* strains imported to Germany were compared to ovine strains from Turkey in a molecular epidemiological study.

3. Material and Method

The samples were divided into two parts

A. Human and camel samples from Dubai

A total of 900 serum samples were received from Central Veterinary Research Laboratory (CVRL); Dubai, UAE (Table 8). From which, 530 samples were collected from apparently healthy camels *(Camelus dromedaries)* which were imported from Sudan at the end of 2008 and the beginning of 2009, as well as 365 samples were collected from local camels, in addition to 5 human samples .

3. A.1 Reference bacterial strains

B. abortus biovar 1 (strain 544) and *B. melitensis* biovar 1 (strain 16M) reference strains were used as positive control.

3. A.2 Antigen

Antigens used for Rose Bengal Test, Slow Agglutination Test and Complement Fixation Test were supplied by Institute Pourquier, France. Positive and negative control sera were national reference sera standardized according to OIE. Positive control sera contain 421 I.U/ml for SAT and 595 ICFTU/ml for CFT.

3. A.3 Chemicals and reaction kits

3. A.4 Reagents for real-time PCR

TaqMan Universal Master Mix ready to use was obtained from Applied Biosystems, New Jersey, USA. The primers and probes were obtained from TIB MOLBIOL (Berlin, Germany).

3. A.5 Reagents for CFT

Complement, haemolysin or amboceptor's, haemolytic system and veronal buffer were supplied by Virion /Serion GmbH, Germany.

3. A.6 Instruments

Table 1. Devices used in this study

Instruments	Type
Vortex Gene 2	**Scientific industries, NC, USA**
Pipettes	**Eppendorf research**
Water bath GFL	**Labortechnik mbH Germany**
Water bath P-DIG	**Medingen., Germany**
Centrifuge (Labofuge 400)	**Heraeus Germany**
Infinite F 500 (Tecan, photometer)	**Switzerland**
Microtitier plates type COS96fb**.**	**Corning USA**
Spectrophotometer	**NanoDrop ND-1000 USA**
Mx3000P thermocycler system	**Stratagene, La Jolla, CA**

3.2 Methods

3.2.1 Rose Bengal Test (RBT)

The test depends on early detection of *Brucella* specific agglutinins by using antigen stained with Rose Bengal and buffered to a low pH, usually 3.65 ± 0.0. The test was conducted as described in the Manual of Standards for Diagnostic Tests and Vaccines **(OIE, 2009)**.

Serum samples and RBT antigen were brought to room temperature then 30 µl of serum were transferred to a clean, dry and non greasy glass slide by micropipette. The antigen bottle was shaken to ensure homogenous suspension then 30 µl of the antigen was added. The antigen and serum were mixed thoroughly with a spreader and then the slide was agitated gently for 4 minutes. The result was noted immediately after 4 minutes. Any visible describe reaction after 4 minute was considered to be positive.

3.2.2 Slow Agglutination Test (SAT)

The test was performed in microplates according to **(OIE, 2009).** Samples showing more than 30 international units per milliliter were considered positive.

3.2.3 Complement Fixation Test (CFT)

The test was done as described in **(OIE, 2009)**. The diluted test sera and appropriate working standards were inactivated for 30 min in a water bath at 61°C ± 2°C. The technique was performed as follows using standard 96-well microtitre plates with round (U) bottoms. 25 µl of diluted inactivated test serum were placed in the well of the first, second and third rows. The first row was an anti -complementary control for each serum. 25 µl of CFT buffer were added to all wells except those of the second row. Serial doubling dilutions were then made by transferring 25 µl volumes of serum from the third row. Onwards; 25 µl of the resulting mixture in the last row were discarded. 25 µl of antigen of working dilution were added to each well except in the first row. 25 µl of complement, diluted to the number of units required, were added to each well. Control wells containing diluent only, complement and diluent and antigen, complement and diluent, were set up to contain 75 µl in each well. The plate was covered and incubated at 4°C overnight. The plate from the first day was prewormed at 37°C for 30 min. in an incubator. 50 µl of the freshly prepared haemolytic system was added into each well and shake carefully. The plate was incubated at 37°C for 15 -30 min. The incubation was stopped when the complement controls with 2 and 1 units complement showed complete haemolysis. The plate was centrifuged for 5 min at 2,000 rpm. The haemolysis was compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity was checked for each serum in the first row.

3.2.3.1 Interpretation

The results were expressed in international CFT unit ICFTU/ml calculated in relation to those obtained in a parallel titration with a working or national standard serum calibrated against the OIE International Standard Serum **(OIE, 2009)** which contains 595 ICFTU/ml. In general, sera giving positive fixation at a titre equivalent to 20 ICFTU or greater were considered to be positive.

3.2.4 Competitive Enzyme Linked Immunosorbent Assay (cELISA)

The competitive enzyme linked immunosorbant assay (cELISA), was done and results were interpreted according to the instructions of the manufacture (SVANOVIR® *Brucella*-Ab c-ELISA, Svanova Biotech AG Uppsala, Sweden).

Samples, reagents and plate(s) were brought to room temperature prior to starting the test. 500 ml wash solution per plate was prepared by adding 25 ml PBST (Phosphate Buffered Saline Tween-20) to 475 ml distilled water. Serum samples were diluted 1:100 with sample dilution buffer. Positive and negative controls were diluted 1:200 in dilution buffer. 50 µl of the prediluted controls and samples were added into each of the appropriate wells, the controls were run in duplicate. 50 µl of the sample dilution buffer was pipetted into two appropriate wells as conjugate control. 50 µl of mAb solution was added into every well used for controls and samples. The plate was sealed and all the reagents were mixed for 5 min. The plate was incubated at room temperature for 30 min. After that the plate was rinsed 4 times with PBS Tween buffer. 100 µl of the conjugate solution were added into each well. Then the plate was sealed and incubated at room temperature for 30 min. The plate was rinsed 4 times again with PBS Tween buffer. 100 µl of the substrate solution were added to each well and incubated for 10 min at room temperature. The time started after the first well was filled. The reaction was stopped by adding 50 µl from the stop solution to each well. The optical densities of the controls and samples were measured at 450 nm in a microplate photometer.

3.2.4.1 Interpretation

The results were expressed as percentage inhibition (PI), calculated from the ODs (Optical density) of the samples and conjugate controls, respectively, using following formula: $PI = 100 - [(\text{mean } OD_{sample} \times 100)/ (\text{mean } OD_{conjugate\ control})]$.

The status of a test samples were determined as follow:

< 30% PI was negative, ≥ 30% PI was positive.

3.2.5 Fluorescent polarization assay (FPA)

The assay was done and results were interpreted according to the instructions of the manufacture (Diachemix Whitefish Bay, WT, USA)**.** FPA was conducted in 96-well flat-bottom black polystyrene microtitre plate. Initially, 180 µl of dilution buffer and 20 µl of test sera were added. The dilution buffer was provided by the

manufacturer in 25 × concentrated form and the working dilution was prepared using ultra clean sterile water. In each microplate, positive and negative control sera of bovine origin, provided by the manufacturer, three negative controls and one positive control were pipetted in the first three wells. Buffer and serum samples were mixed by setting the microplate on a rotating microplate shaker for 2 min. at room temperature. After initial mixing, a background reading was made in fluorescence polarization mode by a multi-mode microplate reader **(Tecan Genios Pro, Switzerland)** connected to a laptop computer. Subsequently, 10 µl of antigen conjugated with Fluorescein isothiocyanate (FITC) were added to every well. After mixing for 3 min. at room temperature, a second reading was made. The reader automatically subtracted the background reading and calculated the value for every sample in millipolarization units (mP). The results of each microplate measurement were interpreted as follow: < 10 mp considered as negative, = 10-20 considered as suspected, > 20 considered as positive

3.2.6 Isolation and identification of *brucella*

For isolation and identification of *Brucella* from camel sera, we followed the standard procedures described by **(Alton *et al.*, 1988; OIE 2009)**.

100 µl of serum were spread on plates of *Brucella* agar medium (BBL TM, Becton, France). The plates were incubated at 37°C for 15 days under 10% CO_2 tension (Carbon dioxide incubator). The plates were inspected every 24 h for growth.

Formula per litre

Panceratic digest casein, 10.0 g; Peptic digest of animal tissue, 10.0 g; Dextrose, 1.0 g; Yeast extract, 2.0 g; Sodium chloride (Nacl), 5.0 g; Sodium bisulfate (Na_2So_4), 0.1 g; Agar, 15.0 g. pH adjusted to 7.0 ± 0.20

3.2.7 DNA extraction

DNA extraction from serum samples was performed using the High pure^{TM} PCR Template preparation Kit (Roche Diagnostics, Mannheim, Germany). Briefly, 200 µl of serum sample mixed with 200 µl of binding buffer and 40 µl proteinase K were mixed immediately and incubated for 10 min. at 70°C. 100 µl of isopropanol was added and mixed. The mixture was applied to the high pure filter tube and centrifuged for 1 min. at 8,000 x g. Flow through and collection tube were discarded. 500 µl inhibitor removal buffer was added and centrifuged for 1 min. at 8000 x g. Flow through and collection tube were discarded. 500 µl wash buffer were added, centrifuged for 1 min. at 8000 x g and the flow through and collection tube were discarded. The last step was repeated again. The column was centrifuged for 10 seconds at 13,000 x g and the collection tube was discarded. A new tube was added. Then 200 µl of the elution buffer at (70°C) were added and again centrifuged for 1 min at 8,000 x g. Subsequently, the concentration of DNA was determined

photometrically using a NanoDrop ND-1000 UV-Vis spectrophotometer **(Nano-Drop Tsechnologies, Wilmington, DE, USA).**

3.2.8 Real-time PCR.

Real-time PCR for the genus specific *Brucella* cell surface salt extractable *BCSP31* kDa protein gene was performed on DNA extracted from camel serum samples using the following primers (**5`GCTCGGTTGCCAATATCAATGC3`)** as forward primer and **(5` GGGTAAAGCGTCGCCAGAAG 3`)** as reverse primer together with genus specific probe **(5`**AAATCTTCCACCTTGCCCTTGCCATCA 3`) **(Probert *et al.*, 2004).** The primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). The real-time PCR assay was prepared using the TaqMan™ Universal Master Mix (Applied Biosystems, New Jersey USA) containing the following components per reaction: 12.5 µl master mix, 0.75 µl of each primer (0.3 µM) and 0.25 µl Taq Man probe (0.1µM). 2 µl of bacterial DNA was used as target and nuclease-free water sum up to a total reaction volume of 25 µl. No template controls that contained 2 µl of water instead of DNA and positive controls that contained DNA of *Brucella* were included in each run to detect any amplicon contamination or amplification failure. The real-time PCR reaction was performed in duplicate in optical 96-well microtitre plates **(q PCR 96-well plates, Micro Amp TM, Applied Biosystem)** using a Mx3000P thermocycler system (**Stratagene, La Jolla, CA)** with the following run conditions: 1 cycle of 50°C for 2 min., 1 cycle of 95°C for 10 min., followed by 50 cycles of 95°C for 25s and 57°C for 1 min. All the examined samples were further examined by second gene IS711 for conformation and typing. *Brucella IS711* species specific real-time PCRs for *B*. *abortus* and *B*. *melitensis* using the primers and probe as described previously by **(Probert *et al.*, 2004)** for typing. Amplification reaction mixtures were prepared in volumes of 25 µl containing 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems) 0.75 µl of each primer (0.3 µM) and 0.5 µl TaqMan probe (0.2µM), 5 µl of template, and nuclease-free water sum up to a total reaction volume of 25 µl. Optimisation resulted in reaction condition of 2 min. at 50°C, 10 min. at 95°C, followed by 50 cycles of 95°C for 15s and 57°C for 1 min.

