# The epidemiology of brucellosis in the Sultanate of Oman

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## DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution

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## ABSTRACT

Brucellosis is a serious disease of cattle in many countries of the world, including Mediterranean and Middle Eastern countries. The study outlined in this thesis was conducted to investigate the epidemiology of brucellosis in the Sultanate of Oman. Thirty of 1267 holdings tested in the Sultanate contained seropositive animals for brucellosis (herd prevalence 2.4%, 95% CI 1.6, 3.4%). The southern governorate (Dhofar) had significantly more seropositive holdings (n = 20, 8.6%, 95% CI 5.3, 13) than did the northern governorates (n = 10, 0.97%, 95% CI 0.5, 1.8) ($p < 0.001$) highlighting the endemic nature of the disease in Dhofar.

Although there were no significant differences between the herd seroprevalence for individual species, the highest herd level seroprevalence was reported in cattle (4.9%) followed by camels (2.3%), goats (1.4%) and sheep (0.6%). The overall individual animal seroprevalence of brucellosis in Oman was generally at a low level (0.4%; 95% CI 0.2, 0.5). The individual seroprevalence level in the different species was also low, being 0.4%, 0.4%, 0.4% and 0.1% in cattle, camels, goats and sheep, respectively. The practice of moving animals without testing between governorates is likely to have allowed the spread of infection throughout Oman. The active importation of live animals from other countries in the Horn of Africa, without prior monitoring of their brucellosis status, inter-species contact, sharing of common pasture, large herd size and the presence of poor biosecurity/unhygienic conditions in herds in the southern governorate may have facilitated the spread of brucellosis in the Dhofar region and from here infection may have been transmitted to other governorates.

A logistic-regression analysis was undertaken to identify risk factors for disease. This analysis indicated associations of breed, age, herd size and production system with seropositivity. A higher seroprevalence was found in imported animals (OR 3.71, 95% CI 0.68, 20.43), and the seroprevalence increased with age. The latter finding is possibly because of a higher risk of contracting the disease after puberty through increased contacts with potentially infected animals.

Only *Brucella melitensis* was cultured from different species of animals and biotype 1 was the only type identified in Oman by molecular means and phage typing. Sequencing of DNA revealed that all isolates had a very similar pattern.

In the current study although there was no significant difference observed in the seroprevalence detected by different diagnostic assays (cELISA, iELISA and RBPT), the ELISAs were capable of detecting more positive samples than the RBPT and Rapid test. This may reflect the better sensitivity of the ELISAs and it is recommended that these tests be used in the control and eradication of brucellosis in Oman, where vaccination is undertaken.

In Oman, human brucellosis was first reported in 1979 in the southern Dhofar governorate. A retrospective analysis of human brucellosis data sourced from the Ministry of Health, Oman from 1995 to 2012 was conducted. Information regarding location, age, gender, nationality of patients and year were included in the analysis. During this period, 2737 human cases of brucellosis were reported, with 96.7% of these in Dhofar. The incidence of disease was highest in young individuals (0-10 years of age), highlighting that these subjects were more at risk of acquiring brucellosis. The incidence of brucellosis was

slightly higher in males (56%) than females (44%). Most of the positive patients were Omani nationals, most likely because of more opportunity for contact with infected animals on privately owned farms.

The failure of disease control programmes in the southern region (2003 until 2012) could be due to a lack of information, inappropriate planning or administrative issues. With the information gathered from this study, it is considered there is a need to build a strategy to control the disease throughout Oman, rather than restricting control to the Dhofar governorate. However it is recommended that the control program adopted in the southern region (Dhofar), where the seroprevalence is high, be different to that implemented in the northern regions, where the disease prevalence is lower and more manageable. In the southern region, implementing a vaccination programme, along with individual animal identification and disease screening with a plan of intensive involvement and extension in the community, should be considered. In contrast in the northern region a test and slaughter program could be implemented.

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Brucellosis is a zoonotic disease (Huddleson, 1943; Boschiroli *et al*, 2001; Hendricks *et al*, 1995; Godfroid *et al*, 2005; Corbel *et al*, 2006) affecting a wide range of mammalian species, including humans (Hall, 1989; Brinley and Corbel, 1990; Capasso, 2002; Glynn and Lynn, 2008), fresh water fish (El-Tras *et al*, 2010), sea mammals (Brew, 1999; Bricker *et al*, 2003a; McDonald *et al*, 2006) and wildlife (Thorpe *et al*, 1965; Godfroid, 2002; Godfroid *et al*, 2010; Van Campen and Rhyan, 2010). In 1887 Colonel David Bruce, a physician in the Royal Army, isolated *Brucella* for the first time (Hall, 1989). The organism was detected in the spleen of British soldiers on the island of Malta, and consequently the disease was known as Malta fever (Hardy *et al*, 1930; Godfroid *et al*, 2005). Subsequently *Brucella melitensis* and *Brucella abortus* were isolated from these patients (Glynn and Lynn, 2008). The first documented case of brucellosis in animals was reported in cattle (Meyer, 1990). The causative agent was subsequently isolated in 1895 by Bernhard Bang, and hence the disease was known as Bang's disease (Sutherland, 1980; Hoffman and Houle, 1995). The organism was initially called *Micrococcus melitensis* (Moreno and Moriyon, 2002), however it was later renamed *Brucella melitensis* (Spink, 1956).

Brucellosis is distributed widely, particularly in the Middle East (Hadad and Al Azawy, 1991; Pappas *et al*, 2006). Meyer and Shaw in 1920 confirmed the relationship between bovine brucellosis and human brucellosis and diagnosed the first human cases in the

United States of America (USA) (Buchanan *et al*, 1974). The World Health Organization (WHO) reported that brucellosis was a significant health and economic problem (Boschiroli *et al*, 2001). It is well accepted today that nearly every case of human brucellosis has an animal origin and, therefore, control is primarily a veterinary problem (Nicoletti, 2002).

Brucellosis is a herd/flock problem (Corbel, 1989; Corbel, 1997 a,b) and is mainly spread through the ingestion of contaminated material (Corbel, 1989; Crawford *et al*, 1990). In females the initial infection is often followed by abortion and subsequent delays in conception or even permanent infertility (Cotton and Buck, 1931; Brinley-Morgan and Corbel, 1990; Enright, 1990, Singh *et al*, 1994). Infected animals shed organisms in colostrum, milk or uterine discharges following abortion or parturition (FAO, 2003).

Humans become infected through ingestion of raw milk and other dairy products (EFSA, 2013), or following direct contact with contaminated tissue, blood, urine, vaginal discharges, aborted foetuses (Sahin *et al*, 2008) or placentas (Flynn, 1983; Hall, 1989; Bercovich, 1998). Airborne infection in laboratories and abattoirs has also been recorded (Hartigan, 1997; FAO, 2003).

Brucellosis can have a major impact on livestock productivity and results in major losses for international trade (Beveridge, 1983; Bridges and Halling, 1994).

## 1.2 Oman overview

### 1.2.1 Location and administrative classification:

Oman is located in the south-eastern quarter of the Arabian Peninsula and covers a total

land area of 309,500 square kilometres. The country contains several topographical features with valleys and deserts accounting for 82%, mountain ranges 15% and coastal plain 3% of the land mass. The coast is 1,700 km long, running from the Strait of Hormuz in the north to the border with the Republic of Yemen. Oman is surrounded by seas: the sea of Oman, Arabian Sea and Arabian Gulf. The country borders the United Arab Emirates (UAE) to the west, the Republic of Yemen to the south and the Strait of Hormuz and the Arabian Sea to the east (Figure 1.1).

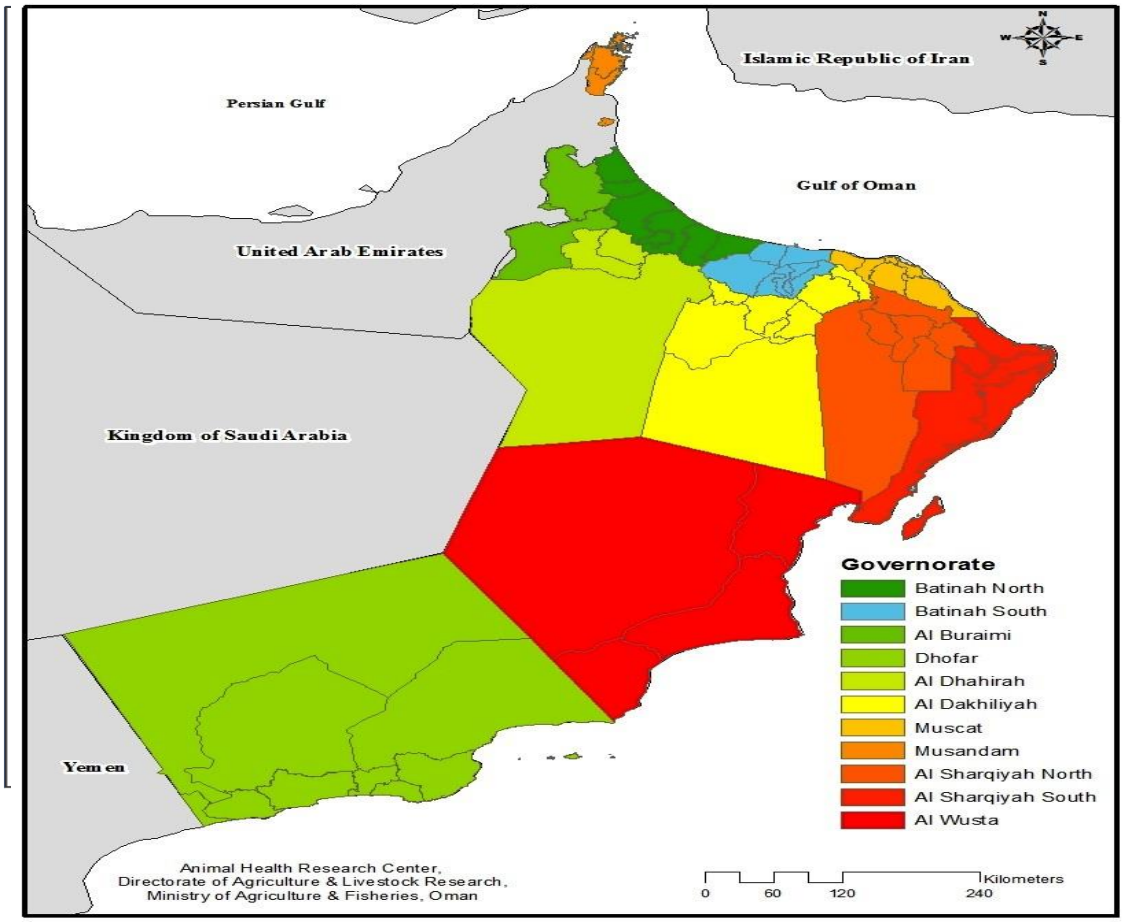
Oman is extremely hot and dry throughout the year, with the exception of Dhofar Governorate, which has a light monsoon climate and receives cool winds from the Indian Ocean. The country is characterized by high summer temperatures, scanty and irregular rainfall, and a high rate of evaporation, high relative humidity and persistent winds from all directions. The hottest months are July and August with mean temperatures reaching 45°C, while December, January and February are the coldest, averaging 17°C (Meteorological Affairs, 2012).

Administratively, the Sultanate is divided into 11 governorates namely: Muscat Governorate in which the affiliated wilayats are Muscat, Muttrah, Al Amerat, Baushar, Seeb and Qurrayat. The centre of this governorate is the Wilayat of Muscat; Batinah South Governorate which contains the affiliated wilayats of Al Rostaq, Al Awabi, Nakhl, Wadi al Maawil, Barka and Musannah. The centre of this governorate is the Wilayat of Rostaq; Batinah North Governorate which includes the wilayats of Sohar, Shinas, Liwa, Saham, Al Khabourah and Al Suwaiq. The centre of this governorate is the Wilayat of Sohar; Musandam Governorate with the affiliated wilayats of Khasab, Diba, Bukha and Madha. The centre of this governorate is the Wilayat of Khasab; Al Buraimi Governorate which

contains the affiliated wilayats of Al Buraimi, Mahdha and Al Sinaina. The centre of this governorate is the Wilayat of Al Buraimi; Al Dakhiliyah Governorate which includes the wilayats of Nizwa, Bahla, Manah, Al Hamra, Adam, Izki, Samayil and Bid Bid. The centre of this governorate is the Wilayat of Nizwa; Al Sharqiyah South Governorate containing the affiliated wilayats of Sur, Al Kamil W'al Wafi, Jaalan Bani Bu Hassan, Jaalan Bani Bu Ali and Masirah. The centre of this governorate is the Wilayat of Sur; Al Sharqiyah North Governorate that includes the wilayats of Ibra, Al Mudhaibi, Bidiya, Al Kabil, Wadi Bani Khalid and Dima W'attayeen. The centre of this governorate is the Wilayat of Ibra; Al Dhahirah Governorate including the affiliated wilayats of Ibri, Yankul and Dhank. The centre of this governorate is the Wilayat of Ibri; and Al Wusta Governorate which contains Haima, Mahout, Al Duqm and Al Jazir wilayats. The centre of this governorate is the Wilayat of Haima. Dhofar Governorate, with the affiliated wilayats of Salalah, Taqah, Murbat, Dhalkut, Rakiout, Thumrayt, Shaleem, Sadah, Muqshin and Mazyounah has its centre in the Wilayat of Salalah.

According to the 2010 census, the total human population of Oman was 2,773,479, with 1,957,336 Omanis and 816,143 expatriates (NCSI, 2010). The agricultural census of 2004/2005 revealed that about 30% of the population was working in the agricultural sector (MAF, 2005).

**Figure 1.1 Map of Oman**




### 1.2.2 Livestock in Oman

Agriculture, livestock and fisheries are among the oldest and most important sectors of the Omani economy. These play a vital part in feeding the population, providing employment for large numbers of Omanis and the economy. The Batinah Region has the most date palms, mango and lime trees, while the Governorate of Dhofar has the most coconut palms. Agricultural advice and guidance programmes have been adopted to promote the use of high-quality fertilizers and seeds, modern irrigation systems have been introduced on the farms and barriers have been built to provide protection against floods.

Livestock play an important role in most national economies through food production, generation of cash returns for urban and rural populations, means of transportation, providing by-products and making employment opportunities for the population (FAO, 1996). Livestock form a major capital reserve for farming households (FAO, 2003). However livestock production is under continuous threat by existing and emerging diseases that may result in direct and indirect losses to the livestock owner as well as to the national economy (FAO, 1996).

According to the agricultural census, the total number of livestock (sheep, goats, cattle and camels) in the sultanate was estimated at 2,327,071 and these were raised on 154,146 holdings (Table 1.1) (MAF, 2005). Following the completion of the camel counting and numbering project in the Governorate of Dhofar, a national strategy was introduced to reduce the number of camels by 50%. This was developed to restore the balance between the number of camels and the natural pastures and to increase the amount of water available by reducing the area used for the production of animal fodder.

### 1.3 Issues

Brucellosis is one of the most economically important worldwide contagious zoonotic diseases. Globally, the disease is endemic in many regions including the Middle East, Sub-Saharan Africa, India, China, Peru, Mexico, central and southwest Asia and the Mediterranean region. Only a few countries are free of the disease including Australia, New Zealand, Japan and Canada (OIE, 2012).

**Table 1.1 Number of livestock present in the Governorates of the Sultanate (MAF, 2005, Agriculture Census)**

| Governorates | Sheep | | Goats | | Camels | | Cattle | |
|---|---|---|---|---|---|---|---|---|
| | No. of animals | No. of holdings | No. of animals | No. of holdings | No. of animals | No. of holdings | No. of animals | No. of holdings |
| Muscat | 13855 | 1699 | 47713 | 3424 | 52 | 21 | 4447 | 1073 |
| Batinah | 110572 | 8183 | 430005 | 23891 | 5626 | 1273 | 66411 | 14426 |
| Musandam | 5609 | 470 | 67977 | 2057 | 38 | 12 | 325 | 76 |
| Al Dhahirah | 95047 | 4517 | 243596 | 8234 | 15641 | 1333 | 22553 | 4953 |
| Al Dakhiliyah | 43499 | 4073 | 203057 | 8756 | 6730 | 1064 | 19245 | 5547 |

| Governorates | Sheep | | Goats | | Camels | | Cattle | |
|---|---|---|---|---|---|---|---|---|
| Al Sharqiyah | 60012 | 8073 | 322858 | 16885 | 12779 | 3585 | 14642 | 6347 |
| Al Wusta | 14867 | 1040 | 71819 | 2043 | 22906 | 1572 | 43 | 20 |
| Dhofar | 7605 | 343 | 170123 | 4650 | 53527 | 6087 | 173892 | 8419 |
| **Total** | **351066** | **28398** | **1557148** | **69940** | **117299** | **14947** | **301558** | **40861** |

In 1986, a study conducted by Ismaily *et al*. (1988) revealed that the seroprevalence of brucellosis in Oman was 2.9, 0.9, 1.6 and 3.6% in cattle, goats, sheep and camels, respectively. However, by 2003 a study conducted in the southern region revealed that the seroprevalence had risen to 3.67 and 4.5% in cattle and small ruminants, respectively but had decreased in camels to 1.07% (MAF, 2003b). In contrast the northern region was considered to be free from disease since no disease had been reported since 1986. To confirm this a pilot serological study was conducted by the Central Veterinary Laboratory (CVL) in 2006 and 1007 samples were collected from small ruminants (306 sheep and 701 goats) in the main areas of the northern region (Batinah, Al Dakhiliyah, Al Dhahirah and Al Sharqiyah). Of these samples 13 were positive (1 sheep from Batinah, 2 samples (1 sheep, 1 goat) from Al Dakhiliyah and 10 samples from goats from Al Dhahirah region). No positive samples were found from the Al Sharqiyah region (MAF, 2006).