3.2.9 Determination of PCR efficiency.

As efficiency is one of the most important PCR parameters, its correct determination makes it possible to increase the precision of real- time PCR tests. Reaction efficiency is best assessed through the generation of a standard curve which was widely used for the purpose of calibrating real-time PCR reaction against known concentration of nucleic acid. A standard curve is generated by plotting a dilution series of template against the C_t value for each dilution **(Larionov *et al.*, 2005).** The quantitative endpoint for real-time PCR is the threshold cycle (C_t). The C_t is defined

as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. The numerical value of the C_t is inversely related to the amount of amplicon in the reaction (i.e., the lower the C_t, the greater the amount of amplicon) (**Schmittgen and Livak 2008**). The template used to generate the standard curve should match (as closely as possible) what is being used for the experiment. The dilution range or dynamic range this is the range over which an increase in starting material concentration give rise to corresponding increase in product should span the concentration range expected for unknown samples. The slope of the curve is used to determine the reaction efficiency. The standard curve also includes an R^2 value (correlation coefficient) which illustrated how well the data fit the standard curve. The R^2 value reflects the linearity of the standard curve. Linearity and linear range are the key evaluations of the accuracy in assay validation, it is the ability of an analytical procedure to produce test results that are proportional to the concentration of analyte in samples within a given concentration range. Using the standard curve the upper and lower limits of linearity were determined as 40. In order to generate a standard curve for the quantification of *Brucella* DNA the concentration of purified *B. abortus* DNA was spectrophotometrically determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Five replicates of six 10 fold serial dilutions of *B. abortus* DNA in negative camel sera were assessed simultaneously in a single run **(Tomaso *et al*., 2010).**

To determine the limit of detection, a probit analysis was performed using (SPSS for windows, Version 17.01, SPSS Inc., Chicago, USA). The probit analysis was done to determine the number of genome equivalents that can be detected with a probability of 95%. The theoretically possible detection limit is free genome equivalents per reaction. This means for *Brucella* spp. around 10 fg DNA. Therefore, probit analysis was performed with the result of 8 replicates of the following amount of DNA per reaction: 1 pg, 100 fg, 50 fg, 20 fg, 10 fg, 5 fg and 1 fg, carried out on three different days. The data were also used to determine repeatability and reproducibility of the assay which refer to the closeness of the agreement between the results of successive measurements of the same measure and carried out under certain conditions of measurement.

3.2.10 Statistical analysis

To compare the sensitivity, specificity and overall agreement between the various tests, the statistical formulas given by **Thrusfield (1995)** were used as described below:

		Gold standared test		Total
Test to be compared		Positive	Negative	
	Positive	a	b	A+b
	Negative	c	d	C+d
	Total	a+c	b+d	a+c+ b+d= N

The notations used above are:

a = Number of samples positive to both conventional and the gold standard tests

b = Number of samples positive to conventional but negative to the gold standard test

c = Number of samples negative to conventional but positive to the gold standard test

d = Number of samples negative to both conventional and the gold standard tests

a + b + c + d = Total number of samples (N)

Definitions and formulas of the indices used for comparing the different assays are described as follows:

Sensitivity: It is the capacity of the test to detect diseased animals, when compared with the gold standard test (a/a+c x 100).

Specificity: It is the capacity of the test to detect non-diseased animals, when compared with the gold standard test (d/b+d x 100).

Overall agreement: Is the proportional similarity of the results of both tests (a+d/N x 100).

In case there were no gold standard for a particular condition and it is necessary to evaluate the diagnosis by the level of agreement between different tests, this assumes that agreement between test is evidence of validity, whereas disagreement suggests that the test are not reliable. The Kappa test can be used to measure the level of agreement beyond that which may be obtained by chance. The kappa statistic ranges within -1 and +1.

Observed agreement = (a+d)/ (a+b+c+d).

This is compared to the expected agreement, which would be obtained by chance, which is given by the formula, [{(a+b)/n} x {(a+c)/n}] + [{(c+d)/n} x {(b+d)/n}].

Kappa is agreement greater than expected by chance divided by potential excess (OA - EA)/ (1-EA). It should be noted that the Kappa value gives no indication which of the tests is better and that a good agreement may indicate that both test are equally good or equally bad.

Evaluation of Kappa statistic (Thrusfield, 1995)

Kappa value	Evaluation
>0.81	Almost perfect agreement
0.61- 0.80	Substantial
0.41-0.60	Moderate agreement
0.21-0.40	Fair agreement
0.01-.020	Slight agreement
0.00	Poor agreement

B. Human and sheep samples from Turkey

20 *B. melitensi*s strains were received from Dr. Ziya Iihan, University of Yuzuncu Y1, Faculty of Veterinary Science, Department of Microbiology, Van, Turkey. These strain were isolated from aborting ewes in 124 different sheep flocks in Van Province, East Anatolia in Turkey during lambing seasons of 2004-2005, 2005-2006 and 2006-2007 (Fig 1: map of Van district). Van Province had approximately 1.528.000 sheep and 89.5000 goats. The total sheep number in these flocks was 14.575 and there were 1.438 aborting ewes in these flocks. The ewes, from which materials were collected, were not previously vaccinated against *Brucella*. Samples of stomach content, pharynx, lung and liver were cultivated and all materials were inoculated onto duplicate plates of blood agar containing 5% defibrinated sheep blood or blood agar base no2 supplemented with *Brucella* selective supplement (Oxoid, Code SR083A). All plates were incubated at 37°C, both in air and microaerobically (5-10 CO_2 %) for 5-7 days. Additionally, 26 strains collected from German tourists, were isolated in Germany and were sent to the National Reference Laboratory, were also included in this study. Turkish and "German" strains were isolated in overlapping time periods, 2004-2007 and 1996-2010, respectively (Table 2). Our investigation was amended with recently published data for 41 *B. melitensis* isolates from German tourists (**Al Dahouk *et al.*, 2007b)**. Data retrieved from medical records confirmed that those patients attract *Brucella* infection during a holiday stay in Turkey. Identification and typing of strains were performed using standard tests, including growth characteristics, catalase, oxidase, urease, H_2S, growth in presence of thionin and basic fuchsine, lysis by Tb phage, and agglutination with monospecific A and M antisera according to **Alton *et al.* (1988).** MLVA data for *Brucella* strains from

human and animals of Turkish origin were also taken from the MLVA home page (http://minisatellites.u-psud.fr/MLVAnet/querypub1.php).

Figure (1): Map of Van district

3. B.1 DNA preparation from bacterial isolate

Genomic DNA was extracted from pure culture by using High pure PCR Template preparation Kit™ (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacture. Subsequently, the concentration of DNA was determined photometrically using a NanoDrop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

3. B.2 Identification of variable number of tandem repeat loci

MLVA was performed to all 46 *B. melitensis* strains according to the scheme initially proposed by **Le Fleche *et al*. (2006)** which includes 15 tandem repeat loci (MLVA 15) and modified by **Al Dahouk *et al*. (2007b)** by including 1 additional locus, Bruce 19 (MLVA 16)**.** PCR amplification of eight minisatellite loci in panel 1 (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, Bruce55 loci), three microsatallite loci in panel 2A (Bruce18, Bruce 19 and Bruce21 loci) and five microsatallite loci in panel 2B (Bruce04, Bruce07, Bruce09, Bruce16, Bruce30) was carried out, the amplification reaction mixtures were prepared in a total volume of 15 µl containing approximately 5 ng of DNA, 7,89 µl of master mix (Qiagen) and 0.3µM of each flanking fluorescence labelled primer. Amplification was performed in an Eppendorf Master cycler. An initial denaturation step at 95 ^{o}C for 15 minutes was followed by 30 cycle of denaturation at 94 ^{o}C for 15 s, primer annealing at 60 ^{o}C for 15 s, and elongation at 72 ^{o}C for 1 min. the final extension step was performed at 70 ^{o}C for 5 min. The genotyping was done according to **(Le Fleche *et al*., 2006)**. Briefly**,** PCR products were diluted 1:100 in PCR water and 1 µl of this pooled mixture was

added to 14 µl deionized formamide. This mixture was denaturated at 95 ^{o}C for 3 min and immediately placed on ice; the final mixtures were subjected to capillary electrophoresis. Fragment analysis was performed using Genetic Analyzer 3130 and Genemapper® Soft ware Ver. 4.0 (Applied Biosystem, Foster City, CA). Repeat numbers were calculated based on published data **(Le Fleche *et al*., 2006).**

3. B .3 Data analysis

The number of repeats was assigned to all isolates and all alleles were imported into Bionumerics software (Applied Maths, Sint-Martens Latem, Belgium) creating a dendogram. The use of the categorical coefficient parameter implies that the character states were considered unordered. The Clustering analyses of genotyping data was performed using the Bionumerics package (categorical and Ward) based on the categorical coefficient and unweighted pair group method using arithmetic averages (UPGMA). The same weight is given to a large and a small number of differences in the repeats at each locus. Taking into account the previously published data **(Le Fleche *et al*., 2006);** polymorphism was quantified by the Hunter Gaston diversity index (HGDI) **(Hunter and Gaston, 1988).** The 16 markers have been divided into two groups; one comprising 8 user-friendly minisatellite markers with a good species identification capability (panel 1) and the other is a complementary group containing 8 microsatellite markers with higher discriminatory power (panel 2). The loci were identified automatically by the software according to their sizes and fluorescence. The use of multiple dyes allowed ease of interpretation of the electrogram

Table 2: Human isolate from the National Reference Laboratory in Germany.

Key	Species	Year
04RB0360_DP249	Brucella melitensis bio1	2004
05RB1047_DP259	Brucella melitensis bio3	2005
05RB0902_DP258	Brucella melitensis bio3	2005
05RB1259_DP436	Brucella melitensis bio3	2005
05RB1416_DP250	Brucella melitensis bio1	2005
05RB1363_DP256	Brucella melitensis bio3	2005
05RB1445_DP438	Brucella melitensis bio3	2005
06RB0375_DP523	Brucella melitensis bio2	2006
06RB0376_DP251	Brucella melitensis bio1	2006
06RB0369_DP434	Brucella melitensis bio2	2006
06RB0393_DP433	Brucella melitensis bio2	2006
06RB0386_DP427	Brucella melitensis bio2	2006
06RB0111_DP248	Brucella melitensis bio1	2006
07RB0718_DP424	Brucella melitensis bio2	2007
07RB0721-DP472	Brucella melitensis bio2	2007
07RB1513_DP524	Brucella melitensis bio2	2007
07RB0001_DP467	Brucella melitensis bio2	2007
08RB3695_DP654	Brucella melitensis bio2	2008
08RB3639_DP642	Brucella melitensis bio2	2008
08RB2138_DP533	Brucella melitensis bio2	2008
08RB2139_DP534	Brucella melitensis bio2	2008
09RB8498_DP786	Brucella melitensis bio2	2009
09RB5269_DP760	Brucella melitensis bio2	2009
10RB9268-DP849	Brucella melitensis bio2	2010
10RB9181_DP833	Brucella melitensis bio2	2010
10RB9247_DP836	Brucella melitensis bio2	2010

4. Results

Table 3. Brucellosis prevalence in cattle and buffaloes based on a survey of studies published between 1978 and 2009.

Species	Country	Year	Number of animals tested	Number of positive animals (%)	Diagnostic test	References
Cattle	Algeria	2006	1,032	9.7	BAPAT	**Aggad and Boukaa(2006)**
				8.2	RBPT	
	Egypt	2007	1,966	5.4	BAPAT	**Samah et al. (2008)**
				4.9	RBT	
	India	1998	23,284	1.9	SAT	**Isloor et al. (1998)**
		2000	110	1.81	Culture	**Verma et al. (2000)**
		2007	150	20.7	ELISA	**Aulakh et al. (2008)**
	Iran	1990	6472	3.9	MRT	**Zowghi et al. (1990)**
		2002-2006	12,113	6.8	RBPT	**Bokai et al.(2008)**
	Jordan	1973	1,064	0.0	MRT	**Schenkel and Abdul Aziz (1978)**
		1977	250	0.4	MRT	**Schenkel and Abdul Aziz (1978)**
		2009	671	10.1	RBPT	**Al-Majali et al.(2009)**
		2009	671	10.1	ELISA	
	Libya	1985	3,753	0.3	RBPT	**El Sanousi (1985)**
		1986	8,607	1. 5	SAT	**Aboudaya (1986)**
				1.8	CFT	
	Syria	1989	12,554	2.9	RBPT	**WHO (1998b)**
					CFT	
	Turkey	2004-2006	407	32.9	RBPT	**Otlus et al. (2008)**
		2001-2006	626	35.3	RBPT	**Sahin et al.(2008)**
				39.5	ELISA	
	Yemen	1992-1993	1,645	0.1	ELISA	**Al-Shamahy (1999)**
Buffaloes	Egypt	2007	916	1.1	RBPT	**Nawal and Ahmed (2008)**
		2007	1,337	3.5	RBPT	**Samah et al. (2008)**
	India	1998	7,153	1.8	SAT	**Isloor et al. (1998)**
		2000	43	0.0	Culture	**Verma etal. (2000)**
		2007	195	16.4	ELISA	**Aulakh et al. (2008)**

BAPAT = buffered acidified plate agglutination test; RBPT= Rose Bengal plate test; CFT = complement fixation test; SAT = standard tube agglutination test; Riv. T = Rivanol test; ELISA = enzyme linked immunosorbant assay; MRT = milk ring test; BCT = Brewer card test.

Table 4. Brucellosis prevalence in sheep, goats and camels based on a survey of studies published between 1948 and 2009.

Species	Countries	Years	Numbers	Positive (%)	Tests	References
Sheep	Egypt	2007	32	31.3	SAT	**Nashwa et al. (2007)**
				25.6	RBPT	
		2007	813	5.4	BPAT	**Samah et al. (2008)**
	Iraq	1979	2,368	0.9	BCT	**Karim et al. (1979)**
	India	2000	163	2. 5	Culture	**Verma et al. (2000)**
	Jordan	1992	206	16.5	Culture	**Aldomy et al. (1992)**
		2003	602	14.3	RBPT	**Al-Talafah et al. (2003)**
				7.2	ELISA	
	Syria	1989	1,827	1.8	RBPT	**WHO (1998)**
					CFT	
	Turkey	2002-2004	37	38.0	Culture	**Unver et al. (2006)**
		2007	167	40.1	SAT	**Otlu et al. (2007)**
		2008	400	36.7	SAT	**Celebi and Atabi (2009)**
			400	35.5	RIV	
			400	34.8	RBPT	
			400	33.8	CFT	
	Yemen	1985	690	0.6	RBPT	**Hoise et al. (1985)**
		1992-1993	2,045	0.6	ELISA	**Al-Shamahy (1999)**
Goats	Egypt	2007	33	3.5	BPAT	**Abdel-El-Razki et al. (2007)**
	India	2000	115	2.6	Culture	**Verma et al. (2000)**
		2004	54	59.0	Serological test	**Gupta et al. (2006)**
		2004	54	88.8	PCR	
	Iraq	1979	3,156	4.4	BCT	**Karim et al. (1979)**
		2007	184 of which:			**Al-Aalim et al. (2009)**
			25 vaccinated	72.0	RBPT	
			17 aborted	52.9		
			142 unvaccinated	20.4		
	Jordan	2001-2003	1,100	27.7	RBPT	**Al-Majali (2005)**
	Iran	2002-2006	7,199	3.4	RBPT	**Bokaie et al. (2008)**
	Yemen	1985	538	0.4	RBPT	**Hoise et al. (1985)**
		1992-1993	2,014	1.3	ELISA	**Al-Shamahy (1999)**
Camels	Egypt	1948	200	20.0	SAT	**Zaki (1948)**
		1993	360	11.6	SAT	**Ahmed and Nada (1993)**
		2004	766	8.7	RBPT	**Abdel Moghney (2004)**
	Iran	2007	1,123	10.5	RBPT	**Ahmed and Nemat (2007)**
				8.5	MRT	
	Libya	1993	967	4.1	RBPT	**Gameel et al. (1993)**
	Saudia Arabia	1987	146	1.4	RBPT	**Hashim et al. (1987)**
		1992	2,536	8.0	RBPT	**Radwan et al. (1995)**
		1999-2000	98	7.1	RBPT	**Hegazy et al.(2004)**
	Yemen	1992-1993	105	0.0	ELISA	**Al-Shamahy (1999)**

Table 5. Summary of literature regarding the occurrence of antibodies to *Brucella* in camels.

country	number	positive %	test	reference
Egypt	200	20.0	SAT	**Zaki 1948**
	175	10.3	SAT	**Hamada et al. (1963)**
	360	11.5	TAT,RBT,BAPAT&Riv.T	**Nada and Ahmed (1993)**
	500	7.0	RBT	**El-Sawalhy et al. (1996)**
		2.3	cELISA	
	592	1.0	STAT	**Abou-Eisha (2000)**
		1.7	card test	
	766	8.7	RBT	**Abdel Moghney (2004)**
		9.3	ELISA	
	340	7.4	CFT	**EL-Boshy et al. (2009)**
Libya	967	4.1	RBT, SAT, CFT	**Gameel et al. (1993)**
	14	14	RBT	**Ahmed et al. (2010)**
Abu Dhabi	392	1.0	RBT	**Afzal and Sakkir (1994)**
		1.5	SA	
	1794	5.8 → (1990-1991)	RBT	**Moustafa et al. (1998)**
	7,899	0.1 → (1995-1996)	RBT	
Kuwait	698	14.8	RBT&CFT	**ALKhalaf and ELKhaladi (1989)**
Nigeria	232	1	RBT&SAT	**Okoh (1979)**
	329	11.4	(RBT,SAT,CELISA)	**Junaidu et al. (2006)**
	480	7.5	MSAT	**Kudi et al. (1997)**
Sudan	740	4.9	RBT,SAT,CFT	**Abu-Damir et al. (1984)**
	238	3.0	SAT	**Abbas et al. (1987)**
	38	84.2	RBT	**Agab et al. (1994)**
	64	0	SAT&TAT	**El-Ansary et al. (2001)**
	3,274	7.8	RBT,SAT&CFT	**Musa and Shigidi (2001)**
	29	13.8→cELISA 10.3→ RBT		
	3549	30.5	RBT	**Omer et al. (2007)**
	21	23.8	RBT&SAT	**Musa et al. (2008)**
Saudi Arabia	146	1.4	RBT	**Hashim et al. (1987)**
	2630	8	RBT, SPA	**Radwan et al. (1992)**
	236	8	RBT, BPAT	**Radwan et al. (1995)**
	98	7.1	RBT& SAT	**Hegazy et al. (2004)**
Somalia	913	1.9	SAT	**Baumann and Zessin (1992)**
		0.3	CFT	
	1,246	3.9	RBT	**Ghanem et al. (2009)**
		3.1	iELISA	
Libya	520	1.4	RBT	**Azwai et al. (2001)**
		1.2	SAT	
		3.0	cELISA	
		3.5	iELISA	
Jordan	412	12.1	RBT& CFT	**Al-Majali et al. (2008)**
	640	14.2	RBT	**Dawood (2008)**
Kenya	172	6.4	RBT,SAT	**Waghela et al. (1978)**
		12.2	CFT	
Iran	953	8.0	RBT, SAT, CFT& ME	**Zowghi and Ebadi (1988)**
	258	1.9	RBT, SAT,ME	**Khadjeh et al. (1999)**
	1,123	10.5	RBT	**Ahmed and Nemate (2007)**
Pakistan	81	2.5	STA	**Ajmal et al. (1989)**
	71	8.0	RBT,SAT	**Straten et al. (1997)**
Ethiopia	1,442	5.7	RBT	**Teshome et al. (2003)**
Republic of Yemen	105	0.0	ELISA	**AL-Shamahy (1999)**

(STAT) Standard Tube Agglutination Test, (RBT) Rose Bengal Test, (CFT) Complement Fixation Test, (SAT) Serum Agglutination Test, (SA) Standard Agglutination, (MSAT) Microtitre Serum Agglutination Test, (TAT) Tube Agglutination Test, (BAPAT) Buffered Acidified Plate Antigen Test, (Riv. T) Rivanol Test, (SAT) Slide Agglutination Test, (SPA) Standard Plate Agglutination Test. (ME) Mercaptoethanol Test.

Table 6: Brucellosis in humans in Turkey

Number of brucellosis cases	Clinical cases of brucellosis (%)			Reference
	Acute	Subacute	Chronic	
233	58.3	14.2	27.5	**Akdeniz et al. 1998**
480	67.1	25.2	5	**Aygen et al. 2002**
283	25	59	16	**Gur et al. 2003**
151	66.2	23.9	9.9	**Roushan et al. 2004**
138	57.2	16.7	26.1	**Kokoglu et al. 2006**
140	53.6	21.4	25	**Savas et al. 2007**
1028	61.6	21.6	13.6	**Buzang et al. 2010**

Table 7: Prevalence of brucellosis in Turkey in sheep

Total number of animals	Number of positive animals	%	Diagnostic test	Reference
58	31	53.4	CT	**Erganis et al. 2002**
37	14	38	Culture	**Unver et al. 2006**
503	44	8.73	RBT & STAT	**Apan et al. (2007)**
167	71	40.11	SAT	**Otlus (2008)**
400	147	36.7	SAT	**Celebi and Atabay (2009)**
	135	33.75	CFT	
162	51	31.4	RBT	**Iihan *et al*. (2008a)**
	45	27.7	SAT	
55	21	38.2	Culture	**Büyükcangaz et al. (2009)**

CT, coagglutination test; RBT, Rose Bengal test; STAT, Standard Tube Agglutination Test; SAT serum agglutination test; CFT , Complement Fixation Test .

4. A.1 Results of camel samples

According to the owners, all camels were apparently healthy at the time of sampling and none of these camels had clinical signs of brucellosis.

Sera from 895 camels were tested with different serological tests as well as real-time PCR to compare their diagnostic efficiency to identify sensitive, rapid and simple combination of tests for detecting *Brucella* infection in camels. Human serum samples were also collected from camel handlers. Bacterial cultivation, RBT, SAT, CFT, cELISA and FPA were used to detect B*rucella* antibodies. Real-time PCR was used as advanced diagnostic technique to confirm the result of serological test.

4. A. 2 Cultivation

In the present study, our attempts to isolate *Brucella* organisms, from 100 randomly selected camel sera that were serologically positive, have not been successful.

4. A. 3 Serological tests

4. A. 3.1 Results of different serological tests

Our findings showed that FPA had the highest number of positive samples 710 (79.3%), while 639 (71.4%), 633 (70.7%), 632 (70.6%) and 616 (68.8%) samples were found to be positive for brucellosis with CFT, RBT, SAT and cELISA, respectively (Table 8). Out of 895 examined sera, 595 (66.5%) were positive and 170 (19.0%) were negative by all serological tests. Only 72 (8.04%) were found to be positive by FPA, wheras 15 samples showed false negative results by cELISA Table (11 & 12).