Historically the reports for brucellosis in the southern region varied from 1997 until 2001 as shown in Table 1.2. In contrast, during the period 1998 until 2002 between 133 and 316 human cases were reported in Oman (both the southern and northern region of the Sultanate) (MOH, 2003). Most cases were in humans living in the southern region (Table 1.3).

**Table 1.2 Passive Seroprevalence (%) of brucellosis in different animals from 1997 to 2001 (MAF, 2001)**

| Year | Sheep | Goats | Cattle | Camels | Total |
|---|---|---|---|---|---|
| 1997 | 0.0 | 8.3 | 16.7 | 0.0 | 9.6 |
| 1998 | 0.0 | 18.2 | 14.3 | 0.0 | 13.3 |
| 1999 | 0.0 | 24.3 | 14.3 | 18.2 | 19.2 |
| 2000 | 0.0 | 19.2 | 6.9 | 9.1 | 11.8 |
| 2001 | 0.0 | 8.3 | 0.0 | 7.1 | 4.8 |

An observational study examining three years of data by the Dhofar Hospital revealed that 63% of cases were due to consumption of raw milk, especially from cattle or camels, and 83% of patients had a history of contact with live animals. The source of infection in 4.5% of cases was unknown. Most (91%) patients had a fever and 70% had arthritis (MOH, 2003).

In Oman, *B. melitensis* biovar-1 is the only type that has been isolated from cattle, camels, sheep and goats in the southern region (MAF, 2003b). However a comprehensive epidemiological study on the strains and types of *Brucella* infecting animal and humans in the Sultanate has not yet been undertaken.

In 2003, a national program to control brucellosis in animals was approved by the government. This 12 year program was divided into four phases each of 3 years. The first phase concentrated on vaccination of cattle and small ruminants using the rev-1 vaccine.

The vaccine was administered at the dose recommended by the FAO (1995) (single dose for young animals at 4-8 months of age and a repeated reduced dose for adults). The aim of the program was to vaccinate 70% of the total population each year. Camels were not included in the program due to a lack of knowledge on the vaccine's efficacy in this species and uncertainty over the safety of milk after vaccination.

In conclusion, brucellosis was reported by Mackinnon in 1979 in the southern region of Oman (Nicoletti, 1986) with both animal and human cases being reported. The highest number of human cases was reported in 1998 with around 300 cases recorded (MOH, 1998).

**Table 1.3 Brucellosis reported in humans (1998 – 2002) (MOH, 2003)**

| Governorate | Year | | | | |
|---|---|---|---|---|---|
| | 1998 | 1999 | 2000 | 2001 | 2002 |
| Dhofar | 305 (99.3%) | 309 (97.8%) | 302 (98.4%) | 159 (98.1%) | 127 (95.4%) |
| Muscat | 1 | 2 | 2 | 1 | 3 |
| N. Batinah | 1 | 3 | 0 | 0 | 0 |
| S. Batinah | 0 | 0 | 0 | 1 | 0 |
| Al Dakhiliyah | 0 | 2 | 0 | 0 | 1 |
| N. Al Sharqiyah | 0 | 0 | 0 | 1 | 1 |
| S. Al Sharqiyah | 0 | 0 | 0 | 0 | 0 |
| Al Dhahirah | 0 | 0 | 2 | 0 | 0 |
| Musandam | 0 | 0 | 0 | 0 | 0 |
| Al Wusta | 0 | 0 | 1 | 0 | 1 |
| **Total** | **307** | **316** | **307** | **162** | **133** |

This study was designed to investigate the epidemiology of brucellosis in Oman from both an animal and human point of view in order to determine the prevalence of disease, to identify the risk factors associated with disease and to type the strains of *Brucella* present in Oman.

**1.4 Objectives:**

The general aim of this project was to undertake a study on brucellosis using different diagnostic tools to determine the distribution of disease in Oman and to identify putative risk factors for disease.

The specific aims of the project outlined in this thesis were to:

1- Map the distribution of disease in Oman using geographical information systems (GIS).

2- Determine the seroprevalence of brucellosis in Oman and to establish the types of *Brucella* present in Oman recently and previously isolated and their genetic relationship.

3- Determine the susceptibility of *Brucella* to the currently available antibiotics.

4- Evaluate different diagnostic tests for the diagnosis of brucellosis in Oman.

In the following chapter the key literature on *Brucella* relevant to the aims of this project is reviewed.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Etiologic Agent of Brucellosis

Brucellosis is caused by a gram-negative coccobacillus organism which belongs to the family *Brucellaceae* (Meyer, 1990; Bridges and Halling, 1994). *Brucella*e are small, non-motile, non-sporing, gram-negative coccobacilli short rods. They grow rather slowly on ordinary nutrient media while their growth is improved by the addition of serum or blood. They are aerobic and there is no growth under strictly anaerobic conditions (Alton *et al*, 1975a). The *Brucella* species are intracellular parasites of humans and animals and can usually be found in the reticuloendothelial and reproductive systems. Typically *Brucella* spp. occur as small gram-negative coccobacilli, but coccal and bacillary forms also occur. The cells are short and slender; the axis is straight; the ends are rounded; and the sides may be parallel or convex outwards. In length they vary from about 0.5 - 0.7 μm, in breadth vary from 0.5 - 1.5 μm occurs in single form (Ray and Steel, 1979) and commonly are found in pairs and rarely in groups (Alton *et al,* 1975a).

All *Brucellae* are fastidious organisms which usually grow in nutrient-rich media within 48-72 hours of incubation at 37ºC in a 5% $CO_2$ atmosphere. The organisms are aerobic, non-encapsulated and catalase and oxidase positive. They do not ferment carbohydrates and have variable urease activity (Young, 1995).

*Brucella* species have a strong host preference, which is evident in their ability to establish chronic infection in individuals and maintain transmission and infection in populations of specific animal species (Glynn and Lynn, 2008). However, almost all *Brucella* spp. can

infect mammalian species other than their preferred host; for example, both *B. melitensis* and *B. suis* are capable of colonizing bovine udders and therefore contaminating cows' milk (Ewalt *et al,* 1997; Kahler, 2000). *Brucella* are facultative intracellular organisms, classified according to the presence or absence of lipopolysaccharide (S-LPS), which is a major component of virulence and is used for differentiating strains into smooth or rough types. The smooth strains of *B. melitensis*, *B. abortus* and *B. suis* are serious pathogens infecting both humans and other animals (Ko and Splitter, 2003). Species which are free of or have little S-LPS, such as *B. ovis*, *B. canis* and *B. neotomae* are classified as rough strains of low virulence. A high level of homology has been identified among *Brucella* species by molecular characterization (Paulsen *et al,* 2002; Halling *et al,* 2005). *Brucellae* are coccoid if culture is undertaken directly from fresh aborted material, however they are pleomorphic on subculture or if culture is delayed. On MacConkey agar the colonies appear in two forms and are 0.1 to 0.2 mm in diameter. The smooth form is glistening, translucent and bluish-green in colour while the rough form has a granular appearance and is yellowish-white in colour. The organism is oxidase, catalase and urease positive and can reduce nitrate to nitrite (Alton *et al,* 1988; Moyer *et al,* 1991). The number of species has increased to 10 over recent years as several new *Brucella* species have been isolated from marine mammals, voles, rodents, and from an infected human breast implant (Foster *et al*, 2007; Scholz *et al*, 2008c, 2010). *Brucella melitensis*, *B. abortus* and *B. suis* are the three species generally associated with human disease. Rare cases of human infection with *B. canis* have been reported, while human cases of *B. ovis* and *B. neotomae* infection have not been reported. Little is known about the capacity of the new *Brucella* species to cause infection. One possible laboratory-acquired infection with a marine mammal isolate has

been reported (Brew *et al*, 1999) and one specific sequence type (ST27) has been associated with three human infections in Peru and New Zealand (Whatmore *et al*, 2008). Interestingly, these patients had no contact with marine mammals; however contact with raw fish was a common feature. Recently, there has been a report of *B. melitensis* biovar 2 found in catfish in Egypt, suggesting that fish may constitute a novel source of infection (El-Tras *et al*, 2010).

## 2.2 History and Zoonotic Importance of Brucellosis

The infectious agent of brucellosis was first isolated by David Bruce from the spleen of soldiers dying of Mediterranean fever on the Island of Malta in 1887 (Alton, 1990a). Bruce named the agent *Micrococcus melitensis*. In domestic animals, brucellosis has been commonly known as enzootic abortion or bovine contagious infection, epizootic abortion, infectious abortion, contagious abortion, slinking of calves, Bang's disease and ram epididymitis. Human brucellosis is also known as undulant fever, Malta fever, Mediterranean fever, gastric fever, Mediterranean gastric fever, Gibraltar-Rock fever, Cyprus fever, Neapolitan fever, intermittent gastric fever, intermittent typhoid fever and pseudotyphus (Ray and Steel, 1979).

Brucellosis is potentially a serious zoonosis and, with few exceptions, infections in humans result from direct or indirect contact with animals or animal products. The main source of infection for the general population is dairy produce prepared from infected milk with *B. melitensis* representing the greatest hazard. The milk of infected sheep and goats may contain large numbers of viable organisms, which become concentrated in products such as soft cheese (Ongör *et al,* 2006). Soft cheese has been recognized as one of the major

vehicles of infection in Turkey (Turgut *et al,* 2006). Direct contact with livestock is a well documented source of infection. Infection may occur through cuts and abrasions on the skin, via the conjunctiva and by inhalation. These routes of infection are important for farmers, veterinarians and butchers, who are all at increased risk of infection through contact with animals and animal products. In 1971, Ogutman carried out a sero-epidemiological study on 2626 individuals in Erzurum, a city in eastern Turkey. In this study 1.5% of people who had been in close contact with animals but who displayed no evidence of clinical brucellosis were seropositive compared with 1.3% in people who had not been in contact with animals and had no evidence of clinical brucellosis, 18% of individuals who had been in close contact with meat or meat products and who had no clinical evidence of brucellosis, 7.4% of individuals who had not been in contact with animals or meat or meat products with brucellosis but who consumed large amounts of milk or milk products, 11.7% of workers who slaughtered cattle and 39.9% of workers who slaughtered sheep (Ogutman, 1972). The overall proportion of individuals seropositive to brucellosis was 13.3%. Another seroprevalence study was carried out in different occupational groups of veterinarians and veterinary assistants, slaughterhouse workers and controls in Kocaeli, a city in northwest Turkey. Serum samples were evaluated using the Rose Bengal Plate test (RBPT), ELISA and standard agglutination tests. The ELISA showed a significantly higher brucellosis seroprevalence (4.8%) in the at-risk groups compared to 0% in the control group. All positive samples were from vets. The highly infectious nature of *Brucella* makes laboratory-acquired brucellosis a common problem in diagnostic and research laboratories. At the Ankara Numune Education and Research Hospital, when 48 healthcare workers with a professional risk of infection were

questioned, 12 were found to have had brucellosis, giving an infection risk of 8% per employee year (Ergonul *et al,* 2004). The main reasons for these infections were the absence of appropriate safety equipment and poor laboratory practices.

The situation of human brucellosis was reviewed in 1995 and findings are summarised in Table 2.1 (Abdou, 1996).

### 2.3 Taxonomy of *Brucellae*

The taxonomy of genus *Brucellae* as a single monospecific genus or multiple species has been a cause of controversial debate among scientists. The biochemical characteristics and host preference differences led to the classification of the genus initially into six species. However, DNA-DNA hybridization studies have revealed a great level of homogeneity between all species and they may be considered as a single species of *B. melitensis* (Verger *et al*, 1987).

Based on DNA homology, it has been proposed that all six members of the genus are actually biovars of a single species (Halling *et al*, 2005). Four members of the genus, *B. abortus*, *B. suis*, *B. canis*, and especially *B. melitensis* are able to cause infection in humans (Young, 1995).

According to their host specificity and phenotypic characters, the genus *Brucella* contains a group of very closely related bacteria. *Brucella melitensis* primarily affects sheep and goats (Sahin *et al*, 2008; Corbel and Brinley-Morgan, 1984), *Brucella abortus* primarily affects cattle, *Brucella suis* primarily affects pigs, *Brucella ovis* affects rams and ewes, *Brucella neotomae* affects desert wood rats, and *Brucella canis* affects male dogs and

bitches (Corbel and Brinley-Morgan, 1984; Verger *et al,* 1985; Alton *et al,* 1988; Corbel and Macmillan, 1998b). However, recently, four more species have been identified *B. ceti* (cetacean), *B. pinnipedialis* (pinnipeds) (Blasco and Molina, 2011), *B. microti* (vole) and *B. inopinata* (humans) (Scholz *et al*, 2008c; Godfroid *et al,* 2011) and 17 biovars have been characterized (Osterman, 2006). (Moved from Etiologic agent)

Common genetic fingerprinting methods, such as pulsed-field gel electrophoresis and multilocus sequence typing analyses, have revealed little variability between isolates of a given species. However, multilocus sequence typing has been useful in identifying the relationship between species and among biovars within a species, and in general, the findings support the classification of *Brucella* into the 6 known species, with at least 1 new species representing the newer marine strains of *Brucella* (Whatmore *et al,* 2007). The genus *Brucella* belongs to the family *Brucellaceae* within the order Rhizobiales of the class Alphaproteobacteria. The closest phylogenetic neighbour of the genus *Brucella* is the genus *Ochrobactrum*, a saprophyte that occasionally infects humans. Until 1985, the genus *Brucella* consisted of 6 species, *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae* and *B. ovis*, known as the six classical species. All these species are genetically highly related. In 1985, it was proposed combining the six species into a single species, *B. melitensis*, with the other species to be recognised as biovars (e.g., *B. melitensis* biovar *Abortus* 1) (Verger *et al*, 1985). In 2003, however, the Subcommittee on the Taxonomy of *Brucella* unanimously agreed on a return to the pre-1986 taxonomic nomenclature of the genus *Brucella*, implying re-approval of the six classical *Brucella* nomenspecies with their corresponding biovars (Osterman, 2006). Since 2007, *B. ceti* and *B. pinnipedialis*, which preferentially infect cetaceans and pinnipeds, respectively, have been recognized as new

*Brucella* species (Foster *et al*, 2007). In 2008, *Brucella microti* was first isolated from the common vole (*Microtus arvalis*) (Scholz *et al*, 2008 a,b,c) and recently *B. inopinata* was isolated from an infected breast implant in a woman displaying clinical signs of brucellosis (Scholz *et al*, 2010). This latter species is the only one that has yet to have been isolated from an animal reservoir. Prospective *Brucella* species have also been isolated from three native rat species in Australia (Tiller *et al*, 2010) and from two cases of stillbirth in non-human primates (Schlabritz-Loutsevitch *et al*, 2009). The preferential hosts and the pathogenicity for humans of the 10 recognized *Brucella* species are depicted in Table 2.1.

**Table 2.1 *Brucella* species, preferred host and pathogenicity for humans (Godfroid *et al*, 2011).**

| ***Brucella* species** | **Biovars** | **Preferential host(s)** | **Pathogenicity for humans** |
|---|---|---|---|
| *B. melitensis* | 1–3 | Sheep, goat | High |
| *B. abortus* | 1–6, 9 | Cattle | High |
| *B. suis* | 1, 3 | Pig | High |
| | 2 | Wild boar, hare | No |
| | 4 | Reindeer, caribou | High |
| | 5 | Rodents | No |
| *B. neotomae* | – | Desert wood rat | No |
| *B. ovis* | – | Ram | No |
| *B. canis* | – | Dog | Moderate |
| *B. ceti* | – | Cetaceans | Unknown |
| *B. pinnipedialis* | – | Pinnipeds | Unknown |
| *B. microti* | – | Soil, vole, fox | Unknown |
| *B. inopinata* | – | Unknown | High |

The organisms continue to affect human populations living in rural areas in the Mediterranean Basin (Portugal, Spain, Southern France, Italy, Greece, Turkey, North Africa), South and Central America, Eastern Europe, Asia, Africa, the Caribbean, the Middle East, and Latin American countries where the organisms are endemic (Gotuzzo *et al,* 1986; Shehabi *et al,* 1990; Yagupsky, 1994).

### 2.3.1 *Brucella abortus*

*Brucella abortus*, initially named as *Bacillus abortus* by Bang in 1897 and eventually renamed in 1920, is the etiological agent of bovine brucellosis, an infection that leads to spontaneous abortion, premature calving, and infertility in cattle. Most species of *Brucella* are primarily associated with certain hosts; however, infections can also occur in other species, particularly when they are kept in close contact. Maintenance hosts for *B. abortus* include cattle, bison (*Bison* spp.) water buffalo (*Bubalus bubalus*) (Longo *et al,* 2009), African buffalo (*Syncerus caffer*), elk (Jensen *et al*, 1995) and camels (Musa and Shigidi, 2001). A feral pig population has recently been reported to maintain *B. abortus*. A variety of other species can become "spill-over" hosts in areas where this organism is enzootic. *Brucella abortus* has also been reported in horses, sheep, Rocky Mountain bighorn sheep, goats, chamois, pigs, raccoons, opossums, dogs, coyotes, foxes, wolves and other species. Moose and llamas can be infected experimentally (Forbes and Tessaro, 1996).

In animals, *B. abortus* is usually transmitted by contact with the placenta, fetus, fetal fluids and vaginal discharges from infected animals. Many infected cattle become chronic carriers and *In utero* infections may also occur (Ray and Steel, 1979; Beveridge, 1983; Alton *et al,* 1988). Venereal transmission seems to be uncommon, however, transmission

by artificial insemination is reported to occur when contaminated semen is deposited in the uterus (WHO, 2006b). Millions of organisms are shed in the afterbirth and in fluids associated with calving and abortions. The disease is spread when cattle ingest contaminated feed or lick calves or aborted fetuses from infected cattle (Alton *et al*, 1988; Hall, 1989; Crawford *et al*, 1990b).This species is able to cross the species barrier affecting other livestock and humans (Young, 1995).

### 2.3.2 *Brucella* melitensis

*Brucella melitensis*, the first species in the genus *Brucella* to be described, causes abortions in female goats and sheep, unilateral orchitis in males and Malta fever in humans (Alton, 1990a). Sir David Bruce, a British army surgeon, discovered the organism in 1887 as the causative agent of Mediterranean or Malta fever (Moreno and Moriyon, 2002). The organism now bears his name coupled with "melitensis," which is Latin for Malta. *Brucella melitensis* is prevalent in Mediterranean and Middle Eastern countries through Central Asia to China and southern areas of the former Soviet Union. Some areas of Africa and India, as well as Central and South America, are also affected. This species' natural hosts are goats and sheep; however the organism is the least species-specific of the *Brucellae* (Alton, 1990a). Sheep and goats and their products are the main source of infection, although *B. melitensis* in cattle has emerged as an important problem in some southern European countries, Israel, Kuwait, and Saudi Arabia. The disease in goats resembles the disease in *B. abortus*-infected cattle (Enright, 1990). *Brucella melitensis* infection is particularly problematic because *B. abortus* vaccines do not effectively protect against the infection. Consequently bovine *B. melitensis* infection is emerging as an increasingly serious public health problem in some countries with the spread of the disease

through unpasteurized dairy products.

The organism is highly pathogenic for humans, making it one of the most serious zoonoses in the world. A vaccine has not yet been developed in humans, and animal vaccines are pathogenic to humans. Although rarely fatal for humans, it is highly contagious, difficult to treat, and easily transmitted, making it ideal for use in bioterrorism (CDC, 2008).

### 2.3.3 *Brucella suis*

Brucellosis caused by *B. suis* was first described by Traum in 1914 in swine herds in Indiana. It was initially thought to be a pathogenic *B. abortus* but was later named *B. suis* by Huddleson (Alton, 1990b). Comparison of the closely-related *B. suis* and *B. melitensis* genomes revealed a set of genomic variations that could be responsible for the differences in virulence and host preference between these organisms (Paulsen *et al*, 2002). Domestic and feral swine are natural hosts of *B. suis* (Norton and Thomas, 1976; Becker *et al*, 1978). Brucellosis caused by *B. suis* is considered to be a venereal disease with the infected boar passing the disease on to uninfected sows (Alton, 1990b). Contraction of the human disease is primarily limited to the occupational hazards of farmers and abattoir workers. *Brucella suis* was the first bio-weapon developed by the U.S.A. military during the 1950s. It is seen as a potential bioterrorism threat that could be used to target military personnel, civilians or food supplies (Paulsen *et al*, 2002).

### 2.3.4 *Brucella ovis*

A rough form of *Brucella, B. ovis* is the primary cause of brucellosis in sheep. *Brucella ovis* was first isolated in New Zealand and Australia (Blasco, 1990). It has also been found

in the USA, Mexico, Canada, South Africa and parts of Asia, Europe and South America (Blasco, 1990).

*Brucella ovis* may be transmitted venereally via an infected ewe. It can also be passed from one ram to another ram by direct contact, sharing of pens or through shearing wounds (Blasco, 1990). Ewes rarely show symptoms and only a small percentage of them actually abort (Grilló *et al,* 1999). However, some ewes may develop placentitis as a result of exposure to the organism which may result in the birth of weak lambs (Thoen *et al,* 1993). In sexually-mature rams, *B. ovis* causes epididymitis, orchitis and infertility. Only approximately 40% of rams with low antibody titers shed the organism, in contrast to 100% of high titer reactors (West *et al,* 2002).

### 2.3.5 *Brucella canis*

*Brucella canis* was first recognized in the late 1960s as a cause of abortions and reproductive failure, and it has since been documented in several countries (Carmichael, 1990). It is especially common in Mexico, Central and South America and in the southern states of the USA. It has been diagnosed in commercial or research breeding beagle kennels in several other countries, including Japan and more recently in The People's Republic of China. The disease has been reported sporadically in Europe (Wanke, 2004). Humans may be infected; however, dogs and other canine species are believed to be the only true hosts. Although canine brucellosis does not typically end in an animal's death it does result in reproductive failure (Hollett, 2006).

Brucellosis in dogs is mainly transmitted through sexual contact. In the female dog, *B. canis* survives in the vaginal and uterine tissues and is often excreted for life. The infected

female is frequently clinically healthy, although she can spread the bacteria through urine, aborted fetuses or most commonly through the act of breeding. Semen from infected males usually contains large numbers of abnormal sperm and inflammatory cells, especially during the first three months following infection. Chronically-infected males may have no sperm or reduced numbers of immature sperm. Infected males harbor organisms in the prostate gland and epididymides (Wanke, 2004).

### 2.3.6 *Brucella neotomae*

*Brucella neotomae* was isolated from the desert wood rat, *Neotoma lepida*, by Stoenner and Lackman in 1957 (Cameron and Meyer, 1958). It was identified as a new species of *Brucella* on the basis of conventional genus speciation, including the organism's behavior on differential dye media, $CO_2$ requirements and $H_2S$ production. The organism was found to be distinctly different from the three main species, *B. abortus*, *B. melitensis*, and *B. suis*, and all sub-classifications within the species (Huddleson *et al*, 1957; Cameron and Meyer, 1958).

### 2.3.7 Marine Mammal Species

Recently, a number of *Brucella* isolates have been described whose properties do not closely agree with the descriptions of the recognized species. The status of most of these strains has not been finalised, and it is possible that some or all of them will eventually be identified as atypical cultures of existing species or biovars. These new *Brucella* species have been isolated from marine mammals, predominantly seals and cetaceans and an otter, from Scotland and the coast around northern England and from a bottle-nosed dolphin from California (Ewalt *et al,* 1994). Identification of these organisms has been based on

serology, staining, metabolic phenotype, culture characteristics and phage typing (Jahas *et al,* 1997; Clavareau *et al,* 1998; Vizcaino *et al,* 2004). Characterization of these strains has failed to assign them to a known species of *Brucella,* and questions have been raised concerning the prevalence of infection, distribution and possible pathogenicity and zoonotic potential of these species (Ewalt *et al,* 1994; Foster *et al,* 1996).

### 2.4 Clinical picture in animals and humans

The incubation period varies with the species and stage of gestation at infection. In cattle, reproductive losses typically occur during the second half of the pregnancy with abortions or stillbirths occurring two weeks to five months after infection. In pigs, abortions can occur at any time during gestation whilst in dogs they occur approximately 7 to 9 weeks during pregnancy, however early embryonic deaths have also been reported after 2 to 3 weeks. Generally, brucellosis is a chronic infectious disease of the reproductive tract leading to abortion, reduced fertility, retained foetal membranes, orchitis, epididymitis and/or impaired fertility in cattle (Huddleson, 1943; Cunningham, 1977; Ray and Steel, 1979; Enright *et al,* 1984; Acha and Szyfres, 1987; Enright, 1990; Cheville *et al,* 1993). Abortion storms can occur with up to 80% of pregnant infected cows aborting (Cunningham, 1977). Subsequently the number of abortions usually decreases, as cows which have aborted in one year may deliver normal calves in subsequent years (Huddleson, 1943; Berman, 1981; Stevenson and Hughes, 1988). Crawford *et al.* (1990) reported that 3% of infected females would lose their calves in subsequent years.

Although much has been written about bovine brucellosis, little has been written about the gross pathological lesions seen in naturally infected animals. Cheville *et al.* (1992) stated

that field infections were typically subclinical in calves and non-pregnant cattle. Palmer *et al.* (1996b) and Cheville *et al.* (1996) failed to observe lesions in aborted foetuses or calves that died within 1 to 2 days of birth after experimental challenge of cows with *B. abortus* strain RB51 and strain 2308. However in the study of Palmer *et al.* (1996a) large numbers of bacteria were isolated from the lung, lymph nodes, allantoic fluid and rectal swabs of foetuses. They observed that 8 of 10 pregnant cattle which were experimentally infected with $1 \times 10^{10}$ colony forming units (cfu) of *B. abortus* strain RB51 were febrile (temperature 39.1 to 41.1$^{\circ}$C) during the first 24 to 48 hours after challenge, although no signs of depression or loss of appetite were apparent. In contrast, Cheville *et al.* (1992) demonstrated that after calves were challenged with $5 - 7 \times 10^{9}$ cfu of *B. abortus* strain 2308, no calves developed fever or other clinical signs, even though they developed high levels of persistent antibody titres.

Retained placentas are frequently reported in cows following abortion (Huddleson, 1943; Beveridge, 1983). Cunningham (1977) considered that this was a result of the prematurity of the parturition rather than from uterine infection. Nevertheless Payne (1959), Mollelo *et al.* (1963) and Palmer *et al.* (1996a) demonstrated that *B. abortus* had a tropism for the bovine placental trophoblasts and could induce placentitis resulting in premature birth. Cheville *et al.* (1993) reported severe acute diffuse purulent placentitis and necrosis with cloudy placental fluid in artificially infected cattle.

In cattle, brucellosis is primarily a disease of females but entire males can also be infected, but they do not readily spread the disease. The organism localizes in the testicles of the bull, resulting in orchitis. In the female, the organism is prevalent in the udder, uterus and lymph nodes adjacent to the uterus. The infected cows exhibit signs which may include

abortion during the last trimester of pregnancy, retained afterbirth and birth of weak calves (Enright, 1990). Typically, infected cows usually abort only once and subsequent calves may be born either weak or healthy. Some infected cows will not exhibit any clinical signs of disease and may give birth to normal calves.

Acha and Szyfres (1987) considered that the presence of metritis following abortion could result in permanent infertility and Huddleson (1943) observed that some infected females subsequently failed to show signs of oestrus. Subclinical mastitis has also been reported and the bacterium may be found in the milk (Beveridge, 1983).

Although infection of male cattle with *B. abortus* often fails to lead to the development of clinical signs (Beveridge, 1983), seminal vesiculitis, epididymitis and orchitis can occur (Huddleson, 1943; Acha and Szyfres, 1987; Stevenson and Hughes, 1988) leading to reduced libido and fertility (Plant *et al*, 1976; Acha and Szyfres, 1987).

Hygromas have been reported in infected cattle (Huddleson, 1943; Beveridge, 1983) and Van der Schaff and Roza (1940) reported that they were common in Zebu cattle in Java, Indonesia. *Brucella abortus* has been isolated from such lesions (Van der Schaff and Roza, 1940; Tounkara *et al*, 1994).

*Brucella melitensis* mainly causes abortions, stillbirths, the birth of weak offspring and retained foetal membranes. Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies. Milk yield is significantly reduced in animals that abort, as well as in animals whose udder becomes infected after a normal delivery. However, clinical signs of mastitis are uncommon. Acute orchitis and epididymitis can occur in males, and may result in infertility. Arthritis is seen

occasionally in both sexes. Many non-pregnant sheep and goats remain asymptomatic (Acha and Szyfres, 1987).

Infection with *B. melitensis* mainly causes abortions, stillbirths, the birth of weak offspring and retained foetal membranes. Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies. The milk yield is significantly reduced in animals that abort, as well as in animals whose udder becomes infected after a normal birth, however mastitis is rare. Acute orchitis and epididymitis can occur in males, and may result in infertility. Arthritis is occasionally seen. Many non-pregnant sheep and goats remain asymptomatic (European Commission, 2001). Moved from B. melitensis

*Brucella canis* can cause abortions and stillbirths in pregnant dogs. Most abortions occur late, particularly during the seventh to ninth week of gestation. Abortions are usually followed by a mucoid, serosanguinous or gray-green vaginal discharge that persists for up to six weeks. Early embryonic deaths and resorption have been reported a few weeks after mating, and may be mistaken for failure to conceive. Some pups are born alive but weak and most die soon after birth. Epididymitis, scrotal edema, orchitis and poor sperm quality may be seen in males. Scrotal dermatitis can occur due to self-trauma. Unilateral or bilateral testicular atrophy can be seen in chronic infections, and some males become infertile (Carmichael, 1990). The bacteria also can infect the developing fetuses resulting in abortion after 45-55 days of gestation, perinatal mortality and subsequent infertility (Carmichael and Joubert, 1988). In males, the bacteria survive in the testicles and seminal fluids with bacteria spread via the urine or semen (Moore and Kakuk, 1969). Infected males often display no clinical signs except in advanced cases where epididymitis,

testicular atrophy, scrotal dermatitis and infertility may be observed (Carmichael, 1990) moved from B. canis

In sows, abortion is the primary indicator of disease, which occurs at any stage of the pregnancy. An infected sow may deliver some healthy live piglets and have some born dead or die shortly after birth. Mastitis may also be observed. In boars, there may be *Brucellae* present in the semen without any visual indications of disease. There may also be unilateral swelling and atrophy of the epididymes and testes usually resulting in infertility. Reports of lameness; swollen joints, bursae and tendons; and paralysis because of abscess formation near the spine have also been documented (Alton, 1990b). Moved from B. Suis

In common, the major clinical sign of brucellosis in animals is infectious abortion as a result of the bacteria invading the placenta and fetus. The infected animals effectively remain carriers for the rest of their lives, even though they may abort only once. During this time they excrete large numbers of organisms in their milk, as well as in the products of subsequent, apparently normal, parturitions. Infection in humans can occur through the ingestion of raw milk or milk products, or by handling infected animals, especially around the time of parturition. Pasteurization effectively protects the urban population in most regions, but stockowners and their families often drink raw milk and are at risk from direct contact with infected animals (Davies and Casey, 1973, Castell *et al,* 1996; Anon, 2014).

Horses are also attacked with *B. abortus* which commonly results in inflammation of the atlantal bursa (poll evil) and paraspinosus bursa (fistulous withers) (Hinton *et al,* 1977; O'Sullivan, 1981; Acha and Szyfres, 1987). Cohen *et al.* (1992) reported that nine (37.5%)

of 24 horses with fistulous withers were seropositive to *B. abortus.* Although abortion has been recorded in horses (McCaughey and Kerr, 1967), Denny (1973) assumed that subclinical infection was the most common form of brucellosis in this species.

There is little information on the effects of brucellosis in marine mammals but *Brucella* has been isolated from the reproductive organs of some marine species. In rare cases, infections have also been linked to lesions or clinical disease. *Brucella*-associated abortions and placentitis have been reported in two captive bottlenose dolphins and a wild Atlantic white-sided dolphin. Recently, *Brucella* has also been isolated from a dead Maui's dolphin calf in New Zealand. *Brucella*-associated epididymitis has been reported in porpoises, and orchitis, suspected to be brucellosis, has been reported in minke whales (Clavareau *et al,* 2009).