4. A.4 Real-time PCR

The real-time PCR assay amplified the *Brucella* cell surface salt extractable genus specific *bcsp31* kDa protein gene in (759/895; 84.8% samples). *B. abortus* was the only species found. Additionally, 534 out of 895 (59.7%) were positive by all serological tests and *bcsp31* real-time PCR; 118 (13.2%) were positive by *bcsp31* but negative in all serological tests; 61(6.8%) samples were positive by serological tests but negative by *bcsp31* genus specific real-time PCR (Table 11). The agreement between the results obtained by FPA and that for real-time PCR targeting *bcsp31KDa* protein gene is illustrated in (Table 10). FPA was positive in 626 samples out of 759 (82.5%) which were positive by *bcsp31*real-time PCR. A probit analysis revealed that real-time PCR assay detect as little as 23fg of *Brucella* DNA per reaction with a probability of 95%. The presence of *Brucella* DNA as demonstrated by *bcsp31KDa* real-time PCR or presence of anti brucella antibody proved by two positive results of different serological tests was considered as proof for a potential risk of consumers when consuming products of theses animals. According to our definition for the positive samples (positive in real-time PCR or in combination of at least two different

serological tests), 828 samples were considered as positive. Real-time PCR detects 759 samples out of 828 with a sensitivity of 91.7%, meanwhile the sensitivity of RBT, cELISA, CFT, SAT and FPA to detect positive samples was 76.5, 74.4, 77.2, 76.3 and 83.9 %, respectively. On the other hand, when we combine real-time PCR with one serological test, the sensitivity will be near 100% (Table 13). The combined sensitivity of FPA with each of the serological test was higher than that obtained when RBT combined with the used serological test (Table 14 &15).

4. A.5 Human samples

Brucella spp were also isolated from animal handlers. The percentage of positive cases among the examined samples was 60, 100, 80, 80 and 100 % by using RBT, cELISA, CFT, SAT and FPA, respectively (Table 17). On the other hand, real-time PCR detected infection among 40% from the examined samples and those samples were positive in most of the used serological tests. Species specific real-time PCR revealed that the infection in human cases was also due to *B. abortus*.

4. B. Samples from Turkey

To trace German cases with respect to the potential animal sources in Turkey, genotyping data of 20 *Brucella* strains isolated from sheep in the highly endemic region of Southeastern Anatolia (province of Van) were compared to 67 strains isolated in Germany either from travellers returning from Turkey or more often from Turkish immigrants (data partially published in **(Al Dahouk *et al*., 2007b).** Standard microbiological methods determined 49 *B. melitensis* biovar 2, 19 biovar 1 and 19 biovar 3 isolates. Genotyping was performed with a Multiple Locus of Variable number of tandem repeats Analysis assay based on 16 markers (MLVA-16) essentially as previously described by **(Al Dahouk *et al*., 2007; Le Flèche *et al*., 2006)**. The assay comprises eight moderately variable minisatellite markers (panel 1) to trace back the geographic origin and eight highly polymorphic microsatellite markers useful for outbreak investigations (divided into two panels, 2A and 2B, according to their diversity index). MLVA-16 has already proven its discriminatory power in *Brucella* strains collected on a global scale **(Maquart et al., 2009)**. In general, the genetic fingerprints of the *Brucella* strains investigated were not associated with the classical biotyping results i.e. the three biovars of *B. melitensis* did not cluster in the same subgroups (Figure 6). The 67 human *B. melitensis* strains isolated in Germany and the 20 sheep isolates from Turkey, Southeast Anatolia were clustered in 68 different genotypes based on the differences in the numbers of repeat units at 16 VNTR loci. The East-Mediterranean genotypes i.e. 42 and 43 and single locus variants were most prevalent. Three main clusters could be distinguished. The panel 1 genotypes 42 (n = 14) and 43 (n = 55 isolates) and their single-locus variants 62 and 57, 58 and 61, respectively. Only a single *B. melitensis* biovar 2 isolate

displayed a completely new genotype. In addition, two Turkish *B. melitensis* biovar 1 ovine isolates were separately clustered due to their genotype.

Table (8): summary on the origin of positive camels to brucellosis

Total No of sample	Positive	Negative	Origin
686	437	249	97% (424) of the positive cases were from Sudan, 3-4 % being local camels
14	4	10	Local
3	3	0	Local
14	14	0	Local
5	5	0	Local
2	2	0	Local
6	6	0	Local
158	158	0	100 camels were Sudanese, the rest believed to be of local origin
6	5	1	Sudanese
1	1	0	Local
5	4	1	Human samples

Fig (2): Summary on the origin of positive camels to brucellosis

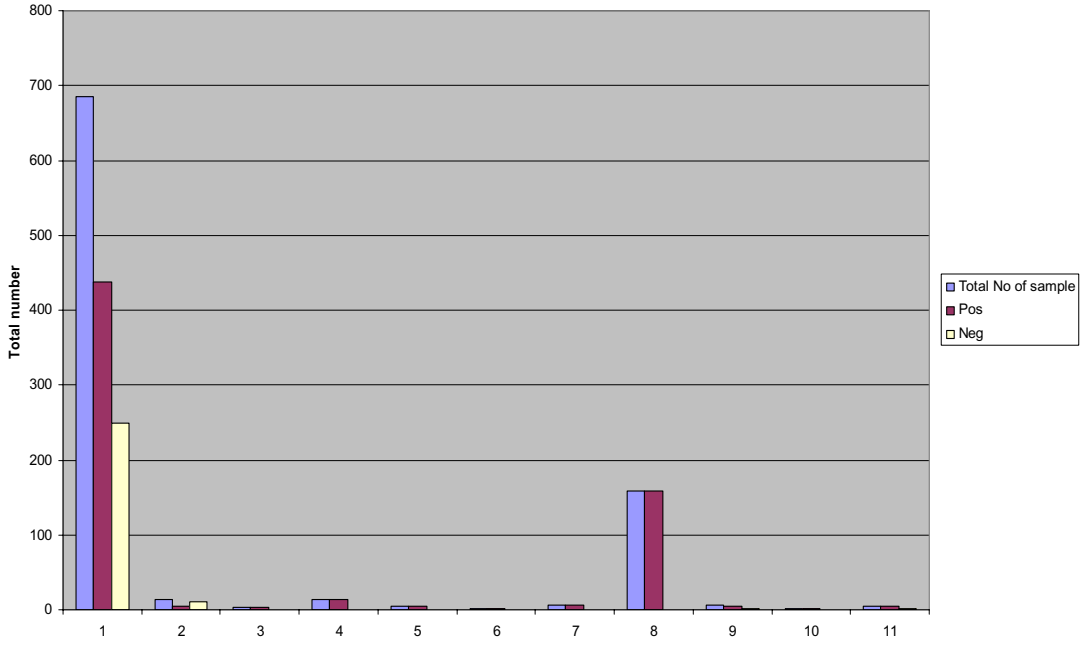

Table (9): Number of positive samples among the examined camel sera

Method	Total samples	Number of Positive sample detected	
		Number	%
RBT	895	633	70.7
cELISA	895	616	68.8
CFT	895	639	71.4
SAT	895	632	70.6
FPA	895	710	79.3
BCSP31	895	759	84.8
IS711	895	695	77.7
BCSP31&IS711	895	687	76.8

Fig (3): Number of positive samples among the examined camel sera

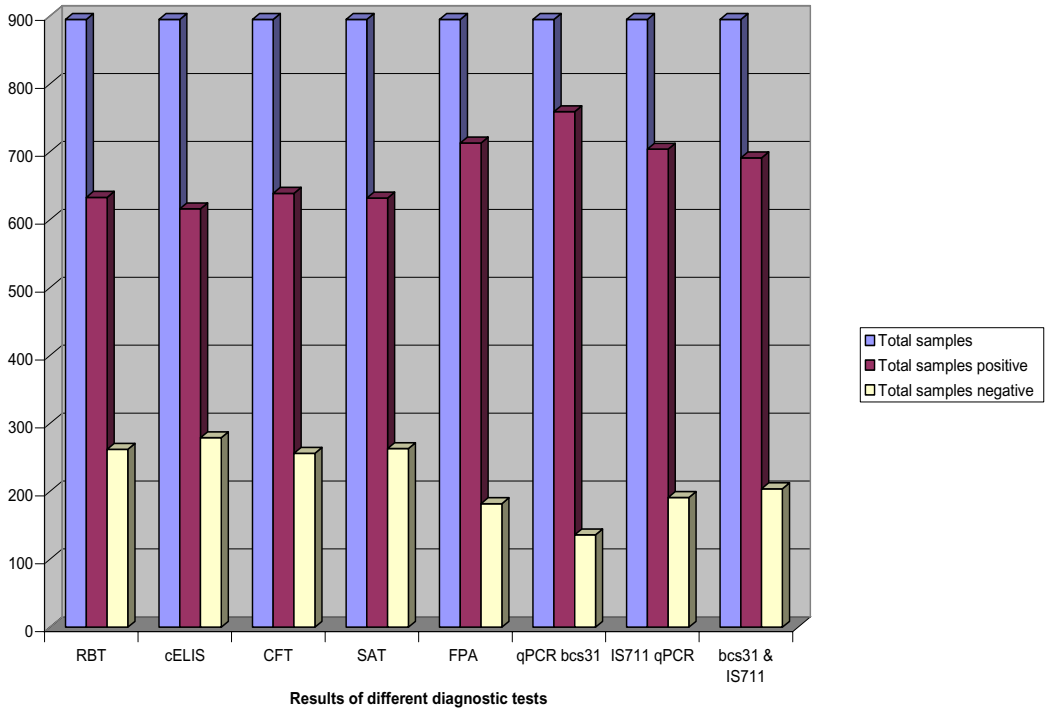

Table (10): Comparison between different diagnostic tests among camel sera

sample		Is711		Bscp31&IS711		RBT		cELISA		CFT		SAT		FPA	
		pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
Bcsp31	Pos (n=759)	687	72	687	72	571	188	552	207	571	188	570	189	626	133
	Neg (n=136)	8	128	-	136	62	74	64	72	68	68	62	74	84	52
Is711	Pos (n=695)			687	8	533	162	515	180	535	160	534	161	583	112
	Neg (n=200)			-	200	100	100	101	99	104	96	98	102	127	73
genus species	Pos (n=687)					528	159	510	177	530	157	529	158	577	110
	Neg (n=208)					105	103	106	102	109	99	103	105	133	75
RBT	Pos (n=633)							609	24	622	11	624	9	619	14
	Neg (n=262)							7	255	7	245	8	254	91	171
cELISA	Pos (n=616)									610	6	608	8	608	8
	Neg (n=279)									29	250	24	255	102	177
CFT	Pos (n=639)											621	18	632	7
	Neg (n=256)											11	245	78	178
SAT	Pos (n=632)													619	13
	Neg (n=263)													91	172