*Brucella*-associated meningoencephalitis has been reported in three stranded striped dolphins and other signs of *Brucella*-associated systemic disease have been seen in Atlantic white-sided dolphins with lesions of hepatic and splenic necrosis, lymphadenitis and mastitis. *Brucella* has also been identified as a possible secondary invader or opportunistic pathogen in debilitated seals, dolphins and porpoises. It has been isolated from several subcutaneous abscesses. In addition, this organism has been found in organs with no microscopic or gross lesions, and in apparently healthy animals (Palmer *et al*, 1996a). Moved from Marine Brucellosis

The infection in humans manifests initially as an acute febrile illness or undulant fever (Pappas *et al,* 2005). However, the clinical signs in humans are not pathognomonic for the disease (Young, 1989a, b), and include fever, chills, weakness, general aches and pains,

neck pain, sweating, headaches, weight loss, anorexia, constipation, nervousness and mental depression (Ray and Steel, 1979; Stevenson and Hughes, 1988). Stevenson and Hughes (1988) considered that, because of the non-specific nature of the symptoms, patients often delayed seeking medical attention and consequently diagnosis was often delayed. Young (1983) considered that fever and lymphadenopathy were the most common clinical signs presented in patients suffering from brucellosis. However the variable symptoms and the occurrence of subclinical and atypical infections in both the acute and the chronic stages make the clinical diagnosis of human brucellosis difficult (Matar *et al,* 1996). In contrast to cattle, abortion in women from brucellosis is uncommon and this may be due to a lack of erythritol in their placenta and uterus (Ruben *et al,* 1991). Local skin lesions have also been described in humans at the site of accidental inoculations with *B. abortus* strain 19 vaccine (Corbel, 1989).

A substantial proportion of patients present with splenomegaly and/or hepatomegaly. When the disease becomes chronic, a wide range of pathological conditions may occur including spondylitis, endocarditis and meningoencephalitis (Young, 1995; Pappas *et al,* 2005). The recommended treatment is a long course (at least 6 weeks) of combinations of antibiotics, notably rifampin plus tetracycline or gentamicin or (parenteral) streptomycin (Solera, 1997b; Ariza *et al,* 2007).

### 2.5 Necropsy findings and microscopic lesions

Granulomatous inflammatory lesions are frequently seen in affected organs and lymphoid tissues (Payne, 1959; Berman, 1981), although lesions such as necrotizing placentitis, testicular alteration, necrotizing orchitis and epididymitis which may be present are not

pathognomonic for brucellosis (Danks, 1943; Lambert *et al*, 1964; Crawford *et al*, 1990b; Cheville *et al*, 1993). In ruminant fetuses, the spleen and/or liver may be enlarged, and the lungs may exhibit pneumonia and fibrous pleuritis (Cheville *et al*, 1993). Abortions caused by *Brucella* spp. are typically accompanied by placentitis. The cotyledons may be red, yellow, normal or necrotic. In cattle and small ruminants, the intercotyledonary region is typically leathery, with a wet appearance and focal thickening (Huddleson, 1943).

In adults, granulomatous to purulent lesions may be found in the male and female reproductive tracts, mammary glands, supramammary lymph nodes and other lymphoid tissues, bones, joints and other tissues and organs (Runells and Huddleson, 1925; Enright, 1990). Mild to severe endometritis may be seen after an abortion (Palmer *et al*, 1996a), and males can have unilateral or bilateral epididymitis and/or orchitis (Danks, 1943; Lambert *et al*, 1964; Crawford *et al*, 1990b). In *B. abortus*-infected cattle, hygromas may be found on the knees, stifles, hock, angle of the haunch, and between the nuchal ligament and the primary thoracic spines (Bracewell and Corbel, 1980).

## 2.6 Epidemiology of the disease

Among the *Brucellae*, *B. abortus*, *B. melitensis*, and *B. suis* are not host-specific (Bridges and Halling, 1994; Corbel, 1997; FAO, 2003) being capable of infecting a wide range of host species, including humans (Alton *et al*, 1988). *Brucella* grows intracellularly, producing a variable bacteraemic phase followed by localization to the tissues of the genital tract and the mammary gland. Abortion is typically the first clinical sign of the pregnant female, with orchitis and epididymitis in males (Huddleson, 1943; Cunningham,

1977; Enright *et al,* 1984; Acha and Szyfres, 1987; Cheville *et al,* 1993; Corbel, 1998a). In particular, female animals that have reached sexual maturity are most susceptible to infection displaying abortions if pregnant (England *et al,* 2004).

Most species of *Brucella* are maintained in a limited number of reservoir hosts including cattle, bison (*Bison* spp.) (Meagher and Meyer, 1994), water buffalo (*Bubalus bubalus*), African buffalo (*Syncerus caffer*), elk and camels (Ray and Steel, 1979; Beveridge, 1983; Alton *et al,* 1988; Hall, 1989; Crawford *et al,* 1990b), and feral pigs. *Brucella suis* contains more diverse isolates than other *Brucella* species, and these isolates have broader host specificity (Priadi *et al,* 1985). Biovars 1 and 3 are found in both domesticated pigs (*Sus scrofa domesticus*) and wild pigs, while biovar 2 has been isolated from domesticated pigs, wild boar (*Sus scrofa scrofa*) and European hares (*Lepus capensis*). Biovar 4 is maintained in caribou and reindeer (*Rangifer tarandus* and its various subspecies) and biovar 5 is found in small rodents.

Other species can become accidental hosts, particularly for *B. abortus*, *B. melitensis* and *B. suis* (Hinton *et al,* 1977; Acha and Szyfres, 1987; Corbel, 1989), and can include buffalo (Ray and Steel, 1979), sheep, goats (Enke *et al,* 1959; Shaw, 1976; Luchsinger and Anderson, 1979), camels (Obied *et al,* 1996), pigs, moose, chamois, alpine ibex, raccoons, opossums (Tessaro, 1986), dogs, coyotes, foxes and wolves (Tessaro, 1986).

### 2.6.1 Worldwide distribution

Although brucellosis is widely distributed in the world (Figure 2.1), information on its distribution in many parts of the world is minimal (Beveridge, 1983; Crawford *et al,* 1990b; Corbel, 1997), primarily due to its insidious nature and a lack of resources to

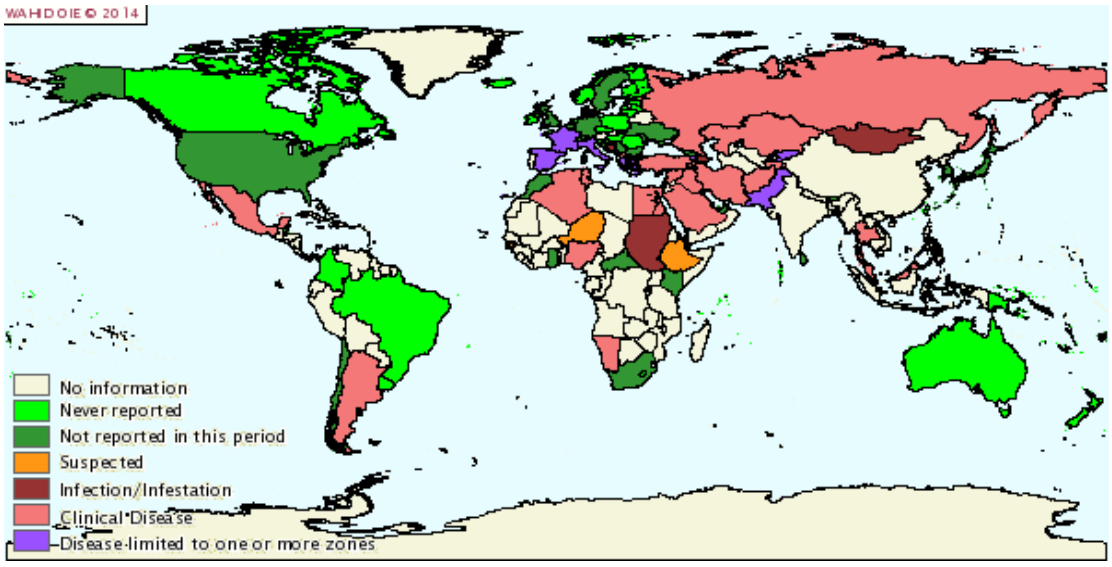
investigate the disease when compared with more spectacular diseases , such as foot-and-mouth disease, sheep pox, Rift Valley fever and peste des petits ruminants.

The disease appears to be endemic in the Mediterranean region (Al-Majali, 2005), central Asia (Baluyut and Dugui-Es, 1977; FAO, 1986), the Arabian Peninsula (Ibrahim, 1986; Qubain, 1986; Corbel, 1997) and as far east as Mongolia. Mexico, Peru and northern Argentina are also seriously affected in Latin America (Corbel, 1997). The disease has also been reported in Africa (Fassi-Fehri, 1975; Johnson *et al*, 1984; FAO, 1986; Corbel, 1989) and India (Corbel, 1989; FAO, 1986; Corbel, 1997). Most of North America is believed to be free of disease as are many countries in Northern Europe (Crawford *et al*, 1990b; Corbel, 1997; OIE, 2012). New Zealand and Australia are also considered free (Corbel, 1997). *Brucella melitensis* biovar 3 is the predominant type in Mediterranean and Middle East, with biovar 1 predominant in Latin America. In Southern Europe, both biovars 1 and 2 have been reported.

Human brucellosis is endemic in Mediterranean countries and in 2003 more than 50,000 human cases were reported in these countries (Pappas *et al*, 2006). An annual incidence of up to 78 cases/100,000 people has been reported in Middle Eastern and Mediterranean countries (Hartigan, 1997). However the true incidence of human brucellosis throughout the world is not known precisely and it would be expected to vary between areas (Alton, 1990). Humans are dead-end hosts and the disease is generally restricted to specific occupational groups including veterinarians, farmers, laboratory technicians, abattoir workers and individuals who work with animals and their products. Fresh dairy products, aborted fetuses, placenta and uterine excretions are considered the major source of organism. Therefore, the most dangerous period for disease transmission is during lambing

or kidding period. For groups without occupational contact with animals, dairy products are the critical source of infection, especially cheese made from unpasteurized sheep and goat milk (FAO, 1995). During the manufacture of cheese most of the organisms are trapped within the clot and hence are concentrated in the cheese. The number of cases contracted from milk and milk products is seasonal and reaches a peak soon after lambing or kidding. Handling of raw wool has also been identified as a potential source of human infection (FAO, 1995). The epidemiology of brucellosis among humans reflects the epidemiology among populations of animals and the public health and economic impact of the disease remains of particular concern in developing countries throughout Africa, West Asia and some parts of Latin America (Glynn and Lynn, 2008).

**Figure 2.1 Global distribution of Animal Brucellosis (OIE, 2012)**



Almost all affected countries have attempted to combat animal brucellosis using different strategies with varying levels of success. While disease prevalence is steadily decreasing in some countries, a dramatic increase in the prevalence has been reported in the Gulf and southern Mediterranean countries. Although brucellosis is a notifiable disease in many countries, the prevalence is likely to be underestimated due to underreporting or misdiagnosis (Hartigan, 1997).

Animal brucellosis poses a barrier to trade of animals and animal products and could seriously impair the socio-economic development of a community, especially for livestock owners. The importance of this disease was evident in the commitment of over half of the total European Commission funding for animal disease control measures in 1997 to the control of brucellosis (Hartigan, 1997).

**Table 2.2 Annual incidence of human brucellosis in some countries**

| Country | Year | Survey area | Sample size | Type of data | Frequency (%)– incidence/100.000 |
|---|---|---|---|---|---|
| Algeria | 1988–1990 | | | | 36–67/100.000 |
| Tunisia | Recent | National | | | 40/100.000 |
| Lebanon | 1984–1986 | | | | 69.6/100.000 |
| Egypt | 1991 | Four governorates | 2720 | All types | 10.5 |
| | 1994 | | 747 | Fever cases | 43.2 |
| | 2000 | | 2236 | | 7 |
| Jordan | 1986–1991 | | 730 | | |
| Iraq | 1988 | | 1187 | | 7.2/100.000 |
| Palestine | 1996 | Gaza | | | 8/100.000 |
| Turkey | 1984–1987 | | 8383 | | 13.9/100.000 |

| Country | Year | Survey area | Sample size | Type of data | Frequency (%)– incidence/100.000 |
|---|---|---|---|---|---|
| Iran | 1988 | | 71051 | | 132.4/100.000 |
| | 1996 | | 30000 | | |
| Saudi Arabia | 1988 | | | | 79.6/100.000 |
| Kuwait | 1985 | | 1168 | | 68.9/100.000 |
| | 1997 | | 112 | Bedouins | 545.7/100.000 |
| Oman | 1997 | Dhofar | | | 200/100.000 |

The seroprevalence in the southern part of Jordan was reported to be significantly higher than that of the central or northern parts. The high level in the southern regions could be due to either a lack of implementation of an effective control program or a high level of uncontrolled animal movement from Saudi Arabia (Al-Majali *et al*, 2007). In Jordan, the incidence of abortions in goat herds from brucellosis was significantly higher than that in sheep flocks (Al-Talafha *et al*, 2003). *Brucella melitensis* biotype 3 was the most prevalent isolate from the cultured aborted fetuses, although *B. abortus* biotype 9 has also been isolated from the lung of one fetus (Aldomy *et al*, 1992; Al-Talafha *et al*, 2003).

#### 2.6.3.1 Age, Species and breed

Brucellosis is commonly a disease of adult animals (sexually mature females and males). Young animals may contract the infection but display no clinical signs, although a transient and weak serological response may be detected. The susceptibility increases with pregnancy and after sexual maturity (Corbel *et al*, 2006).

Goats seem to be the principal host for *B. melitensis*, with few sheep infected (Corbel & Brinley-Morgan, 1984); however in some areas the disease is more important in sheep (Corbel, 2006). Different breeds of sheep shows a great variation in their susceptibility to infection, with milking breeds being more susceptible than meat breeds (Corbel & Brinley-Morgan, 1984). The Maltese breed and other breeds from South America appear to have strong resistance to infection. However Awassi's are very susceptible and act as an important reservoir of infection for humans. Consequently for most countries bordering the Mediterranean Sea and in Southwest Asia, brucellosis is mainly focused on sheep, while in Latin America goats are the predominant species (European Commission, 2001). The behavioral patterns of sheep, including gathering at lambing time or during the evening, may play a role in disease transmission (Alton, 1982).

There is significant evidence suggesting the high susceptibility of camels to *B. melitensis* and consequently consuming raw camel milk is regarded a major risk factor for humans (European Commission, 2001). Cattle and pigs are also susceptible to infection, especially where these are in contact with infected small ruminants (Garin-Bastuji and Hars, 2000). Carnivores, such as, cats, dogs and foxes, can also transmit the infection mechanically.

### 2.6.3.2 Mode of infection and disease transmission

Two major modes of infection are recognized. The direct mode occurs through the ingestion of the bacterium, transmission via contaminated semen or infected embryos or through inhalation in aerosols (European Commission, 2001). Dogs can act as mechanical and biological vectors (FAO/WHO Expert Committee on Brucellosis, 1986). The organisms are rarely spread through waterways (European Commission, 2001). The number of bacteria excreted in milk is low but is sufficient to infect lambs and kids and humans (Philippon *et al,* 1971). Although the majority of infections are acquired through consumption of colostrum and milk, a small proportion of lambs and kids may be infected *in utero*. These animals then may subsequently shed the bacterium in their faeces. However a self-curing mechanism has been suggested resulting in the animals being subsequently susceptible to infection when they reach sexual maturity (Grilló *et al,* 1997). Immunotolerance to infection with *B. melitensis* has been reported and this may account, in part, to the difficulty in eradicating the disease (Dolan, 1980).

In vaccinated animals, infection may be rapidly eliminated and sheep are reported to have a strong resistance to reinfection following recovery from infection with *B. melitensis* (Alton, 1990a). Long lasting immunity has also been demonstrated in experimentally infected sheep (Durán-Ferrer, 1998).

Placenta, foetal fluids and vaginal discharges expelled by infected ewes after abortion are the primary route of dissemination of bacteria (European Commission, 2001; Corbel, 2006) with a large number of organisms shed at the time of parturition or abortion (Alton, 1990a, Durán-Ferrer, 1998). Aborted goats shed the organisms for a prolonged period (2-3

months). In contrast the organism is discharged by sheep for a maximum of three weeks after abortion or parturition. The organisms can also be shed from milk, colostrum and semen and may be isolated from various tissues, including lymph nodes and arthritic lesions. Infection of the mammary glands or supramammary lymph nodes may result in intermittent or continuous shedding of the pathogen (European Commission, 2001).

The environmental conditions and type of husbandry greatly influence the dispersal of the disease such as, lambing in enclosures and an overcrowded environment. The disease is most likely introduced to a previously free area through the introduction of infected animals (European Commission, 2001). Communal grazing and the practice of transhumance are strongly correlated with disease (Corbel, 2006).