Fig (4): Comparison between different diagnostic tests among camels

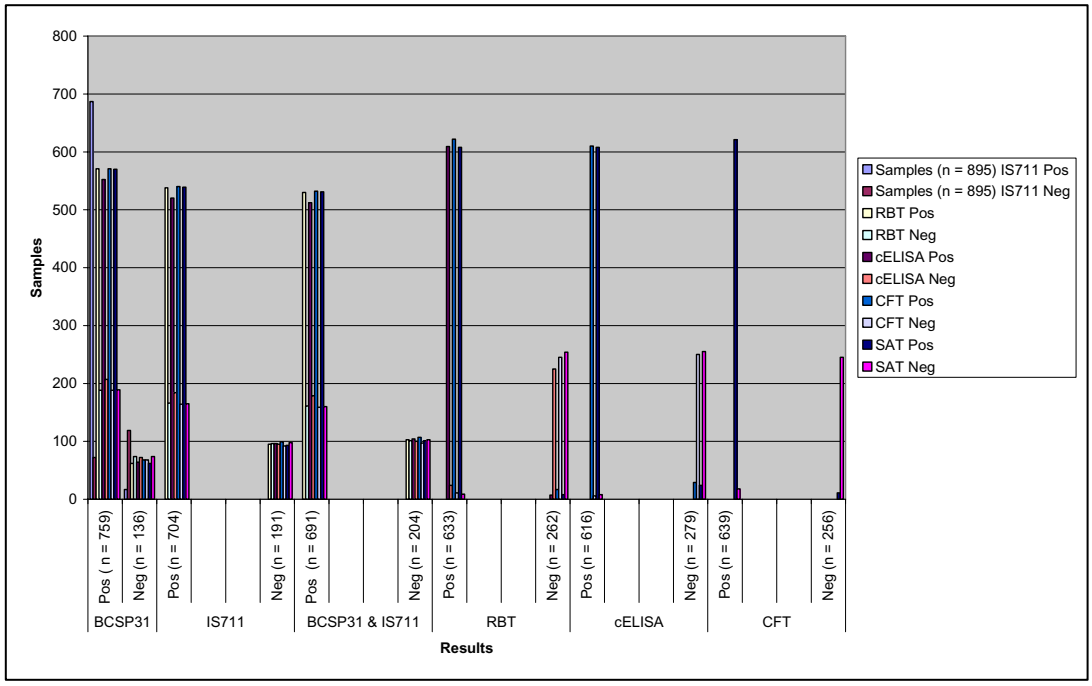

Table (11): Results of test used to detect brucellosis in asymptomatic camels

Sample n = 895	BSCP31	IS711	RBT	CELISA	CFT	SAT	FPA
495	495	495	495	495	495	495	495
96	96	96	-	-	-	-	-
51	-	-	51	51	51	51	51
50	50	50	-	-	-	-	50
50	-	-	-	-	-	-	-
39	39	-	39	39	39	39	39
22	22	-	-	-	-	-	-
14	14	14	14	-	14	14	14
13	-	-	-	-	-	-	13
10	-	5	10	10	10	10	10
7	7	-	-	-	-	-	7
4	4	4	4	4	4	4	-
4	-	-	-	-	4	-	4
3	3	3	-	3	3	3	3
3	3	3	-	-	3	-	3
3	3	3	3	3	3	-	3
3	3	3	3	3	-	3	-
2	2	2	-	-	-	2	2
2	2	2	-	-	2	-	2
2	2	2	2	-	-	2	-
2	2	2	2	-	2	2	-
2	-	2	-	-	-	-	-
2	-	1	-	-	-	-	2
2	-	-	-	2	2	-	2
1	1	1	-	-	1	1	1
1	1	1	1	-	-	-	1
1	1	1	1	-	1	-	1
1	1	1	-	1	1	-	1
1	1	1	1	1	-	1	1
1	1	1	-	-	-	1	-
1	1	1	1	-	1	-	-
1	1	1	1	-	-	1	-
1	1	-	1	-	1	1	1
1	1	-	1	1	-	1	1
1	1	-	1	1	1	-	1
1	1	-	1	1	-	-	-
1	-	-	1	-	-	-	1
1	-	-	-	1	1	1	1
895	759	695	633	616	639	632	710

RT-PCR BCSP31 (Real Time Polymerase Chain Reaction, Brucella Cell Surface Protein 31 kDa, RBT (Rose Begnal Test), SAT (Slow Agglutination Test), CFT (Complement Fixation Test), cELISA (Competitive Enzyme Linked Immunosorbant Assay), FPA (Fluorescence Polarization Assay).

Table (12): Results of test used to detect brucellosis in asymptomatic camels (without IS711RT-PCR)

Sample n= 895	RT-PCR	RBT	cELISA	CFT	SAT	FPA
534	534	534	534	534	534	534
118	118	-	-	-	-	-
61	-	61	61	61	61	61
57	57	-	-	-	-	57
52	-	-	-	-	-	-
15	15	15	-	15	15	15
15	-	-	-	-	-	15
5	5	-	-	5	-	5
4	4	4	4	4	-	4
4	4	4	4	4	4	-
4	-	-	-	4	-	4
3	3	-	3	3	3	3
3	3	3	3	-	3	-
3	3	3	-	-	3	-
2	2	2	2		2	2
2	2	-	-	-	2	2
2	2	2	-	2	2	-
2	-	-	2	2	-	2
1	1	1	-	-	-	1
1	1	1	-	1	-	1
1	1	-	-	1	1	1
1	1	-	1	1		1
1	1	1	-	1	-	-
1	1	1	1	-	-	-
1	1	-	-	-	1	-
1	-	1	-	-	-	1
1	-	-	1	1	1	1
Total n= 895	759	633	616	639	632	710
%	84.8	70.7	68.8	71.4	70.6	79.3

Table (13): Sensitivity of different diagnostic tests and the combination of RT-PCR and a serological test

	RT-PCR	PCR +RBT	PCR+cELISA	PCR + CFT	PCR+SAT	PCR+FPA
Goldstandard*	828	828	828	828	828	828
Number of positives in RT-PCR	759	759	759	759	759	759
Number of positives in the serological test		62	64	68	62	69
Number of positives in PCR and serological test		821	823	827	821	828
Sensitivity of the combination		99,2	99,4	99,9	99,2	100
Sensitivity of the single method	91,7	76,4	74,4	77,2	76,3	85,7

*positive in RT-PCR or positive in at least two different serological tests

Table (14): Sensitivity of different serological tests and the combination of RBT and a second test

	RBT	RBT+cELISA	RBT + CFT	RBT+SAT	RBT+FPA
Goldstandard*	828	828	828	828	828
RBT positiv	633	633	633	633	633
Positive by the second serological test only		7	17	8	76
Number of positives in the combination of the two methods	633	640	650	641	709
Sensitivity combination	76,4	77,3	78,5	77,4	85,6
Sensitiviy of one method	76,4	73,6	75,1	75,4	74,8

Table (15): Sensitivity of different serological tests and the combination of FPA and a second test

	FPA	FPA+cELISA	FPA + CFT	FPA+SAT
Goldstandard	828	828	828	828
FPA positiv	695	695	695	695
Positive by the second serological test only		8	7	13
Number of positives in the combination of the two methods	695	703	702	708
Sensitivity combination	83,9	84,9	84,8	85,5
Sensitivity of one method	85,7	74,4	77,2	76,3

Table (16): Test agreement (K value) with different test results.

Serological test	**K value**
CFT vs RBT	0.92
CFT vs. cELISA	0.91
CFT vs. SAT	0.92
CFT vs. FPA	0.68

Table (17): Comparison between different diagnostic tests among human sera.

Samples (n=5)		IS711		RBT		cELISA		CFT		SAT		FPA	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
BCS31	Pos (n= 2)	2	-	1	1	2	0	2	0	2	0	2	0
	Neg (n= 3)	-	3	2	1	3	0	2	1	2	1	3	0
IS711	Pos (n= 2)			1	1	2	0	2	2	2	0	2	0
	Neg (n= 3)			2	1	3	0	0	1	2	1	3	0
BCS31& IS711	Pos (n= 2)			1	1	2	0	2	2	2	0	2	0
	Neg (n= 3)			2	1	3	0	1	-	2	1	3	0
IS711	Pos (n= 3)					3	-	2	1	3	0	3	0
	Neg (n= 2)					2	-	2	0	1	1	2	0
cELISA	Pos (n= 5)							4	1	4	1	5	0
	Neg (n= 0)							0	0	0	0	0	0
CFT	Pos (n= 4)									3	1	4	0
	Neg (n= 1)									1	0	1	0
SAT	Pos (n= 4)											4	0
	Neg (n = 1)											1	0

Fig (5): Comparison between different diagnostic tests among human sera.

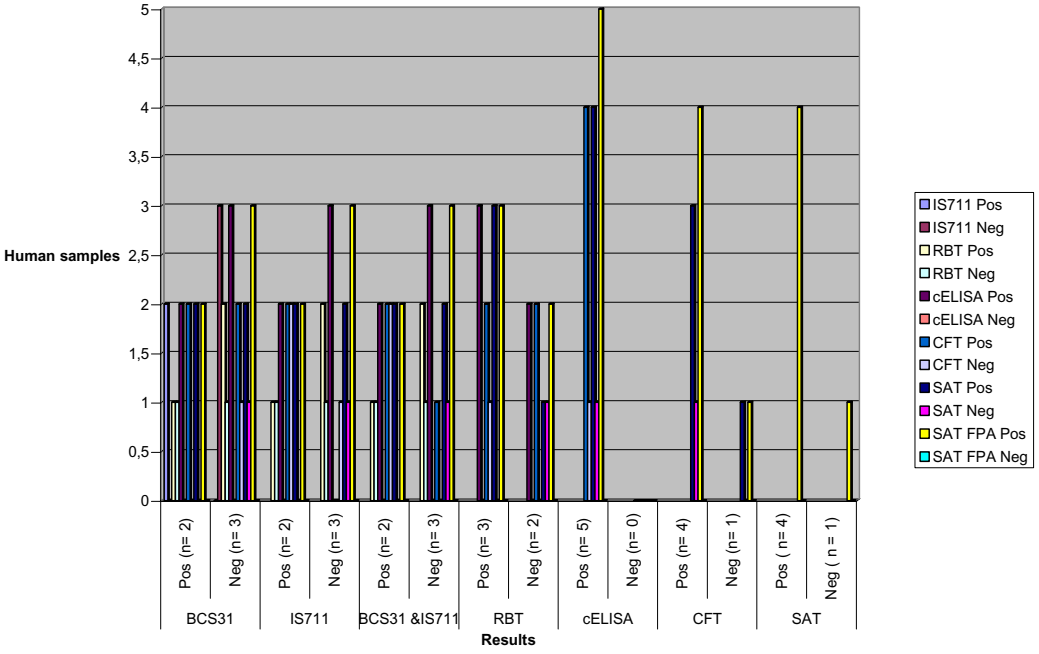

Table 18: Distribution of genotypes in side the similar clusters

Clust	Genotyp	MLVA profiles**	No of isolates	species	Host	Year	Origin
A	1	5 13 2 3 1 3 3 2 4 20 8 4 4 4 3 4	2	*B. melitensis* b2	Human	2003	Germany
				B. melitensis b3		2005	
B	2	5 13 2 3 1 3 3 2 4 20 8 4 5 4 3 8	2	*B. melitensis* b2	sheep	2005	Turkey
				B. melitensis b3		2006	
C	3	5 13 2 3 1 3 3 2 4 20 8 4 5 4 3 7	2	*B. melitensis* b2	Sheep	2006	Turkey
					Human	2008	Germany
D	4	5 13 2 3 1 3 3 2 4 20 8 4 5 4 3 4	5	*B. melitensis* b2	3 Sheep	(2)2004, 200	Turkey
				B. melitensis b3	2 Human	(1996,2005)	Germany
E	5	5 13 2 3 1 3 3 2 4 20 8 4 5 4 3 6	2	*B. melitensis* b2	Human	2000	Germany
					Sheep	2005	Turkey
F	6	5 13 2 3 1 3 3 2 4 20 8 4 5 4 3 5	2	*B. melitensis* b2	Human	2003	Germany
				B. melitensis b3		2005	
I	9	5 13 2 3 1 3 3 2 4 20 8 5 5 4 3 4	3	*B. melitensis* b1	Human	2002	Germany
				B. melitensis b2	sheep	2004	Turkey
J	10	5 13 2 3 1 3 3 2 4 20 8 6 4 4 3 4	2	*B. melitensis* b2	human	2006	Germany
						2007	
M	13	5 13 2 3 1 3 3 2 4 20 8 4 5 5 3 4	2	*B. melitensis* b2	Human	2002	Germany
						2006	
N	14	5 13 2 3 1 3 3 2 4 20 8 5 5 5 3 7	2	*B. melitensis* b2	Human	2003	Germany
						2006	
O	15	5 13 2 3 1 3 1 2 4 20 8 3 4 4 3 5	2	*B. melitensis* b1	sheep	2005	Turkey
						2007	
P	16	5 13 1 3 1 3 3 2 4 20 8 7 6 4 3 5	2	*B. melitensis* b1	Human	2002	Germany
						2004	
Q	17	5 13 2 3 1 3 3 2 5 18 8 5 6 4 3 5	2	*B. melitensis* b2	human	2007	Germany
S	19	5 13 2 3 1 3 2 2 4 20 8 5 5 9 3 6	3	*B. melitensis* b2	Human (2)	2002,2005	
				B. melitensis b3	sheep	2004	
T	20	5 13 2 3 1 3 2 2 4 20 8 9 6 6 3 6	3	*B. melitensis* b2,b3	sheep	2004 (2), 200	Turkey
				B. melitensis b3		2006	
U	21	3 13 2 3 1 3 2 2 4 20 8 5 4 5 3 6	2	*B. melitensis* b2	Human	2008	Germany
Total			38				

*They were grouped according to 100 % for all clusters except the last two groups with 90% similarity via clustering analysis using UPGMA, ** the TRS copy numbers were arranged in the following order: Bruce 08, 12,43,45,06,11,42,55,18,19,21,30,04,07,09,16.