#### 2.6.3.3 Host-Parasite interactions

Within mammalian hosts, *Brucella* spp. are intracellular organisms and infect phagocytes. The VirB operon, a type IV secretion pathway that is induced on phagosomal acidification, plays a key role in intracellular parasitism and is essential for pathogenicity (O'Callaghan *et al,* 1999; Ugalde, 1999; Boschiroli *et al,* 2002). *Brucella* spp. resist different environmental stresses within the phagocytic cells, modify their intracellular trafficking (the ability to modulate and evade fusion with lysosomes) and eventually reach their replicative niche (Kohler *et al,* 2002). *Brucella* spp. survive and multiply in dendritic cells, resulting in interference with their maturation so that antigen processing is impaired resulting in a compromised host immune response (Roop *et al,* 2009). *Brucella* spp. prevent apoptosis within macrophages and their long-term survival in the reticuloendothelial system of the spleen, liver and bone marrow results in chronic infection

(Gorvel and Moreno, 2002). During gestation, *Brucella* spp. replicate in large numbers in placental trophoblasts. The integrity of the placenta may be disrupted and abortion induced. The pregnant uterus is an immunological privileged site and the local immune response is modulated to prevent rejection of the fetus. However this may allow the *Brucellae* to replicate extensively (Neta *et al*, 2010). The *Brucella* lipopolysaccharide (LPS) is a weak inducer of the host inflammatory cytokines IL-1β, IL-6 or TNF-α compared to LPS molecules from many other Gram-negative bacteria (Roop *et al*, 2009). Although *Brucella* LPS stimulates TLR4, it has lower immunostimulatory activity compared to other Gram-negative bacteria such as *Salmonella enterica* serotype Typhimurium (Rittig *et al*, 2003). IFN-γ is a key cytokine controlling *Brucella* infection. One of its major functions is the stimulation of *Brucella*-killing effector mechanisms in phagocytic cells (Baldwin and Parent, 2002).

The global picture emerging from what is known about *Brucella* virulence is an extremely efficient adaptation to shield itself from immune recognition and to manipulate key aspects of host cell physiology (apoptosis, vacuolar trafficking) (Gorvel and Moreno, 2002; Letesson *et al*, 2002; Gorvel, 2008). It is evident that one of the *in vivo* adaptation keystones is the ability to fine tune metabolism according to the various nutrients encountered during the infectious cycle (Brown *et al*, 2008; Lamontagne *et al*, 2010). Quorum sensing (QS) is also known to be involved in the regulation of *Brucella* virulence determinants mostly linked to the cell surface (type IV secretion system, flagellum, Omps and exopolysaccharide) (Letesson *et al*, 2002; Weeks *et al*, 2010). Both the BvrR/BvrS TCS and the QS system could contribute to the adaptation of the metabolic network during the nutrient shift faced by *Brucella* along its intracellular trafficking. Furthermore the

phosphoenolpyruvate phosphotransferase system (PTS) of *B. melitensis* senses the metabolic state of the cell leading to a coordinated regulation of C and N metabolisms, and as well as some key virulence genes, e.g., the *virB* operon (Dozot *et al,* 2010) and flagellar genes (Fretin *et al,* 2005).

In the placenta, *Brucella* invade trophoblasts and utilize iron for their replication which may lead to placental disruption resulting in abortion or weak offspring. Erythritol is also assumed to play a major role to determine tissue tropism for *Brucella* (Williams *et al,* 1964; Keppie *et al,* 1965; Acha and Szyfres, 1987) and this may be linked with iron acquisition for virulence in ruminants. Most *Brucella* species are highly virulent, causing an acute infection in both their natural and accidental hosts. The bacterium has a reputation for being a 'stealth pathogen' that can infect without inducing a massive inflammatory response. Its ability to survive and multiply in host cells, such as macrophages in the reticuloendothelial system and trophoblasts in the placenta, is a key aspect of its virulence. Over the last 20 years, considerable advances have been made in understanding the genetics and cell biology of *Brucella* virulence, and these have been reviewed recently (Roop *et al,* 2009; Martirosyan *et al,* 2011). The bacteria enters cells via lipid rafts, which help in the avoidance of defense mechanisms. The bacterium's lipopolysaccharide and periplasmic cyclic β-glucan are essential for the first steps in the establishment of an intracellular replication niche, in which *Brucella* survives and multiplies. Acidification of the phagosome induces the expression of several virulence factors including the VirB type IV secretion system. The VirB system is thought to translocate effectors' proteins into the host cell, which modulate host cell biology to create the intracellular replication niche. *Brucella* creates its replication vacuole by capturing membrane vesicles at endoplasmic

reticulum exit sites, a tactic used by some other intracellular pathogens, including *Legionella* (O'Callaghan *et al*, 1999; Kohler *et al*, 2002).

Compared to other non-sporing bacteria, *B. melitensis* has a relatively higher ability to persist outside the host (European Commission, 2001). Favourable environmental conditions include high humidity with a pH>4, low temperatures and the absence of direct sunlight. The infectivity of the organisms may persist for several months in contaminated water, aborted materials, liquid manure, wool, hay, contaminated equipment and clothes. Under dry conditions, *B. melitensis* may remain viable in dust and soil (European Commission, 2001). Contaminated equipment and utensils can be sterilized by autoclaving at 121°C and liquid manure treated by xylene and calcium cyananaide for 2 to 4 weeks. Caustic soda, 2% formaldehyde and 2.5% sodium hypochlorite can destroy the organisms on contaminated surfaces (European Commission, 2001). In milk and dairy products, survival of *Brucella* depends on the type and age of products, their pH, humidity, temperature and storage conditions (Carrère *et al*, 1960). Prolonged boiling and pasteurization inactivates the bacteria (Davies and Casey, 1973). In fermented cheese, *Brucella* do not survive for long, however the optimal time of fermentation to ensure safety is not exactly known although it has been estimated to be around three months (Nicoletti, 1989). In acidified soft cheeses the survival time of *Brucella* is enhanced, hence the only means to ensure their safety is pre-processing pasteurization of the milk (European Commission, 2001). Radiation of colostrum by gamma rays is effective in inactivating *Brucella* (Garin-Bastuji *et al*, 1990). In contrast to dairy products, the life span of *Brucella* in meat is short due to the lower pH that occurs post mortem.

Disinfectants, including phenol (10 g/l), formaldehyde and xylene (1ml/l), are effective in

inactivating the bacterium. For exposed skin, ethanol, diluted hypochlorite solution and iodophores are used for decontamination. On pasture *Brucella* can survive for up to 4 days if there is direct sunlight and up to 6 days in the shade (WHO, 1986).

### 2.7 Laboratory diagnosis

Generally, brucellosis can be diagnosed by serological tests, culture and PCR assays. *Brucella* species and biovars are usually identified by phage-lysis, biochemical criteria and molecular techniques.

#### 2.7.1 Serodiagnosis

Serological tests have been used widely to detect the humoral response in cattle with *B. abortus* infection, although false positive and false negative reactions, associated with a specificity and sensitivity less than 100%, can be a problem. False positive reactions have been reported in animals infected with other microorganisms including *Yersinia enterocolitica* 0:9 as their O-chain in the LPS are identical. Animals exposed to other bacteria, such as *E. coli* O:157, *Streptomonas maltophilia*, vibrio cholera O:1, *Francisella tularensis*, *E. hermani* and *Bordetella bronchiseptica*, can also produce conflicting serological results (Weynants *et al*, 1996b). False positive reactions arising from antibodies induced by vaccination and the failure to develop an immune response until animals become pregnant are further diagnostic challenges. Interferon gamma tests and indirect ELISAs using rough strain antigen have been reported to be promising tools to differentiate brucellosis from diseases caused by other cross-reacting microorganisms (Nielsen *et al*, 1989; Weynants *et al* 1996a).

No serological test has been developed solely for *B. melitensis*. Diagnostic assays developed for detecting infection of cattle with *B. abortus* have been used to detect *B. melitensis* infection in small ruminants. Accordingly, the RBPT and the CFT are the most widely used tests for the serological diagnosis of brucellosis in sheep and goats (MacMillan, 1990). These tests detect antibodies raised against the smooth lipopolysaccharide (S-LPS) (OIE, 2008). However, the sensitivity of the CFT test is poorer than that of both the RBPT and the indirect ELISAs in small ruminants (Blasco *et al*, 1994a; Blasco *et al*, 1994b). In addition, both the RBPT and CFT tests lack specificity when used for testing sera from sheep and goats recently vaccinated with Rev-1, the only available vaccine against *B. melitensis* (Fensterbank, *et al*, 1982; De Bagués *et al*, 1992; Díaz-Aparicio *et al*, 1994). Although this issue is less if the Rev-1 vaccine is applied via the conjunctival route (De Bagués *et al*, 1992; Díaz-Aparicio *et al*, 1994).

#### 2.7.1.1 Rose Bengal Plate Test (RBPT)

In ruminants the RBPT is often used to screen entire herds for evidence of infection with brucellosis (Alton *et al*, 1975b; Sutherland, 1980; Alton *et al*, 1988; MacMillan, 1990). The principle of the test depends on an antigen-antibody reaction resulting in agglutination. Smooth *Brucella* culture stained with Rose Bengal dye is mixed in a buffered acidic suspension and mixed with an equal volume (drops) of serum (Sutherland, 1980; Alton *et al*, 1988). The acidic buffer is used to decrease problems associated with non-specific agglutination (Corbel, 1972). One studied tested the sera of 300 cows that had aborted and which had been cultured for *B. abortus* with the RBPT, CFT and SAT. Of the sera 91.4%, 92.7% and 66.9% were positive, respectively (MacMillan, 1990). Sutherland and Searson (1990) reported that the sensitivity of the RBPT was 78% and the specificity 71%. In

contrast, MacMillan (1990) reported a sensitivity and specificity of 97 and 92.7%, respectively in an automated RBPT. Koh and Morley (1981) reported a specificity of 97.9 to 99.1% in vaccinated and non-vaccinated herds. As a consequence of this specificity, the RBPT is not recommended for testing individual animals (Alton *et al,* 1988).

Similar to other serological tests, the RBPT can give incorrect results and cannot distinguish between vaccinated and infected cases (Brinley-Morgan *et al,* 1969; Alton *et al,* 1975b; Sutherland, 1980; Alton *et al,* 1988). Furthermore the lower specificity of the test may result in more false positive reactions, which are test negative on other assays (Brinley-Morgan *et al,* 1969; Mylrea, 1972; Browne, 1974). The RBPT may be interfered by infection with Salmonella, *E. coli* O:157 (Nielsen *et al,* 1980) and *Y. enterocolitica* O:9 (Mittal and Tizard, 1979).

The RBPT primarily detects immunoglobulin G (IgG) (Corbel, 1972) however it can also detect IgM (Allan *et al,* 1976). The high sensitivity of the test is one of its key features with only 0.4 to 1.8% of RBPT seronegative animals testing positive on the CFT (Mylrea, 1972; Brinley-Morgan *et al,* 1978). False negative results can occur to the RBPT during the first weeks of infection (MacMillan, 1990).

#### 2.7.1.2 Compliment Fixation Test (CFT)

The CFT has been stated to be the most accurate and definitive of the serological tests (Sutherland, 1980). It has been widely used in control and eradication programs (Alton *et al,* 1988; MacMillan, 1990). The test usually utilizes the whole cell of *B. abortus* (MacMillan, 1990). Several authors have revealed that compliment fixing antibodies in infected animals are mostly IgG and IgM (Anderson *et al,* 1964; Brinley-Morgan *et al,*

1969). However, several authors have reported that IgM has reduced ability to fix compliment when sera is heated at 56°C which may restrict the early detection of infection (Sutherland, 1980; Alton *et al,* 1988). The prozoning phenomenon is also an obstacle associated with sera where the ratio of the two types of IgG ($IgG_1$ and $IgG_2$) is high in sera. This may result in blocking the fixation of compliment (Hobbs, 1985; Alton *et al,* 1988). The sensitivity of the CFT is high compared with culture and this test has been considered to be the most superior serological test (Nicoletti, 1969; Alton *et al,* 1975b). Furthermore the CFT's specificity is high compared with the SAT (serum agglutination test), RBPT, and Indirect ELISA (Dohoo *et al,* 1986). Huber and Nicoletti (1986) reported that in adult vaccinated cows, the CFT had the highest sensitivity and specificity compared with the rivanol and milk ring tests. The CFT is very sensitive to changes, is not easy to perform requiring experienced and skilled scientists, may exhibit prozone and anticomplimentary reactions and can fail to detect latent carrier infections (Christie *et al,* 1968). False negative reactions may occur as the test only can detect antibody at least two weeks after infection (Sutherland, 1980).

#### 2.7.1.3 Enzyme linked Immunosorbent Assay (ELISA)

The ELISAs are dependent upon the detection of antibodies in the serum of infected or vaccinated animals; however they can detect all antibody isotypes (Hobbs, 1985). For over two decades, ELISAs have been used to supplement other serological tests for the diagnosis of *B. abortus* (Engvall and Perlmann, 1971). The tests may utilize whole cell antigen, crude and semi purified LPS or non-LPS antigens (Letesson *et al,* 1997). Several ELISA types have been developed including the direct, indirect and competitive forms. ELISA's have also been used to detect antibodies in milk (Thoen *et al,* 1995; Nielsen *et al,*

1996), and have been evaluated in naturally infected animals, vaccinated animals (Abalos *et al*, 1996; Uzal *et al*, 1996) and in humans (AlShamahy and Wright, 1998).

Although the ELISA is not a cheap test, several authors have highlighted several advantages in using this assay. Firstly, it has high sensitivity and specificity (Saunders and Clinard, 1976; Cargill *et al*, 1985; Sutherland *et al*, 1986). Secondly, and unlike the CFT, the ELISA is not affected by haemolysis, prozone and anticomplimentary effects (Reynolds, 1987) and finally the technique is not complicated and is commercially available.

The sensitivity and specificity of the ELISA has been found to be much better than the Milk Ring Test (MRT) when testing milk for evidence of infection in lactating cows. It also has a higher sensitivity and specificity than the CFT (Sutherland *et al*, 1986). Although Cargill *et al.* (1985) reported that the ELISA and CFT had similar specificities. Nicoletti and Tanya (1993) reported that the ELISA was an efficient test for the early detection of brucellosis, although its use for field diagnosis was not as efficient.

Although cross-reactions have been highlighted with *Y. enterocolitica* O:9, use of a competitive ELISA with monoclonal antibody to *B. abortus* LPS eliminates this problem. Depending on its sensitivity and its ease of performance, Nielsen *et al.* (1995) concluded that the ELISA is the most suitable assay for the diagnosis of brucellosis in individual animals. However, as with other assays, caution must be taken with its use in areas where vaccination has been undertaken.

In an investigation carried out by Hornitzky and Searson (1986), the usefulness of the ELISA was highlighted in cattle that were culture positive, non-vaccinated RBPT negative

reactors or low CFT titer animals. Heck *et al.* (1984) reported that the ELISA and Haemolysis In Gel Test (HIGT) were able to detect antibodies in 92 to 96% of animals, respectively, within four weeks of experimental challenge of vaccinated cows, and almost 100% were detected ten weeks after inoculation. However with the SAT, CFT and rivanol tests, less than 88.6% were positive after 24 weeks of infection. In contrast, in non-vaccinated challenged cows, 93 and 90% were positive on the ELISA and HIGT, respectively, four weeks post infection while less than 62% were positive by other tests.

In general, the indirect ELISAs are good tests for surveillance purposes in countries in the latter phases of eradication where vaccination is no longer used. However, these ELISAs lack specificity when used in vaccinated animals, particularly when Rev-1 is used in adult animals. In these conditions, only the Native Hapten (NH) gel precipitation test is useful for determining infection in vaccinated animals (Díaz, *et al,* 1979; OIE, 2008). Although the competitive ELISA is promising, this test also lacks specificity in vaccinated animals and those infected with *Y. enterocolitica* O:9 (Marín *et al,* 1999; Muñoz *et al,* 2005). The World Health Organization has recently classified brucellosis among the 7 top neglected zoonoses, a group of diseases that are simultaneously a threat to human health and a cause of poverty perpetuation (Maudlin and Weber, 2006).

#### 2.7.1.4 The Serum Agglutination Test (SAT)

The SAT was used as a standard test for the diagnosis of brucellosis before the RBPT was developed (Brinley-Morgan, 1967; Davies, 1971; Alton, 1977a, b; Sutherland, 1980; Nicoletti, 1969). The test was found to detect IgM more efficiently than $IgG_1$, and consequently was reported to be more effective in the early detection of infection (Allan *et*

*al,* 1976). In cattle vaccinated with strain 19, SAT was also found to detect more IgM than IgG antibody and it was recommended by Alton (1977 a, b) to take this feature into account when using the SAT in areas where strain 19 had previously been used. However, several disadvantages with using the SAT have been demonstrated. These include the prozone phenomenon which can result in false negative reactions, especially where the $IgG_1$ concentration is high (MacMillan, 1990). Cross-reaction with *Y. enterocolitica* O:9 and *E. coli* O:157 have also been reported (Mittal and Tizard, 1980; MacMillan, 1990). In culture positive animals Sutherland and Searson (1990) demonstrated that the SAT had a sensitivity and specificity of 70% and 95%, respectively. As a consequences of these limitations, several countries test samples with confirmatory tests such as the CFT (Sutherland, 1980). In contrast, Jiwa *et al*. (1996) recommended the use of the SAT as it is a simple, inexpensive technique that can be performed by untrained personnel.

#### 2.7.1.5 Milk Ring Test (MRT)

The milk ring test is a simple test that can detect antibodies in the milk of infected cows (Beveridge, 1983; MacMillan, 1990). It detects antibodies attached to fat globules of the milk by using whole cell haematoxylin-stained killed *Brucella* antigen (MacMillan, 1990). The antigen is added to the milk sample and the antigen-antibody complex rises to the surface of the milk forming a ring in the cream layer (Sutherland, 1980; MacMillan, 1990). The sensitivity of the test has been reported to be high (Beveridge, 1983), however false negative reactions have been described (Christie *et al,* 1968; Brinley-Morgan *et al,* 1978), although testing of bulk milk samples from dairy farms helps decrease the false negative reactions. Thoen *et al.* (1995) reported that the MRT was less useful in areas where the prevalence of brucellosis was low and Cunningham (1968) reported that the test was also

less effective where animals had been vaccinated with strain 19 and in animals with mastitis.

Although the MRT has several disadvantages, it is a simple, inexpensive test which can be used for screening dairy herds by non-skilled personnel (Sutherland, 1980; Beveridge, 1983; Nielsen *et al,*1996).

**2.7.1.6 Rivanol Test**

The Rivanol test uses rivanol dye to precipitate serum protein, other than gamma globulins (Brinley-Morgan, 1967). The test has been shown to be promising in differentiating infected from vaccinated cattle (Nicoletti *et al*, 1978a, b; Alton *et al*, 1988). However, false negative reactions have been reported, especially when animals are vaccinated at a young age (Huber and Nicoletti, 1986). Many authors have studied the sensitivity and specificity of the test, although the results have been variable (Nicoletti, 1969; Anczkyowski and Murat-Skwarek, 1972). In a study undertaken by Huber and Nicoletti (1986) which involved testing over 1000 culture–positive adult cows, 99.3% were positive on the rivanol test, however only 24.8% of 2,417 culture-negative adult vaccinated cows were negative by this test. Nicoletti (1969) reported that the test had a good specificity (80%) and high sensitivity (96%) and as a result of the low false positive rate it was concluded that the test was useful for detecting infected cows. Nevertheless, the sensitivity of the rivanol test is lower than the CFT and is time-consuming and not easy to perform or interpret (Brinley-Morgan, 1967).

#### 2.7.1.7 Other tests

**The Mercapto-ethanol Test (ME Test), Coombs Test and The Indirect Haemolysis Test (IHLT)**

The mercapto-ethanol test is based on inactivation of IgM by mercapto-ethanol and then detection of IgG only. However it requires experienced technicians and the results can be difficult to interpret and hence it is rarely used today (Brinley-Morgan, 1967).

Coombs test has mainly been used as an assay to detect brucellosis in humans and has also been called the Antihuman globulin test (AHG). It also has been used to confirm the results of the CFT in cattle (Sutherland, 1980). Although, the test has been demonstrated to be effective in detecting chronic carriers, it can have a high percentage of false positive reactions in vaccinated animals (Brinley-Morgan, 1967). Sutherland (1980) also reported that the test was not easy to perform or interpret.

In the indirect haemolysis test (IHLT), LPS sensitized erythrocytes are used to overcome the prozone phenomenon (Plackett *et al*, 1976; Sutherland, 1980). Corner *et al.* (1983) stated that the IHLT was characterized by low sensitivity (47%) when compared with the CFT (89.5%) and RBPT (89.5). In one study, 54% of culture negative cattle were positive on the CFT, 45% to the RBPT and 68% to the IHLT. This was assumed to be associated with the low specificity of the IHLT, leading to a high proportion of false positive reactions (Sutherland and MacKenzie, 1983).

### 2.7.2 Antigen detection

Bacterial culture is a useful tool for confirming brucellosis, as well as differentiating

bacteria for taxonomic purposes and producing vaccines. *Brucella* can be isolated from several sources including foetal membranes and tissues (spleen, lungs, testes, stomach contents and liver), aborted materials, vaginal excretions, hygromas, testes, lymph nodes (supramammary and iliac lymph nodes in particular), uterine cotyledons, mammary glands and colostrum and milk (Alton *et al,* 1975a). Isolation of the bacterium from udder secretions was suggested to be a method to differentiate field strains from vaccinal strains (Huber and Nicoletti, 1986). Although Alton *et al.* (1975a) were able to isolate *Brucella* for up to six weeks *post partum*, others were unable to isolate the organism five days after abortion. In a study undertaken by Hornitzky and Searson (1986), *B. abortus* was isolated from the supramammary lymph nodes of 79.6% of the culture-positive cows, and the detection proportion increased to 89.8% when the results for culturing of the prescapular lymph nodes were included with those of the supramammary lymph node. Including the retropharyngeal lymph nodes increased this to 93.9% and 100% when the results for the submandibular and iliac lymph nodes were included in the results. Weynants *et al.* (1995) isolated *B. abortus* from lymph nodes adjacent to the inoculation site after challenging 10 cattle, highlighting the role of the lymph nodes in filtering the bacterium.

Although bacterial culture is considered the definitive diagnosis (Cunningham, 1977; Crawford *et al,* 1978; Hornitzky and Searson, 1986; Huber and Nicoletti, 1986; Mayfield *et al,* 1990; OIE, 2009) it has many disadvantages. Firstly contamination can make successful culturing difficult. Secondly it takes time, reagents and equipment and hence requires significant laboratory experience and it is expensive. Thirdly the infected materials or culturing process may expose personnel to the agent (Mikhail *et al,* 1983; Alton *et al,* 1988; Gaviria-Ruiz and Cardona-Castro, 1995; Ouahrani-Bettache *et al,* 1996).

Consequently serological assays are key for successful eradication and control programmes (Sutherland, 1980).

### 2.7.2.1 Culturing of *Brucella* on media

Although several media have been developed for culturing *Brucellae* (Mayfield *et al*, 1990), standard basal medium is considered to be the ideal medium for this pathogen. Corbel and Brinley-Morgan (1984) found that the primary isolation of *Brucella* could be accelerated by adding 5-10% normal serum to the medium. Atmospheric conditions required for growth is 10% $CO_2$, except for strain 19, at 37°C, although growth can be seen between 20 and 40°C. The optimal pH range for growth is between 6.6 and 7.4 (Corbel and Brinley-Morgan, 1984).

Farrell's medium, a selective medium containing antibiotics such as bacitracin, cycloheximide, nalidixic acid, nystatin, polymixin B and vancomycin with 5% horse serum has been used for the isolation of *Brucella* from contaminated tissues. Culturing on solid media limits the interference by faster growing microbes as the media discourages dissociation which facilitates the recognition of colonies. Alton *et al.* (1988) reported that colonies were visible on nutrient agar after three days of incubation, although routinely examination is not undertaken until the fourth or fifth day of culturing. *Brucella* colonies appear transparent or pale honey colored on serum dextrose agar. The colonies are raised and convex with a smooth, shiny surface.

Alton *et al.* (1988) demonstrated that the growth of *Brucella* in liquid medium was poor and culturing on static liquid medium accelerated the dissociation of smooth to non-smooth forms. Furthermore culturing in liquid medium has reported to require a longer incubation

period (Mayfield *et al.* 1990).

Biphasic medium of Farrell's medium and a liquid phase of Bordie Sinton's medium are commonly used as additional media for the isolation of *Brucella* from heavily contaminated samples. It has been found that culturing on biphasic medium increases detection (positive cultures) by 64.8%, compared to isolation on solid medium (Corner *et al*, 1985; Hornitzky and Searson, 1986).

### 2.7.2.2 Biotyping of brucellosis

The importance of biotyping *Brucella* is to provide epidemiological information, to establish the agent's characteristics and to facilitate control programmes (Luchsinger *et al*, 1973; Crawford *et al,* 1990). Each type of *Brucella* consists of several biovars or biotypes. *Brucella abortus* is composed of eight biovars and *B. melitensis* nine (Corbel and Brinley-Morgan, 1984). Each biovar may contain many different strains. Biovar 1 of *B. abortus* is the most prevalent biovar in cattle but is also found in other species including sheep and goats, buffalo, horses, camels, and humans (Kerr *et al*, 1966; Hendricks and Meyer, 1975; Shaw, 1976; Crawford *et al,* 1990). Biovar 2 has also been isolated from cattle in New South Wales (Hornitzky and Searson, 1986).

The two common ways for biotyping are phage typing, which depends on lysis of the bacterium by phages, and a comparison of oxidative metabolic profiles on selected amino acids and carbohydrate substrates (Alton *et al*, 1988). However the latter method can be hazardous, time consuming and requires specific facilities. Biovars of *B. abortus* can also be differentiated by their utilization of $CO_2$, production of $H_2S$, growth on media with dyes and reactions with monospecific antisera (Corbel *et al*, 1983; Crawford *et al,* 1990).

Corbel and Brinley-Morgan (1984) demonstrated a significant amount of DNA homology in species within the genus and similar polynucleotide sequences have been detected. As a result, the usual biotyping tests may not always reveal the full extent of differences between biovars, especially where the differences rely upon a single characteristic. Aldrick (1968) highlighted the importance of biotyping isolates as soon as possible after culturing due to the unstable nature of colonies which may not be visible after repeated subculturing.

#### 2.7.2.3 Molecular detection and identification

Classically the detection and identification of *Brucella* spp. has been based on cultural and phenotypic analysis (biotyping). Although providing valuable information, biotyping is a highly specialized and time-consuming approach requiring experienced staff using well-optimized non-commercial reagents which is ideally conducted in a securely biological contained laboratory (Bridges and Halling, 1994; Mercier *et al*, 1996).

The polymerase chain reaction (PCR) is a common diagnostic tool used worldwide. The technique has been used to detect *Brucella* DNA, to differentiate between species and strains and to study the epidemiology of the disease (Allardet-Servent *et al*, 1988). In contrast with other conventional techniques, the PCR has several advantages. It takes less time with the results being available within a few hours; it minimizes the need to handle potentially infectious samples; it can be automated; and it is not expensive.

Initial PCR methods were based on the 16S rRNA and *bcsp31* genes (Baily *et al*, 1992; Herman and Deridder, 1992). PCR methods based on the 16S rRNA amplify a DNA fragment common to all *Brucella* species; however the method cross-reacts with members of the closely related genus *Ochrobactrum* (Velasco *et al*, 1998; Scholz *et al*, 2008a). The

IS*711* molecular element has become the preferred target for general identification purposes due to its restricted occurrence in *Brucella* and the presence of multiple copies, allowing for unparalleled sensitivity and direct testing on clinical samples (Halling *et al*, 1993; Ouahrani-Bettache *et al*, 1993). Other molecular markers such as *recA* (Scholz *et al*, 2008a), *omp2* (Leal-Klevezas *et al*, 1995) and 16S–23S intergenic transcribed sequence (Rijpens *et al*, 1996) can also be used to correctly identify members of the genus *Brucella*. Until recently the most popular PCR assay developed for differentiating *Brucella* at the species level was the AMOS PCR (Bridges and Halling, 1994). This technique is based on the insertion site of the IS*711* element which is species-specific and results in a unique PCR profile for *Brucella* strains belonging to each of the *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* species (hence the name AMOS). However, *B. canis*, *B. neotomae*, some biovars of *B. abortus* and *B. suis* and the *Brucella* species isolated from marine mammals cannot be detected by the AMOS PCR Novel IS*711*. Chromosomal locations specific to marine mammal *Brucella* isolates have been identified allowing their identification and classification based on the same principle as AMOS PCR (Cloeckaert *et al*, 2003; Maquart *et al*, 2008; Zygmunt *et al*, 2010). Real-time PCR assays, based on some of the genetic markers described above, have been developed to identify *Brucella* species (Al Dahouk *et al*, 2007b), although these tests have the same limitations regarding *B. suis* and *B. abortus* detection. AMOS-PCR has provided the basis for other multiplex PCR assays, such as the Bruce-ladder-PCR which is able to successfully discriminate between isolates of the six classical species and the marine mammal *Brucellae* (Lopez-Goni *et al*, 2008; Mayer-Scholl *et al*, 2010). These tests, which are remarkably robust and require no expensive laboratory equipment, display specificity at the species level, except for some strains belonging to the

closely related *B. canis* and *B. suis* species (Koylass *et al,* 2010). For many years, most epidemiological data was based on the division of the three most significant species, *B. melitensis*, *B. abortus* and *B. suis*, into a number of biovars. Several other molecular techniques have been used to divide isolates into molecular groups such as PFGE, IS*711* fingerprinting, *omp* typing, IRS-PCR and AFLP that corresponded well with the classical species divisions (Vizcaino *et al,* 2000; Moreno *et al,* 2002; Whatmore, 2009). However, none of these techniques has significant resolution at the subspecies level. The use of multilocus sequence analysis (MLSA) opens the way to detailed characterization of the global population structure of *Brucella* (Whatmore *et al,* 2007). These analyses confirmed the status of the classical species as distinct genetic entities, allow indexing of intra-species diversity and relating this to historical biovar designations, and have provided a framework for the placement of atypical or emerging *Brucella* isolates (De *et al,* 2008; Schlabritz-Loutsevitch *et al,* 2009; Tiller *et al,* 2010). Furthermore, these analyses and the availability of more robust phylogenetic histories, have allowed the identification of canonical single nucleotide polymorphisms (SNPs) that could be exploited as the basis of rapid diagnostic tests. A number of SNP-based assays have recently been described that can rapidly identify *Brucella* isolates to the species level (Foster *et al,* 2008; Gopaul *et al,* 2008; Gopaul *et al,* 2010), identify vaccine strains (Gopaul *et al,* 2010) or even identify the biovar (Fretin *et al,* 2008). A further major recent genome-driven advance has been the identification and exploitation of tandem DNA repeats as typing tools. These repeats have been exploited in many bacteria to develop a new generation of Variable Number of Tandem Repeat (VNTR) based typing approaches but are likely to prove particularly valuable in *Brucella* which previously lacked any epidemiological tools with adequate resolution to facilitate

reliable epidemiological trace-back (Bricker *et al,* 2003; Le Flèche *et al,* 2006; Whatmore *et al,* 2006). Both MLSA and VNTR based analyses question the validity of some of the biovars established by classical microbiological typing, particularly those of *B. melitensis* (Al Dahouk *et al,* 2007a; Whatmore *et al,* 2007). Such analyses applied to local epidemiological scenarios shall allow progress in a number of areas previously hampered by the lack of tools with adequate discriminatory capacity. Application of these approaches should ultimately allow rapid detection of nationally or internationally dispersed clusters, detection of transmission chains, detection of new and emerging strains and trace-back to sources of outbreaks. The ability of VNTR analysis to link genotypes to background epidemiological data should also facilitate the identification of risk factors and help understand differential virulence or pathogenic properties of individual genotypes. VNTR has recently proven highly efficient in confirming laboratory or other professionally acquired infections (Marianelli *et al,* 2008; Valdezate *et al,* 2010), in distinguishing relapse from re-infection (Al Dahouk *et al,* 2005a; Kattar *et al,* 2008), in characterizing outbreaks (Valdezate *et al,* 2007; Lucero *et al,* 2010), in identifying associations of different genotypes with different pathogenic profiles (Nöckler *et al,* 2009), and in assessing the stability of vaccine preparations (Garcia-Yoldi *et al,* 2007).

Although Bridges and Halling (1994) reported that the sensitivity of the PCR could be reduced by contamination, Da Costa *et al*. (1996) considered that its sensitivity was its main advantage. However the assay has to be performed under strict standardized conditions which are not always available in laboratories.

Pulsed field gel electrophoresis (PFGE) is a restriction endonuclease analysis that has been used to study the relationship between strains of *Brucella* species. The technique has been

found to be helpful in classifying the genus as it detects differences between DNA fingerprints of species and biovars. Unlike other techniques which produce a large number of small DNA segments, Tcherneva *et al*. (1996) demonstrated that the PFGE could split DNA into a small number of large segments. The enzymes (Xho I) and (XbaI) have been shown to divide the DNA into more than 25 bands of different intensities (Allardent-Servent *et al,* 1988). After digestion by XbaI, *Brucella* can be clearly differentiated into species based on their unique DNA fingerprints. However, the technique was unable to clearly differentiate biovars (Allardent-Servent *et al*, 1988). However in the study by Jensen *et al*. (1995) it was demonstrated that the PFGE could distinguish field isolate of *B. abortus* biovars 1, 2 and 4 from that of RB51 vaccine strain.

Although PFGE has several advantages, it suffers from the disadvantage that the concentration of DNA cannot be altered after the agarose is prepared and therefore the migration of the DNA molecules is influenced by the total DNA concentration (Li *et al*, 1989). Consequently the technique has been modified by immersing cells in agarose prior to lysis. This keeps the large DNA molecules intact during the diffusion of the detergent and protease (Schwartz and Cantor, 1984).