Fig (6): Dendogram of clustered MLVA-16 genotypes

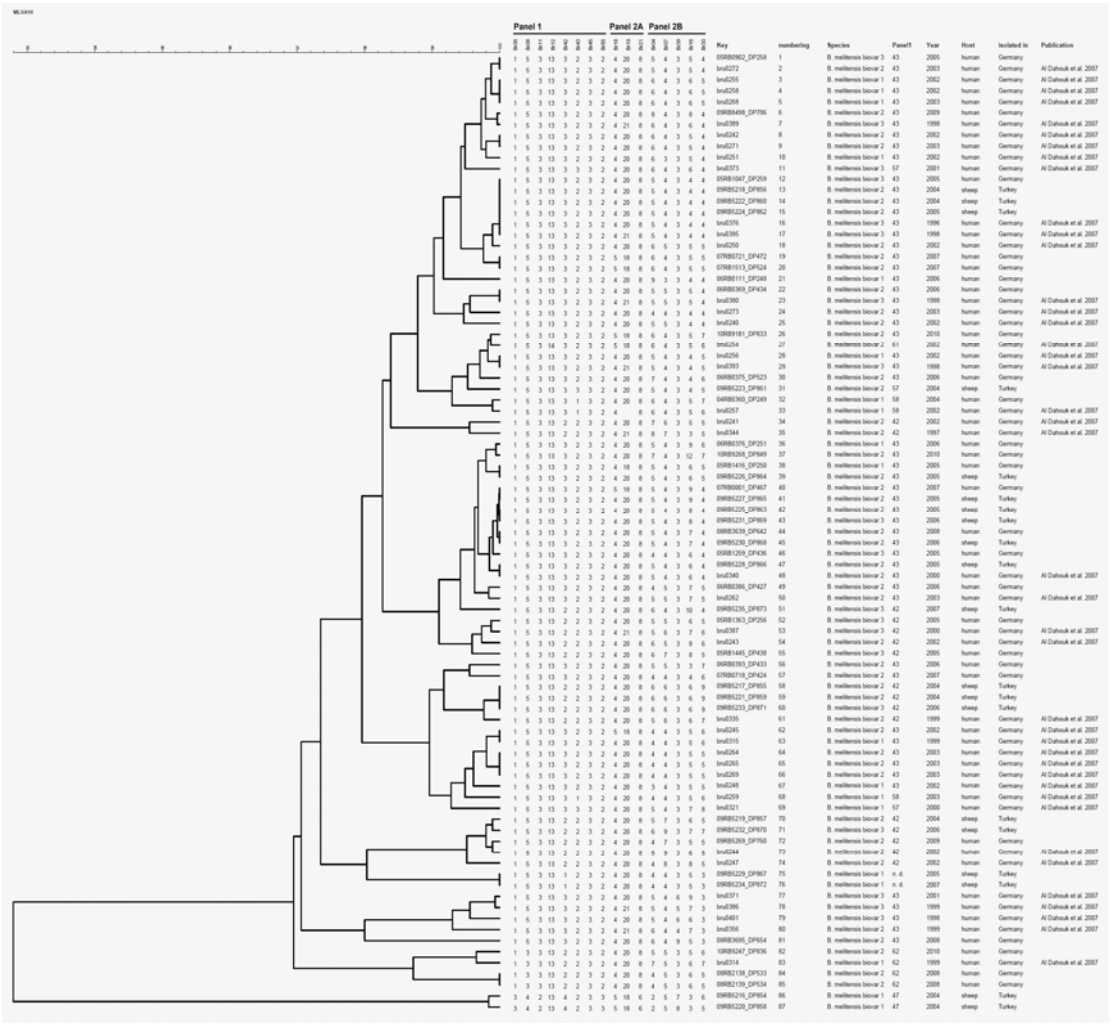

Figure legend

Figure 6. Dendrogram of clustered MLVA-16 genotypes (panels 1 and 2). The 67 human *B. melitensis* strains isolated in Germany and the 20 sheep isolates from Turkey, Eeastern Anatolia were clustered in 68 different genotypes based on the differences in the numbers of repeat units at 16 VNTR loci. The East-Mediterranean genotypes (panel 1) i.e. 42 and 43 and single locus variants were most prevalent (*n.d.:* not yet defined). In the columns the following data are presented: numbers of tandem repeat units at each locus, DNA batch ('key'), numbering, species and biovars, panel 1 genotypes, year of isolation, host, and geographic origin. The clustering analysis was based on the categorical coefficient and unweighted pair group method using arithmetic averages (UPGMA). The same weight was given to a large and small number of differences in the repeats at each locus. Three different character data sets were defined and combined using the composite data set tool provided by Bionumerics. A different weight was given to the markers depending on the panel they belong to i.e. panel 1 markers got an individual weight of 2, panel 2A markers got a weight of 1, and markers of panel 2B got a weight of 0.2

5. Discussion and Conclusions

With respect to a survey on prevalence of brucellosis in different animal species published between 1948 and 2009, it has been shown that the retrieved studies were heterogeneous, especially in the number of samples and laboratory tests. Thus, a meta-analysis or comparison of these datasets is impossible. Nevertheless, the studies show that the prevalence of brucellosis in different animal species in Mediterranean countries, the Middle East and India varies widely. High prevalence appears to be due to insufficient preventive measures and the lack of adequate control programs in some countries as well as uncontrolled animal transportation across "open" borders. Due to armed conflicts and political instability in various countries, it is very likely that previously successful eradication programs have no long lasting effects and brucellosis has become a severe hygiene problem again. Therefore, there is an urgent need for the strict implementation of brucellosis eradication programs for cattle, small ruminants and camels in affected regions. Given the huge economic and medical impact of brucellosis such control programms are cost-effective **(Roth *et al*., 2003).** The impact of control programs and the consequence of their subsequent neglection can be demonstrated by the situation in Iran. The prevalence of animal brucellosis in Iran reached 44% in 1956 and decreased to only 5% following a control program starting in 1958. Because of reluctance in control, the prevalence increased again to 17.4% in 1977. A new control program was established in 1983 and the prevalence decreased again to 1.25% in 1987 and to 0.85% in 1991. Nevertheless, the number of human cases of brucellosis recorded in 1988 was [710,521 (132.4/100,000)], suggesting that the low animal prevalence reported was not representative for the total animal population **(Refai, 2002).** More recent country-wide data are not available. In Iraq, several studies on the seroprevalence of brucellosis have been conducted in recent decades**.** The Northern provinces of Iraq share an extensive border with Iran, Turkey and Syria. Other provinces of Iraq share borders with Jordan, Saudi Arabia and Kuwait. This geographic situation highlights the need for a strategic planning of control measures. In fact, due to its geographical location the prevalence of brucellosis in Jordan may be an indicator of the status of the disease in neighbouring countries in the Middle East region **(Al-Majali, 2009)**. In many countries, there is a lack of recent data about the seroprevalence of animal brucellosis and further studies are required to identify areas where tight brucellosis control is crucial to prevent cross-border transmission.

The prevalence of brucellosis in animal reservoirs is the key to its control in humans. Eradication programs for bovine brucellosis markedly reduce the incidence in humans (**Acha and Szyfer 2001).** Worldwide reported incidence of human brucellosis in endemic areas varies widely, from <0.01 to >200 per 100,000

inhabitants**.** However, the true incidence of human brucellosis is unknown due to misdiagnosis, underreporting, lack of proper laboratory facilities in remote areas as well as poor cooperation and exchange of information between veterinary and public health services **(Mantur *et al*., 2007)**.

Brucellosis has been recognised as an important zoonotic disease due to sever human disease and considerable losses in animal production due to abortion and infertility in cattle, small ruminants and pigs. This disease persists in regions where little attention is given to the control of the disease. Brucellosis in camels has received comparatively little attention from researchers and scientists. It seems that *B. melitensis* is the most frequent isolated Brucella species of camels in Middle East whereas both species, *B. melitensis* and *B. abortus* are from camelids in Africa **(Abbas and Agab, 2002).** Natural infection with other Brucella species (*B. ovis* and *B. suis*) has not been investigated yet (**Azwai et *al*., 2001).** From the epidemiological point of view, it is of utmost important to trace the source of infection to by investigating the prevalence of brucellosis in camels and humans. This work was initiated to throw a strong light on the efficiency of different serological test and real-time PCR for diagnosis of camel brucellosis with special reference to its public health hazards as a basis for designing an effective control strategy. On the light of the aforementioned point of view, this work was also carried out to elucidate the risks of camel brucellosis for human beings.

5. A. 1 Brucellosis in camels diagnosed by conventional methods.

Control of brucellosis in livestock and humans depends on the reliability of the methods used for detection and identification of the causative agent. However, diagnosis of brucellosis in camels is frequently difficult. The disease can mimic many infectious and non infectious diseases. Characteristic clinical signs of brucellosis in camels are often lacking and diagnostic methods are not evaluated yet. In the present study, our attempts to isolate *Brucella* organisms from 100 camel sera that were serologically positive have not been successful. Our results were in agreement with that previously reported by **Agab (1993); Al Khalaf and El Khaladi (1989); Obeid *et al*. (1996)** who failed to isolate *Brucella* organisms from milk of seropositive camels. In contrast, other researchers isolated *Brucella* spp. from some but not all of the milk samples obtained from seropositive camels **(Radwan *et al*., 1995; Abou-Eisha, 2000)**. It is also possible to isolate *Brucella* spp. from internal organs particularly: lymph nodes, testes and vagina **(Agab *et al*., 1994; Zowghi and Ebadi, 1988; Ramadan *et al*., 1998; Abu Damir *et al*., 1989).** However, a more-successful isolation of *Brucella* was reported from lymphoid tissues than any other organ **(Abu Damir *et al*., 1989).**

All serologically positive camels were clinically normal at the time of sampling. According to camel owners, none had previously shown clinical signs of brucellosis. Our results indicated that many infected camels might be silent carriers for brucellosis. This finding was in harmony with reports by **Abu Damir *et al.*, (1989)** who mentioned that non pregnant camels experimentally infected with *B. abortus* had no clinical manifestations and only negligible pathological changes were found. On the contrary, individual cases of abortion, placental retention, fetal death, mummification, delayed sexual maturity, infertility, stillbirth, mastitis, orchitis and joint disease might be encountered in naturally infected camels with *B. abortus* **(Higgins, 1986; Obeid *et al.*, 1996; Ramadan *et al.*, 1998; Musa and Shigidi, 2001).** Having in mind these facts, a camel posing a risk for consumers was considered either to have *Brucella* DNA in its blood samples or being positive for the presence of antibodies confirmed by two independent test systems. Animals being positive for DNA only, may be in incubation period before antibody titer develops or may be unable to produce specific antibodies at all. Because of the biology of *Brucellae*, chronic infection and sporadic shedding of the agent is common, human infection is consequently to be expected. Serological tests can detect infection when sepsis has passed and the agent has found its niche in the host. An arithmetic safety to identify animals which pose a risk for human consumers is nearly 100% when real-time PCR combined with one of the serological tests is used. The panel of sera of my collection which fulfil at least one of the criteria was considered to be the "gold standard".