#### 2.7.2.4 Skin Delayed-Type Hypersensitivity (SDTH)

The skin delayed-type hypersensitivity (SDTH) test has been used widely for the diagnosis of brucellosis, especially in ruminants, and it is a valuable addition to serological tests. Similar to the tuberculin test for tuberculosis, the principle is to evaluate the cell-mediated immunity after inoculation of 0.1 ml of crude extract of *B. (brucellin) abortus* intradermally into the neck. In a positive result a hypersensitivity reaction is observed 24 to

72 hours after injection. The degree of skin swelling reveals the intensity of the reaction and an increase in the thickness of the skin fold by 2 mm or more is considered a positive reaction (Bercovich *et al,* 1993).

The SDTH can also detect latent carriers and can confirm the status of false negative results arising from serological tests. A purified protein brucellin (P39) produced negative results in cattle infected with other microorganisms, such as *Y. enterocolitica* O:9, and *E. coli*. This confirms the high specificity of this test (Denoel *et al*, 1997). In contrast another study by Cheville *et al*. (1994) demonstrated that vaccinated heifers reacted to standard brucellin, and consequently it was concluded that the test was unable to distinguish between vaccinated and naturally infected animals. In a study undertaken by Bhongbhibat *et al*. (1970), allergenic fractions of *Brucella* species were purified and could distinguish between infections with *B. abortus* and *B. melitensis*.

However the test is time-consuming, animals need to be handled twice and the results are not easy to interpret. Weynants *et al*. (1995) highlighted that the SDTH could alter the animal's immune status if used repeatedly and this may interfere with subsequent serological tests. Consequently an interval of seven weeks after the SDTH test is recommended before testing with serological tests (Muskens *et al*, 1995, 1996).

**2.7.2.5 Gamma Interferon Assay (IFN-¥)**

The gamma interferon assay was developed as an *in vitro* alternative to the SDTH (Weynants *et al,* 1995). It involves mixing cytoplasmic protein from *B. melitensis* B 115 with whole blood from cattle. The test offers the advantage of being able to distinguish between true and false positives, although like most tests it cannot distinguish between

vaccinated and infected animals.

## 2.8 Treatment, prevention and control of Brucellosis

### 2.8.1 Treatment

As an intracellular bacterium, the treatment of animal brucellosis is challenging (Metcalf *et al,* 1994). Although there is no effective treatment for bovine or swine brucellosis, canine brucellosis has been treated successfully through the administration of combinations of antibiotics. Streptomycin with sulphadiazine and oxytetracycline are the commonly used antibiotics. Infusing the udder of infected cattle with these antibiotics has also been attempted, although the infection was not successfully eliminated (Corbel, 1977; Radostits *et al*, 1994). Few authors have demonstrated the efficacy of oxytetracycline and streptomycin to cure animals from natural infection with *B. abortus* or *B. melitensis* (de Bagues *et al,* 1991; Radwan *et al,* 1993). Nicoletti *et al*. (1987) found that giving an oral subtherapeutic dose of chlortetracycline in parallel with injection of strain 19 (S19) vaccine did not interfere with the formation of antibodies. However, others have demonstrated the positive effect of oxytetracycline when administered with S19 (Smith *et al*, 1983; Nicoletti, 1990). In conclusion, treatment of infected animals is not practical or feasible from an economic point of view unless the animals have significant value.

Two treatment regimens are recommended in humans: a combination of oral doxycycline 100 mg twice a day and rifampicin 600–900 mg/day (15 mg/kg/day) in a single oral dose over a 6-week course; and a combination of streptomycin 1 g intramuscularly once a day

for 2 weeks or an alternative aminoglycoside, instead of the administration of rifampicin. The latter regime has the advantage of lower relapse rates (Corbel, 2006; Ariza *et al*, 2007). For a successful therapy of focal complications and chronic disease, combination of three or four of the antimicrobial drugs listed previously and longer treatment courses (>45 days) are recommended (Robson *et al*, 1993; Hendricks *et al*, 1995; Solera *et al*, 1997a; b; Ariza, 1996; Solera, 1997b; Ariza *et al*, 2007).

### 2.8.2 Prevention and control of animal brucellosis

The live-attenuated *B. melitensis* Rev-1 vaccine is the only vaccine available for *B. melitensis*, and this vaccine has been shown to be effective in preventing brucellosis in sheep and goats (Blasco, 1997). However, when administered by the classic subcutaneous route (individual doses of $1\times10^9$–$2\times10^9$ cfu), a long-lasting serological response is induced, which makes an eradication program based on test and slaughter impractical. When the same vaccine is administered by the conjunctival route (at the same dose but in a smaller volume), the immunity conferred is similar to that induced by the classic subcutaneous method, although the serological response is significantly reduced making it suitable for use in an eradication program (Blasco, 1997). However, this type of program is still out of the reach of many countries that have only elementary veterinary services and limited economic resources. In these cases, a mass vaccination campaign is the only reasonable alternative to control brucellosis. Unfortunately, the vaccination of pregnant animals with Rev-1 subcutaneously can result in abortions and the excretion of Rev-1 strain in milk (Blasco, 1997). Reduction of the Rev-1 dose ($10^3$ to $10^6$ cfu administered subcutaneously) has been reported to avoid these significant adverse reactions while still inducing effective protection (Al Khalaf *et al*, 1992). However, field and experimental data suggests

otherwise, and reduced doses of Rev-1 should not be recommended as an alternative to vaccination with standard doses (Fensterbank *et al*, 1982; Blasco, 1997). Due to the risk of abortion, there is no entirely safe strategy for mass vaccination. Even conjunctival vaccination is not safe enough to be applied regardless of the pregnancy status of the animals (Blasco, 1997). It is recommended that Rev-1 should not be used in mid-gestation animals, the main critical period for abortions. However, this is impractical under field conditions, and some of the risks have to be assumed if the objective is to control the disease. Conjunctival vaccination of animals before the start of the mating season, during the late stages of the lambing season, or during lactation are the safest approaches to performing a whole-flock/herd vaccination program (Blasco, 1997). This modified-live vaccine also has a very slight chance of infecting humans (Blasco and Díaz, 1993) as well as being resistant to streptomycin which, in combination with doxycycline, constitutes the most effective treatment of brucellosis in humans (Ariza *et al*, 2007).

Accordingly some biosafety measures (wearing protective glasses and gloves) and educational campaigns are needed to reduce the risk of infection in humans. In the case of accidental injection with Rev-1, a combined doxycycline-gentamicin (or doxycycline-rifampin) treatment should be administered (Blasco and Diaz, 1993; Ariza *et al*, 2007).

One of the key disadvantages of vaccination is the potential interference with serological assays. The diagnostic epitopes involved are located in the *O*-polysaccharide section (a homopolymer of *N*-formylperosamine) of the *B. melitensis* S-LPS immunodominant surface antigen (González *et al*, 2008). Research to improve the vaccines by removing these S-LPS epitopes (ie, to develop rough—R—vaccines) has been conducted. Among the live rough *Brucella* strains obtained by classic attenuation methods, is the *B. abortus* RB51

vaccine. However, its efficacy and safety with regard to bovine brucellosis is questionable (Moriyón *et al*,2004; Mainar-Jaime *et al,* 2008) and it is not effective against *B. melitensis* or *B. ovis* infections in sheep (Moriyón *et al,* 2004). Finally, Other research efforts in developing R vaccines have resulted in candidates of low overall efficacy (Moriyón *et al*, 2004; Barrio *et al,* 2009). Whereas, R candidate vaccines do not interfere with the classic serological tests (RBPT and CFT) this is not the case for the ELISAs. Using S-LPS or its hydrolytic polysaccharides as antigens, a proportion of ewes vaccinated with R candidates have been classified as seropositive to an indirect ELISA (Barrio *et al*, 2009). This result is not unexpected, because R mutants elicit antibodies to the core epitopes also present in the wild-type S-LPS and its hydrolytic polysaccharides. Core epitopes are not readily accessible on the whole S *Brucellae* (used as antigen in the RBPT and CFT), but they can become exposed on adsorption to ELISA plates and, therefore, prevent a clear-cut distinction of the antibody responses to S and R *Brucellae*. This problem is likely to affect all R vaccines (Mainar-Jaime *et al*, 2008). In conclusion, the potential advantages for R vaccines are questionable and there is increasing evidence demonstrating that these vaccines interfere in S-LPS–based ELISAs, are not safe in pregnant animals, may be excreted in the milk of vaccinated animals, may infect humans and have reduced efficacy when compared with the Rev-1 and S19 vaccines in small ruminants and cattle (Moriyón *et al*,2004).

Other approaches to develop new-generation vaccines, such as the construction of recombinant strains with missing relevant diagnostic proteins or DNA-based vaccines, are also being investigated (Blasco, 2006). In fact, the Rev-1 vaccine strain with a deletion of the gene coding for BP26 protein (that can be used as a differential marker) has been

shown to induce the same protective efficacy as Rev-1 in sheep (Jacques *et al*, 2007). It also showed efficacy against *B. ovis* infection in rams, however evaluation of the performance of the BP26-based differential diagnostic test is limited (Grilló *et al*, 2009).

However none of the new-generation vaccines have been shown to have improved efficacy and safety over the classic Rev-1 vaccine and it has been recommended that Rev-1 should remain the reference vaccine for the prevention of brucellosis in sheep and goats (Blasco, 2006).

Independent of their origin, the Rev-1 vaccine and the tests used to diagnose the disease should always be submitted for quality control to internationally recognized laboratories, and should fulfill the minimal requirements described by the World Organization for Animal Health (OIE, 2008). A country's veterinary services must select a control or eradication approach compatible with the socioeconomic conditions and infection status of that country. The effect of brucellosis on both the livestock economy and human health as well as the costs of the different strategies must be evaluated as part of this practice. Several aspects, such as knowledge of livestock management and breeding practices, the habits of the community and the availability of adequate human resources to carry out the program, must also be evaluated. Moreover, cooperation between all related stakeholders is of paramount importance and should be promoted. Collaboration between the public health and veterinary services has to be encouraged through the establishment of a national zoonoses body (Metcalf *et al*, 1994).

Although vaccination can interfere with serological testing, this strategy is central to protect susceptible livestock. However, this interference is minimized when animals are

vaccinated at a younger age. In endemic countries, several strategies have been designed to eradicate and or control the disease. The implementation of any sanitary strategy requires considerable technical training, and an awareness campaign aimed at farmers and the general population. The most common strategies to control infection with *B. melitensis* in small ruminants include blanket whole-flock/herd vaccination or testing and slaughtering with or without vaccination. In both cases, the use of adequate vaccination procedures and diagnostic tests is of paramount importance (Blasco and Molina, 2011).

The use of vaccination to increase disease resistance in herds is important in these strategies and it has been demonstrated that, in cattle, vaccination was the most effective control measure. In California the prevalence in dairy and beef herds was nearly 87% lower after vaccination for ten years (Nelson, 1977). However, a study has shown that for any successful prevention, 70% of a herd's population needs to be immunized (Berman, 1981). However Metcalf *et al*. (1994) reported that vaccination alone, without the adoption of any other control measures, was of doubtful value. Consequently other measures, including movement restrictions and management changes, are also required to be adopted in conjunction with a vaccination campaign.

Numerous attempts to eradicate *B. melitensis* infection or, at least, to reduce its prevalence to an "acceptable" level have been made in several countries within the region. In Algeria, an approach based on test-and-slaughter of goats was initiated in 1995, however it was replaced after a 3-year period with a mass vaccination campaign. In Tunisia, mass vaccination with Rev-1 by the conjunctival route, irrespective of age, was implemented throughout the country in order to stop the spread of the 1991 epizootic. A similar programme was implemented in eastern Morocco in 1996. Simultaneously, an

epidemiological survey was conducted and confirmed the absence of brucellosis in small ruminants in the rest of the country (Benkirane, 2006). In Iran, sheep and goats raised under an intensive husbandry system were individually identified and tested with the RBPT and SAT in 1983. Positive animals were then slaughtered and compensation paid to their owners. Following this campaign young animals were vaccinated with Rev-1. This resulted in a dramatic drop of infection from 3.2 to 0.5% between 1983 and 1996. For flocks raised under an extensive husbandry system young animals were vaccinated with Rev-1, although seropositive animals were not removed. In this group the proportion of seropositive animals had decreased from 3% in 1994 to 2.2% in 1998. Kuwait also initiated an annual mass vaccination campaign in 1993, using a reduced dose (1/50) of Rev-1, administered subcutaneously (Al-Khalaf *et al*, 1992; Crowther *et al*, 1977). Between 1993 and 1997 the proportion of animals vaccinated each year had increased to 75%.

Several vaccines have been produced to protect cattle, sheep and goats and swine against infection with *Brucella*. In endemic areas, RB51 and S19 are the most common vaccines to control *B. abortus* infection while Rev-1 vaccine is mostly used to control infections with *B. melitensis*.

#### 2.8.2.1 Control of *B. melitensis*

Although the application of a test and slaughter strategy can be an effective way for the control and eradication of *B. melitensis*, this method is not always applicable in areas where the prevalence of disease is high and where socio-economic obstacles exist to the diseases control. Consequently control programs based on vaccination are suitable to reduce the prevalence of disease to an acceptable level prior to implementing an

eradication campaign. It is now recommended that a combination of vaccination of young animals and culling of infected adults is the most practical way to control *B. melitensis* (Blasco and Moriyon, 2005).

#### 2.8.2.1.1 Classical *B. melitensis* REV-1 vaccine

Rev-1 is an attenuated strain of virulent *B. melitensis* obtained in the 1950's (Elberg and Faunce, 1957) and is reported to be the best isolate of *B. melitensis* for incorporation into a vaccine (Blasco, 2006). Alton and Elberg (1967) have demonstrated the efficacy of the vaccine after vaccination of cattle 3 to 6 months of age, as well as in adult animals. The vaccine has been shown to induce a high and durable immune response (Blasco, 1997).

The vaccine can be administered via the subcutaneous (S/C) or conjunctival route in both young and adult animals. However the S/C vaccination of young animals (3 to 6 months of age) with a standard full dose can result in persistent infection which interferes with the interpretation of results of serological assays (Fensterbank *et al,* 1982). However, this is not an issue if the aim is to induce the highest level of immunity in animals and not eradication of infection. In contrast vaccination via the conjunctival route confers adequate protection in young animals without interfering with serological assays (Marin *et al,* 1999).

Although Rev-1 has some advantages it can result in infection of humans if accidentally inoculated (Blasco and Diaz, 1993) and has the potential to infect rams (European Commission, 2001). The vaccine may also induce abortions in sheep and goats if animals are vaccinated during pregnancy (Blasco, 1997). Even reduced doses of Rev-1 are not totally safe and may not induce effective protection in sheep (Fensterbank *et al,* 1982). Blasco (1997) recommended the use of a standard (full) dose via the conjunctival route to

minimize the risk of abortion (Blasco, 1997).

In conclusion, vaccines (S19 and Rev1) are useful for the control of *Brucella melitensis*, especially if used in young animals (3 to 6 months) in countries with a high prevalence and limited resources (Blasco, 1997). However the level of protective immunity developed, safety issues in males and females, interference with serological assays, duration of immunity and the standardization of the vaccine are potential areas of concern with brucellosis vaccines.

#### 2.8.2.2 Vaccines of *B. abortus*

##### 2.8.2.2.1 Strain 19 vaccine (S19)

Strain 19 is the mostly widely used vaccine against *B. abortus* in the world and it is considered the reference vaccine to which all other vaccines are compared. It was initially produced in the USA in 1939 and has been used in the field since 1941. The vaccine contains a live attenuated strain that was initially cultured from milk and then subcultured 19 times (Nicoletti 1990). Jones *et al.* (1965) and Sangari *et al.* (1996) demonstrated that the S19 isolated could not grow in the presence of erythritol, was highly immunogenic, was less virulent than field strains and retained its viability during lyopholisation. Normally the vaccine is administered as a single subcutaneous injection containing 5-8 x $10^{10}$ cfu to female calves 3 to 6 months of age. In contrast adult cattle receive a lower disease of 3 x $10^{8-9}$ cfu also through the subcutaneous route. It has also been reported that the vaccine can be administered to any age group through the conjunctival route at the reduced dose (3 x $10^{9}$ cfu). The conjunctival route has the advantages over the subcutaneous route in that there is a lower risk of abortion, it stimulates protection without

the presence of persistent antibodies which may interfere with the interpretation of serological tests and is not excreted in the milk.

Reduced dose S19 can induce a similar immune response to that of full dose in adult cattle (Corner and Alton, 1981) and consequently has been recommended in areas where the disease is severe. However Bartone and Lomme (1980) considered that care has to be taken when handling the vaccine to ensure sufficient viable organisms were present to stimulate immunity in the vaccinated animals.