All camel sera were tested by RBT, SAT, CFT, cELISA and FPA to compare their ability in detecting antibodies of *Brucella* spp. My findings showed that 70.7, 70.6 and 71.4% were positive by using RBT, SAT and CFT, respectively. This high percentage of positive cases might be due to the fact that most of camels were imported from Sudan where a high prevalence of camel brucellosis is known **(Agab *et al*., 1994; Omer *et al*., 2007; Musa *et al*., 2008).** Another reason might be the transmission of *Brucella* infection from infected camels to healthy ones during transport i.e. in an extreme crowding situation. CFT detected higher number of positive cases than agglutination tests. This finding was in agreement with that previously reported by **Waghela *et al*. (1978); Abu Damir *et al*. (1984); Abbas *et al*. (1987); Yagoup *et al*. (1990); Gameel *et al*. (1993); Agab *et al*. (1994); Radwan *et al*. (1995); Abdel Moghney (2004) ; Alshaikh *et al*. (2007).** The authors regard the complement fixation test as being the most widely used test for brucellosis screening in camels. CFT is recommended by OIE as a test prescribed for international trade **(Nielsen, 2002).** It is also recognized as a good test when correctly performed, but it

has various practical drawbacks: it is cumbersome, time consuming and difficult to standardize **(Uzal *et al*., 1995).**

Only little published information was available on the application of cELISA for diagnosis of brucellosis in camel **(Azwai *et al*., 2001; Abdel Moghney, 2004; Alshaikh *et al*., 2007)**. However, several studies have confirmed the superiority of ELISA technique over other conventional methods used for serological screening of brucellosis in other animal species **(Singh *et al*., 2000; Rivera et al., 2003; Muma et al., 2007)**. Our result revealed that 616 camel serum samples out of 895 (68.8%) were positive by cELISA demonstrating the lowest sensitivity when compared to other serological tests (Table 13). This may be attributed to the fact that the test was specially standardized to work with bovine sera or the very special presentation of brucellosis in camels. Although the performance of the cELISA in my study was not superior to that of the CFT or FPA, the cELISA can be used as an alternative to the CFT without reducing the diagnostic effectiveness. The cELISA is fairly simple, can be performed in a relatively short time (approximately 1 h and 30 min) and can be readily adapted to non-reference laboratories. Additionally, the cut-off for the cELISA can be adjusted so that a desirable combination of diagnostic sensitivity and diagnostic specificity can be achieved in different epidemiological situations.

To the best of my knowledge, FPA has not yet been used for the diagnosis of camel brucellosis. My results revealed that FPA detects more positive cases i.e. 710 (79.3%) out of 895. Thus, FPA seems to be a valuable tool for the diagnosis of brucellosis in camels especially when taking into consideration speed, objective of result interpretation, and cost factor. It is suggested that FPA can be considered as a sensful replacement for other established methods. Further studies are now needed to assess FPA's reproducibility.

A perfect agreement between CFT, RBT, SAT and cELISA and substantial agreement between CFT and FPA according to Kappa value are illustrated in (Table 16). A kappa statistic was used to measure the agreement between the various serological tests. In general, the kappa statistics were quite low, suggesting that various serological tests may detect different antibody isotypes. It is difficult to decide which test should be adopted for routine screening of brucellosis in camels. Because, until now, there has been no positive or negative international standard reference serum that could be used to calibrate tests for diagnosing brucellosis infection in camels and most serological tests have been directly transposed, without validation, from their use in other domestic animals. Thus, no single test is capable of giving conclusive diagnosis in detecting all positive cases **(Morgan et al., 1978; Sutherland, 1980)**. It is advisable to combine at least two serological test methods to screen and confirm brucellosis on herd level combine i.e. RBT and CFT. This finding

is in accordance with the procedure of monitoring in other animal species. Thus, the sensitivity will be increased. Nevertheless, serological methods used solitary or in combination carry the risk to miss seronegative carriers of *Brucella*.

According to my definition for positive samples (positive in real-time PCR or in combination of at least two different serological tests), 828 samples were considered as positive. Real-time PCR detect 759 samples out of 828 with a sensitivity of 91.7% meanwhile, the sensitivity of RBT, cELISA, CFT, SAT and FPA to detect positive samples was 76.5, 74.4, 77.2, 76.3 and 83.9 %, respectively. On the other hand, when real-time PCR combined with one serological test sensitivity is near 100% as illustrated in Table 12. The sensitivity of different serological tests when combined with each other (Table 13 & 14). From the practical and economical point of view, screening by RBT confirmed by CFT or ELISA might be a possible option with due regarding to the veterinary system in the respective countries.

5. A. 2 Brucellosis in camels diagnosed by real-time PCR.

From the aforementioned results, nucleic acid amplification methods might circumvent the drawbacks associated with bacterial isolation and serological tests. PCR assay has been shown to be a valuable method for detecting DNA of different microorganisms and provides a promising option for diagnosis of brucellosis. Interestingly, real-time PCRs targeting *bcsp31* and species specific targeting *IS711* were positive in 759 (84.8%) and 695 (77.7%) out of 895, respectively. The difference between both types of real-time PCR is presented in Table 3. Real-time PCR proved to be a valuable tool when culture failed or serological results are inconclusive **(Queipo *et al*., 2005)**. Real-time PCR has several advantages over the traditional culture methods, since it is faster and more sensitive. The risk of transmission of the disease to laboratory worker can be minimized. To the best of authors knowledge, real-time PCR has not been previously used for diagnosis of camel brucellosis. Interestingly, real-time PCR targeting the genus specific *bcsp31* was positive in 84.8% out of 895 samples demonstrating the presence of the agent within the animal population and the single animal as well. It can not be concluded that those camels are permanently infected. Brucellosis tends to have a chronic course. These animals may be asymptomatic carriers or shedders. They pose a permanent risk to other animals and humans and have to be removed from the herd. 534 samples out of 895 (59.7%) were positive in all serological test simultaneously with *bcsp31* real-time PCR. The high percentage of positive animals detected by real-time PCR could be attributed to the ability of real-time PCR assay to detect as little as 23fg of *Brucella* DNA per reaction with a probability of 95%. Real-time PCR detected also infection in 118 out of 759 (15.5%) of the seronegative camels which prompts us to conclude that these were probably acute or chronically infected animals

with antibody levels not yet or no longer detectable. While PCR directly detects the DNA of the pathogen, serology is dependent upon the rising and falling titres of antibodies during the different clinical phases of brucellosis. Consequently, real-time PCR is the test to complement diagnosis of camel brucellosis in developing countries. It is assumed that conventional PCR, as species specific PCR, can be used because of the lower cost. On the other hand, 15.2% (136/895) of samples were negative by real-time PCR. Those samples include 61 out of 136 which were seropositive in my study. Small number of false positives cannot be ruled out. Species specific real-time PCR system revealed that only *Brucella abortus* was present in the investigated camels. **Al Khalaf and El Khaladi (1989)** isolated *B. abortus* biovar 1 from aborted fetuses of camels in Kuwait, **Agab *et al.* (1994)** isolated *B. abortus* biovar 3 from camels in eastern Sudan. **Musa *et al*. (2008)** recorded the first isolation of *B. abortus* biovar 6 from camels in Sudan. It can be supposed that a spill over from cattle was the origin for this massive outbreak and that the spread of disease was promoted by the crowding situation during traveling.

5. A. 3 Brucellosis among camel workers in a farm

Keeping in mind the low number of examined samples, the obtained results showed that the percentage of positive cases among the examined samples were 60, 100, 80, 80 and 100 % by using RBT, cELISA, CFT, SAT and FPA, respectively (Table 17). Other researchers identified seropositive cases among the nomads being involved in camel caring. The percentages described were 18.75, 10.6 and 0.9% by **Amer (1989); Soliman (1998); Abou-Eisha (2000**), respectively. These results indicate that the rate of incidence of the disease in humans is greatly affected by the rate of disease in animals (**Alton, 1990**). On the other hand, real-time PCR detected infection among 40% of the examined samples and those samples were also positive in most of the used serological tests.

Species specific real-time PCR revealed that the infection in human cases was also due to *B. abortus*. This result confirmed the hazards associated with occupational exposure through direct contact with infected animals. Generally, real-time PCR assay has several advantages over the current microbiological diagnostic methods including speed, safety, high sensitivity and specificity. Therefore, it should be considered for evaluation of asymptomatic occupationally exposed persons.

5. B. Samples from Turkey

Sheep and goat brucellosis caused by *B. melitensis* has a major impact on human health and is also causing significant economic losses in animal husbandry **(Cetinkaya et al., 2005).** *B. melitensis* biovar 3 is believed to be the most virulent biotype for humans **(Sayan *et al*., 2009).** In Turkey, sheep and goat brucellosis is investigated by bacteriological, serological, and molecular methods and the

occurrence of the disease is monitored by epidemiological studies **(Güler *et al*., 2003)**. In a 10-year retrospective study by **Güler *et al*. (1998)**, brucellosis was found to be the primary cause among all infectious abortus agents in sheep as evidenced by bacteriological and serological methods. The province of Van, an area of intensive animal husbandry and a major hotspot of ovine brucellosis in Turkey, was supposed to be the source of disease distribution within the country, across its borders and potentially throughout Europe. Once a year at the Islamic "Festival of Sacrifice", sheep from this province are distributed all over Turkey and consequently *Brucella* with them. It can be supposed that our panel of strains from the Anatolian Van area may include clones to be found not only in human patients in Turkey but also in tourists importing the disease to their home countries e.g. Germany after being infected via sheep products e.g. raw cheese in Turkey. It is of interest that, some genotypes remained stable over time proving their long-term persistence in Turkey e.g. the MLVA-16 genotype of two *B. melitensis* biovar 3 strains which were isolated from different German travellers to Turkey in 1996 and 9 years later(no.12/16 Fig 6). Despite the restricted area where most of the isolates had their assumed geographic origin, the *B. melitensis* strains under study turned out to be very heterogeneous and three main clusters could be distinguished. The panel 1 genotypes 42 (n = 14) and 43 (n = 55 isolates) and their single-locus variants 62, 57, 58 and 61, respectively, being endemic in the East Mediterranean region. These master clones may have displaced other genotypes leading to reduce the genetic diversity of *Brucella* strains in Southeast Anatolia. The slight differences observed between these isolates of the same origin can be explained by micro-evolution from step-wise mutation events of individual loci. Only a single *B. melitensis* biovar 1 isolate displayed a completely new genotype (no. 75/76). In addition, two Turkish *B. melitensis* biovar 1 ovine isolates separately clustered due to their genotype (47) which so far predominated in Non-European strains (American genotype) **(Al Dahouk et al., 2007b).** Genotypes which are atypical for strains of Turkish origin can be easily silhouetted against the genetic background. It seems to be that the two strains can be allocated to a remote area outside of Europe. A continuous molecular surveillance may help detect the accidental import or intentional release of such strains. Thus, countermeasures can be implemented very early to prevent a further spread. Most of the genotypes observed in Turkish sheep isolates, i.e. 42, 43, and 57, were also isolated from humans, reflecting the epidemiological concordance of these strains. In two doubles of *B. melitensis* biovar 2 strains isolated from Turkish sheep and patients from Germany (no. 44/45 and 47/48), identical MLVA-16 genotypes were detected proving their common origin in the Van province **(Al Dahouk et al., 2007b).**

Conclusions

Wars in the Middle East, insufficient preventive measures, the lack of adequate control programs in some countries as well as uncontrolled animal transportation through "open" borders increase the risk that brucellosis will spread in a new non endemic region. We found that new seroprevalence data are needed urgently to evaluate the current situation and for continuous monitoring of necessary control programs as the published studies are scarce, some are more than 20 years old, and they use different laboratory tests, making the datasets impossible to compare. Only the initiation of continuous monitoring programs will allow an evaluation of the current status of brucellosis seroprevalence and the effectiveness of control measures.

In addition, it will be nearly impossible to control this important zoonosis without reimbursement of farmers for their financial losses due to removal of infected animals as part of an effective herd and individual animal registration system. Farmers, the dairy industry, breeding companies, consumers, veterinarians, and politicians in each country must work together to find a suitable eradication strategy. It is also important for countries to coordinate their control and eradication programs, especially the countries in the same region of the world.

In conclusion, with regard to the veterinary system in the respective countries, it is suggested that a combination of real time PCR with FPA can be used to detect camel brucellosis in developed countries, meanwhile a combination of conventional PCR with one of the commonly used serological tests (i.e. RBT, SAT, CFT) is recommended in less developed countries. Camels have to be included in national programs for control and eradication of brucellosis endemic countries in order to eliminate the hazard of infection among human begins.

Animal migration between Turkey and neighbouring countries such as Iraq, Iran and Syria where brucellosis is known to be highly endemic cannot be easily controlled. Furthermore, sheep and goats are traditionally kept on small-scale family farms in Turkey which may also hamper the control of ovine/caprine brucellosis. The genotyping data of *Brucella* strains isolated from patients living in Germany and from animals raised in Van province revealed a continuous epidemiological link over the last decade. The molecular tracing of *Brucella* isolates confirmed that brucellosis is of overall European public health concern and not only a regionally emerging zoonotic disease. Southeastern Anatolia has to be regarded as a region of high priority to take precautions for the prevention of brucellosis both in Turkey and in Europe. Since threats posed by biological agents are not confined to geographical barriers physicians in non-endemic European countries have to be aware of brucellosis in patients who suffer from fever of unknown origin and travelled to endemic countries. In summary, fast and accurate molecular typing procedures such as the MLVA-16 may provide a

deeper insight into disease epidemiology and will allow fighting back the re-emergence of brucellosis in Europe.

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6. Summary

Isolation, Identification and Typing of *Brucella* Species as Zoonotic Pathogens by Using Conventional and Molecular Biological Methods

Representative studies from countries including North Africa, the Middle East, and India which are neighbours or are important trading partners of the European Union and on trade animals and their product. In our review, published data on seroprevalence of brucellosis from 1948 to 2009 were retrieved. Based on the collected literature, we found that new seroprevalence data are needed urgently to evaluate the current situation and for continuous monitoring of necessary control programs.

Sera of 895 apparently healthy Camels *(Camelus dromedaries)* in addition to 5 human samples from Central Veterinary Research Laboratory (CVRL); Dubai, UAE was investigated. Rose Bengal Test (RBT), Complement Fixation Test (CFT), Slow Agglutination Test (SAT), Competitive Enzyme Linked Immunosorbant Assay (cELISA) and Fluorescence Polarization Assay (FPA) as well as real-time PCR were used. Our findings revealed that *bcsp31* kDa real-time PCR detected *Brucella* DNA in 84.8% (759/895) of the examined samples, of which 15.5% (118/759) were serologically negative. Species specific real-time PCR system revealed that only *Brucella abortus* was present in the camels investigated in this study. A probit analysis revealed that real-time PCR assay detect as little as 23fg of *Brucella* DNA per reaction with a probability of 95%. Our results show no relevant difference in sensitivity between the different serological tests. FPA detected the highest number of positive cases (79.3%) followed by CFT (71.4%), RBT (70.7%), SAT (70.6%) and cELISA (68.8 %). A perfect agreement between CFT, RBT and SAT was proven by calculating Kappa values but sensitivity of all tests is low when compared to FPA or real-time PCR. A combination of real-time PCR with one of the used serological tests identified brucellosis in more than 99 % of the infected animals. 59.7% of the examined samples were positive in all serological tests and real-time PCR. On the other hand, *Brucella* spp was also isolated from animal handler. The percentage of positive cases among the examined samples were 60, 100, 80, 80,100 by using RBT, cELISA, CFT, SAT, FPA, respectively. On the other hand, real-time PCR detected infection among 40% from the examined samples and those samples were also positive in most of the used serological tests. Species specific real-time PCR revealed that the infection in human cases was also due to *B. abortus.* This result confirmed the hazards associated with occupational exposure through direct contact with infected animals.

A total of 46 *B. melitensis* isolate obtained from sheep and human from Turkey were studied. From which, 20 were isolated from aborting ewes in 124

different sheep flocks in Van Province, East Anatolia in Turkey during lambing seasons 2004-2005, 2005-2006 and 2006-2007, as well as 26 strains from German tourists were isolated in Germany and were sent to the National Reference Laboratory, were also included in this study. They were collected during 2004 to 2010. Our investigation was amended with recently published data for 41 *B. melitensis* isolates from German tourists (**Al Dahouk *et al.*, 2007b)**. The 67 human *B. melitensis* strains isolated in Germany and the 20 sheep isolates from Turkey, Southeast Anatolia were clustered in 68 different genotypes based on the differences in the numbers of repeat units at 16 VNTR loci. It is concluded that brucellosis is highly prevalent in sheep and humans in several district in Turkey.

7. ZUSAMMENFASSUNG

Isolierung, Identifizierung und Typisierung von Brucella Spezies als Zoonose-Erreger mit Hilfe konventioneller und molekularbiologischer Methoden

In einer Übersicht wurden Veröffentlichungen zur Seroprävalenz von Brucellose bei Tieren aus Nordafrikanischen Ländern, aus Ländern des Mittleren Ostens und Indien ausgewertet. Diese Länder sind entweder wichtige Handelspartner der EU oder grenzen direkt an diese an. Berücksichtigt wurde ein Zeitraum von 1948 bis 2009. Dabei wurde festgestellt, dass v.a. Daten aus dem letzten Jahrzehnt fehlen und deshalb eine Beurteilung der aktuellen Brucellose-Situtation in diesen Ländern nicht möglich ist. Ebenso lässt sich keine Ausage zum Erfolg der dort durchgeführten Kontrollprogramme zum jetzigen Zeitpunkt machen.

In einer Studie zur Verbreitung von Brucellose bei Kamelen (*Camelus dromedarius*) wurden 895 Seren von klinisch gesunden Kamelen, die vom Central Veterinary Research Laboratory in Dubai zur Verfügung gestellt wurden, untersucht. Zusätzlich wurden fünf Humanseren in die Untersuchung eingeschlossen. Zur Anwendung kamen: Rose Bengal-Test (RBT), Komplement Fixations-Test (CFT), Langsam Agglutinations-Test (SAT), Competitiver ELISA (cELISA), das ‚Fluorescence Polarization Assay' (FPA) sowie eine real-time PCR. Dabei wies die genus-spezifische *bcsp31* kDa real-time PCR in 84, 8% (759/895) der Proben *Brucella* DNA nach, wobei 15,5% (118/759) der Seren vorher serologisch negativ befundet wurden. Eine species-spezifische real-time PCR bewies, dass nur *Brucella abortus* DNA in den Proben vorhanden war. Eine sogenannte ‚Probit-Analyse' ermittelte, dass die verwendete PCR 23fg *Brucella* DNA in eine Probe mit eine Sicherheit von 95% nachwies. Die verschiedenen serologischen Testsysteme zeigten keinerlei gravierende Unterschiede in Sensitivität oder Spezifität. Der FPA identifizierte die meisten positiven Seren (79,3%) gefolgt von CFT (71,4%), RBT (70,7%), SAT (70,6%) und cELISA (68,8 %). Eine perfekte Übereinstimmung der Kennwerte gab es zwischen CFT, RBT und SAT, wie mit dem Kappa Test gezeigt werden konnte. Allerdings sind die Sensitivitäten von CFT, RBT, SAT und cELISA sehr niedrig, wenn man sie mit denen von FPA oder PCR vergleicht. Eine Kombination aus real-time PCR mit einem der untersuchten serologischen Tests kann Brucellose in mehr als 99 % der untersuchten Tiere identifizieren. 59,7% der untersuchten Proben waren positiv in allen serologischen Tests und der PCR. Betrachtet man die Anzahl der positiven Tiere gestaffelt nach den verwendeten Tests, so waren diese positiv in 60% der Fälle mit RBT, in 100% mit dem cELISA, in 80% mit dem CFT, in 80% mit dem SAT und in 100% mit dem FPA. Die real-time PCR bewies das Vorhandensein von Brucellose bei 40% der untersuchten Tiere, wobei diese Tiere auch regelmässig mit serologischen Tests positiv getestet wurden. Auch bei den humanen Seren konnte *B. abortus* DNA nachgewiesen werden. Diese

Untersuchung zeigt die grosse Gesundheitsgefährdung für den Menschen, der in Kontakt mit selbst nicht klinisch erkrankten Kamelen kommt.

In einer Studie zum epidemiologischen Kontext zwischen von Patienten in Deutschland (türkische Migranten und Touristen) isolierten *Brucella melitensis* Stämmen und Stämmen, die von türkischen Schafen aus der Ost-Anatolischen Provinz Van stammten, kamen 26 Stämme humanen und 20 ovinen Ursprungs zur Untersuchung. Die Schaf-Stämme waren während der Ablammsaison 2004 – 2005, 2005-2006 und 2006-2007 aus 124 Herden isoliert worden. Die deutschen Stämme waren in der Zeit von 2004 bis 2010 an das Nationale Referenzlabor für Brucellose zur Typisierung eingesandt worden. Zusätzlich konnte auf Datensätze aus einer Publikation von Al Dahouk et al. von 2007 zurückgegriffen werden, in der weitere 41 *B. melitensis* Stämme des NRLs mit türkischem Ursprung mittels molekularer Typisierung untersucht worden waren. Die angewendete Multi Locus Variable Number of Tandem Repeats Analyse erbrachte insgesamt 68 unterschiedliche, wobei mehrere Human- und Schafstämme sogar identische Genotypen aufwiesen. Diese Untersuchung zeigt, dass Brucellose in der Türkei immer noch endemisch vorkommt und durch ‚Touristen' in nicht endemische, europäische Länder wie z.B. Deutschland regelmässig eingeschleppt wird.

8. List of Publications

1. Title	Brucellosis serology in camel
Authors	M. Gwida, F. Melzer, H. Tomaso, R. Wernery, A. El-Gohary, H.Neubauer, U Rösler.
Conference	Deutsche Veterinärmedizinische Gesellschaft (DVG) Fachgruppentagung 22. bis 24. Juni 2010, Jena, Deutschland.
2. Title	Brucellosis – Regionally Emerging Zoonotic Disease?
Authors	Mayada Gwida, Sascha Al Dahouk, Falk Melzer, Uwe Rösler, Heinrich Neubauer, and Herbert Tomaso.
Journal	Croat Med J. 2010 August; 51(4): 289–295. doi: 10.3325/cmj.2010.51.289.

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DEDICATION

TO

MY PARENTS, SISTERS, DEAR HUSBAND

AND TO MY KIDS

RAHMA AND RAWAN

10. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Der Inhalt der vorliegenden Arbeit wurde bereits in folgenden Publikationen veröffentlicht oder zur Veröffentlichung eingereicht:

1. Brucellosis – Regionally emerging zoonotic disease?
2. Comparison of diagnostic tests for the detection of *Brucella* spp. in camel ser.
3. Brucellosis in camels.
4. Cross-border molecular tracing of brucellosis in Europe.

Jena , den 22 September, 2010
Mayada Gwida