Although S19 has many advantages, several limitations have been documented. Firstly, the vaccine may result in infection if given late in pregnancy (Crawford *et al*, 1978; Nicoletti *et al*, 1978a). Secondly, Breitmeyer *et al.* (1992) reported that the organism could be excreted in the milk of vaccinated cattle, resulting in increased probability of transmission to humans or other animals. Thirdly, persistent infections can occur after vaccination (Nicoletti, 1977; Corner and Alton, 1981). Although Crawford *et al.* (1978) cultured the organism from the milk of one of 245 dairy cattle five months after vaccination; the organism was not cultured from the lymph nodes of vaccinated adult beef cattle. Finally, administration of S19 in pregnant cows by the subcutaneous route may result in abortions (Nicoletti, 1977; Beckett and MacDiarmid, 1985). However, others failed to detect an influence on the pregnancy level in vaccinated animals (Corner and Alton, 1981).

The side effects after using S19 are believed to be as a result of erythritol tolerant mutants derived from the vaccine strain culture. These mutants are believed to cause persistent infection or abortions after inoculation into pregnant cattle. Although a few cases of abortion may result from vaccination with S19, several authors have recommended that

S19 can be used at any stage of gestation as the benefits gained from vaccination outweigh any adverse effects or abortion risk (Barton and Lomme, 1980; Nicoletti, 1976).

Another limitation of S19 is the presence of persistent antibodies which may interfere with serological assays. Nicoletti (1985) reported that the presence of these antibodies was influenced by the age and pregnancy status of the animal at vaccination and the dose administered. Alton *et al*. (1980) found that approximately 0.5% of animals vaccinated at a young age developed persistent antibodies. However, Beckett and MacDiarmid (1985) demonstrated that cattle vaccinated as calves had lower titres than did those vaccinated as adults. As a consequence of these persistent antibodies, false positive reactions must be considered when interpreting the results of serological surveys. Therefore, Nicoletti (1990b) recommended a reduction in the number of bacteria in the vaccine in order to decrease these undesirable antibodies. Several authors have demonstrated that the antibody level decreases six months post-vaccination eventually reaching non-detectable levels. Consequently it is recommended that the time of vaccination be accounted for when considering serological results and that all vaccinated animals should be identified (Worthington *et al*, 1973; Nicoletti *et al*, 1978a).

The pathogenicity of S19 vaccine for humans is also a disadvantage of the vaccine as it can lead to necrosis and swelling of infected tissues. It also causes orchitis in male cattle during the first 10 days of vaccination and post vaccinal arthritis in calves. However the protective nature of S19 has been highlighted by many authors (Alton 1978; Erasmus 1995) if administered at 4 to 8 months of age.

In conclusion, although S19 has some limitations, the vaccine is ideal for the control of

bovine brucellosis and it is relative safe, easy to use and has high immunogenicity leading to stimulation of immunity in a range of animals.

#### 2.8.2.2.2 Strain RB51 vaccine

RB51 is an O-antigen deficient mutant of a virulent strain of *B. abortus* (S2308). The vaccine was proposed to overcome the disadvantages of S19 and has been officially used in several countries to prevent brucellosis in cattle. However there have been conflicting results about the vaccine's efficacy. Schuring *et al*. (1995) and Palmer *et al*. (1996a) demonstrated that this vaccine resulted in fewer abortions when compared with S19. The immunity induced by RB51 is mostly cellular, resulting in fewer false positive reactions with serological tests. Furthermore the lack of antibodies against O-antigen enables differentiating between naturally infected and vaccinated cases.

The route of administration and the dose used has varied from country to country. In the USA, the vaccine has been used mainly in calves at 4 to 12 months of age and injected subcutaneously with a dose of 1-3 x $10^{10}$ cfu. However, in other countries both calves and adult cattle have been vaccinated with two doses one year apart to boost the animal's immunity. Vaccination with RB51 can result in the organisms being detected in milk, and abortions have been reported in cattle and bison post-vaccination (Palmer *et al*, 1996b). As a result, vaccination of early pregnant cattle is not recommended, unless the dose is reduced. Although vaccination with reduced dose during late stage pregnancy has resulted in no side effects, the pathogen can still be shed by a significant proportion of the vaccinated animals.

Although studies on the effect of RB51 in humans are limited, it is likely that RB51, like

S19, can induce infection in humans. The diagnosis of humans infected with RB51 requires a specific test and it is not easy to treat as RB51 is resistant to rifampicin (Villarroel *et al*, 2000).

#### 2.8.2.2.3 *Brucella abortus* strain 45/20 vaccine

The inactivated vaccine 45/20 was initially made through dissociation of a rough strain after passaging strain 45 in guinea pigs. Although the vaccine has good immunogenicity, it is not widely used due to several drawbacks. Firstly, the vaccine organism can revert to a smooth form resulting in infections. Secondly, non-agglutinating antibodies play a role in blocking the antigen of smooth strains resulting in delayed clearance and increasing the likelihood of chronic cases developing. Finally, a lesion can develop at the injection site.

After the priming vaccination no serologically response is detectable, although low levels of antibodies are stimulated within 10 days of administering a second dose (Hall *et al*, 1976). The vaccine can be used at any age and during pregnancy (Alton, 1978).

### 2.8.2.3 Other preventive measures

Brucellosis is usually introduced to a herd through contact with infected animals and or semen of infected males. To prevent its introduction new animals should be purchased from *Brucella*-free herds and new animals should be isolated and screened before they are added to the herd. Semen also should be evaluated or collected from disease free bulls before it is used for artificial insemination. However managing the disease in endemic areas where animals co-graze can be difficult unless a vaccination program is also implemented.

Understanding the epidemiology of the disease in a country, in particular the distribution of the disease is critical prior to developing or assessing a disease control program. In the following chapter the results of a cross-sectional study are reported to further the knowledge on brucellosis in livestock in Oman.

# CHAPTER THREE

# SEROPREVALENCE OF BRUCELLOSIS: CROSS-SECTIONAL STUDY

## 3.1 Introduction

As outlined in Chapter 1 brucellosis was detected in small percentage of animals in 1986 but the seroprevalence subsequently increased (Ismaily *et al,* 1988; MAF 2003b) and evidence of infection in humans was evident (Nicoletti, 1986). However a thorough study on the seroprevalence in animals had not been undertaken in Oman. A cross-sectional serological study was undertaken in Oman to better understand the epidemiology of brucellosis in the country. The results of that study are reported in this chapter.

## 3.2 Materials and Methods

### 3.2.1 Samples

#### 3.2.1.1 Sampling plan for Seroprevalence:

A cross-sectional serological study was undertaken to determine the prevalence of brucellosis in cattle, sheep, goats and camels in the Sultanate. Past reports indicated the presence of brucellosis in the southern Dhofar governorate (prevalence approximately 6%), however the status of brucellosis in the northern areas was unknown.

The Sultanate was divided into two areas South (Dhofar with known expected prevalence of brucellosis) and North (Batinah, Al Buraimi, Al Dhahirah, Al Dakhiliyah, Al Sharqiyah, Al Wusta, Musandam and Muscat Governorates where the expected prevalence was unknown). A two stage sampling plan was adopted. The number of herds selected for

sampling from the north of Oman (387 herds) was based on an expected herd prevalence of 50% (95% desired absolute precision - DAP) and a 5% margin of error. Within these herds, animals were randomly selected on the basis of the minimum sample size to detect the disease at an expected prevalence of 50% with the probability of finding at least one infected animal at a 95% confidence limit. Since there were some previous studies available for the seroprevalence (less than 10%) of brucellosis in different animals species in Dhofar, 138 herds were randomly selected based on an expected herd prevalence of 10% (95% DAP) and 5% error. Animals from Dhofar were randomly selected by assuming the expected percentage of the diseased animals to be 10% at a 95% confidence limit and 5% margin of error (Thrusfield, 2005).

The number of samples thus calculated was subjected to the following formula for the estimation of required sample size ($n_{adj}$):

$$n_{adj} = (N \times n) \div (N + n)$$

Where:

N = total population

n = calculated sample size through formula

The sampling units were stratified and selected in proportion to the livestock population in the selected areas based upon the Agricultural Census in 2004/2005 (MAF, 2005). Random selection methods were used for the selection of areas to be sampled (random village selection through Survey Toolbox available at http://www.ausvet.com.au/content.php?page=software#st) within each Wilayat. The

selection of individual animals was also conducted through simple random sampling on-site by either tossing a dice to determine which animal to sample or by using random number allocation.

The minimum number of animal holdings required were 525 cattle, 529 sheep, 527 goat and 525 camel farms from all the governorates of Oman (Table 3.1 to Table 3.4). The number of individuals sampled at each holding varied according to the population of the species present at each holding. Number of herds to be sampled from a governorate was selected according to the population proportion of respective livestock species. No data were available regarding the animal population of two newly constituted wilayats [Al Sinainah (Al Buraimi Governorate) and Mazyounah (Dhofar Governorate)]. The sampling plan for these was made after acquiring animal population information from local animal health representatives and after interviewing livestock owners.

#### 3.2.1.2 Collection of samples and epidemiological information

Field sampling was carried out between July 2009 and April 2010 throughout the Sultanate. For the data collection and mapping ArcPad™ (ESRI, USA) on Juno™ SB Handheld computers (Trimble Navigation Limited, USA) were used (Figure 3.1). In case of the herds belonging from the Dhofar governorate, the possible vaccination status of the animal was determined (ear-tagged or not / history of vaccination of herd from local veterinary staff) and only non-vaccinated animals were selected for sampling.

Information regarding the husbandry (management and cohorts), animal characteristics (age, breed, gender and physical examination) and history (abortion, still birth at individual and herd level) was recorded on a predesigned proforma (Appendix 2). A herd was

categorized as positive for abortion history if any of the sampled animals or non-sampled cohorts had abortion history. Most of the sampled livestock was kept under the mixed management system where two or more species were kept together at same location with extensive or limited contact. Coding on the basis of presence of different cohorts was performed as single / alone (when only one species was found at a location), or multiple species contact (with camel, with cattle, with goats, with sheep or with other ruminants).

Samples were collected into labeled sterile vacutainers. To produce serum, blood was drawn into a 10mL clot activator vacutainer without anticoagulant. Samples were transported to the Veterinary Research Center (VRC) for further processing while observing the critical temperature requirements (4°C). Upon arrival each sample was given a unique identity (VRC Number) and serum was removed. Later these samples were stored at -20°C until further testing was performed.

**Table 3.1 Sampling plan to determine the seroprevalence of brucellosis in cattle in Oman**

| Governorate | Total Population (Heads) | % of total population | Herds selected for sampling | Adjusted No. of herds sampled |
|---|---|---|---|---|
| Muscat | 4447 | 3.48 | 13.38 | 14 |
| Batinah | 66411 | 52.02 | 199.75 | 200 |
| Musandam | 325 | 0.25 | 0.98 | 1 |
| Al Buraimi | 5971 | 4.68 | 17.96 | 18 |
| Al Dhahirah | 16582 | 12.99 | 49.88 | 50 |
| Al Dakhiliyah | 19245 | 15.07 | 57.89 | 58 |
| Al Sharqiyah | 14642 | 11.47 | 44.04 | 45 |
| Al Wusta | 43 | 0.03 | 0.13 | 1 |
| Total for North of Oman | 127666 | 42.33 | 384 @ 50% expected prevalence & 95% CI & 5% error | 387 |
| Total for South of Oman (Dhofar) | 173892 | 57.66 | 138 @ 10% expected prevalence & 95% CI, 5% error | 138 |
| Grand Total | 301558 | | | 525 |

**Table 3.2 Sampling plan to determine the seroprevalence of brucellosis in sheep in Oman**

| Governorate | Total Population (Heads) | % of total population | Herds selected for sampling | Adjusted number of herds sampled |
|---|---|---|---|---|
| Muscat | 13855 | 4.03 | 15.49 | 16 |
| Batinah | 110572 | 32.19 | 123.62 | 125 |
| Musandam | 5609 | 1.63 | 6.27 | 7 |
| Al Buraimi | 54005 | 15.72 | 60.38 | 61 |
| Al Dhahirah | 41042 | 11.95 | 45.89 | 47 |
| Al Dakhiliyah | 43499 | 12.66 | 48.63 | 50 |
| Al Sharqiyah | 60012 | 17.47 | 67.09 | 68 |
| Al Wusta | 14867 | 4.33 | 16.62 | 17 |
| Total for North of Oman | 343461 | 97.83 | 384 @ 50% expected prevalence & 95% CI & 5% error | 391 |
| Total for South of Oman (Dhofar) | 7605 | 2.17 | 138 @ 10% expected Prevalence & 95% CI, 5% error | 138 |
| Grand Total | 351066 | - | - | 529 |

**Table 3.3 Sampling plan for seroprevalence of brucellosis in goats of Oman**

| Governorate | Total Population (Heads) | % of total population | Herds selected for sampling | Adjusted number of herds sampled |
|---|---|---|---|---|
| Muscat | 47713 | 3.44 | 13.21 | 14 |
| Batinah | 430005 | 31 | 119.05 | 120 |
| Musandam | 67977 | 4.9 | 18.82 | 19 |
| Al Buraimi | 95202 | 6.86 | 26.36 | 27 |
| Al Dhahirah | 148394 | 10.69 | 41.08 | 42 |
| Al Dakhiliyah | 203057 | 14.64 | 56.22 | 57 |
| Al Sharqiyah | 322858 | 23.28 | 89.38 | 90 |
| Al Wusta | 71819 | 5.18 | 19.88 | 20 |
| Total for North of Oman | 1387025 | 89.07 | 384 @ 50% expected prevalence & 95% CI & 5% error | 389 |
| Total for South of Oman (Dhofar) | 170123 | 10.92 | 138 @ 10% expected Prevalence & 95% CI, 5% error | 138 |
| Total | 1557148 | - | - | 527 |

**Table 3.4 Sampling plan for seroprevalence of brucellosis in camels of Oman**

| Governorate | Total Population (Heads) | % of total population | Herds selected for sampling | Adjusted number of herds sampled |
|---|---|---|---|---|
| Muscat | 52 | 0.08 | 0.31 | 1 |
| Batinah | 5626 | 8.82 | 33.88 | 34 |
| Musandam | 38 | 0.06 | 0.23 | 1 |
| Al Buraimi | 6947 | 10.89 | 41.83 | 42 |
| Al Dhahirah | 8694 | 13.63 | 52.35 | 53 |
| Al Dakhiliyah | 6730 | 10.55 | 40.52 | 41 |
| Al Sharqiyah | 12779 | 20.04 | 76.95 | 77 |
| Al Wusta | 22906 | 35.92 | 137.96 | 138 |
| Total for North of Oman | 63772 | 54.37 | 384 @ 50% expected prevalence & 95% CI & 5% error | 387 |
| Total for South of Oman (Dhofar) | 53527 | 45.63 | 138 @ 10% expected Prevalence & 95% CI, 5% error | 138 |
| Total | 117299 | - | - | 525 |

**Figure 3.1 ArcPad (ESRI, USA) ArcPad™ (ESRI, USA) mounted Juno™ SB Handheld computer (Trimble Navigation Limited, USA) used for the field data collection and mapping**

### 3.2.1.3 Population dynamics of samples

In this study, to determine the seroprevalence of brucellosis in livestock from the Sultanate, 10697 serum samples were collected from all governorates of Oman. These samples were collected from 1479 geographically marked animal holdings (Figure 3.7) distributed throughout the Sultanate. Sera were collected from 2209 cattle, 2457 sheep, 3776 goats and 2255 camels. Although the aim of the study was to sample herds not previously vaccinated against brucellosis, a lack of cooperation by farmers in the brucellosis endemic region (Dhofar governorate) prevented reaching the desired number of unvaccinated herds to sample. Herds containing any vaccinated animals were excluded from the analyses. Consequently data from 1267 unvaccinated herds comprising 1704 cattle, 2215 sheep, 3308 goats and 2250 camels were used in the final analyses.

All samples were collected according to the proportion of livestock present in each governorate as shown in Figure 3.2 and the distribution of the livestock sampled is presented in Table 3.7. In total 442 (29.8% of those available) holdings were sampled in the Dhofar governorate. Animals sampled in these holdings included 703 cattle, 554 sheep, 1676 goats and 909 camels. The vaccination histories of the herds and animals were examined carefully and 232 (18.3% of unvaccinated) herds comprising 198 cattle, 312 sheep, 1208 goats and 904 camels were confirmed not to have been vaccinated against brucellosis. From the north of Oman, 274 herds (21.6%) were sampled from Batinah governorate (753 cattle, 659 sheep, 676 goats and 118 camels) followed by 179 (14.1%) from Al Sharqiyah governorate (172 cattle, 272 sheep, 406 goats and 247 camels), 172 (13.6%) from Al Wusta governorate (4 cattle, 98 sheep, 105 goats and 547 camels), 164 (12.9%) from Al Dakhiliyah governorate (260 cattle, 278 sheep, 367 goats and 138

camels), 101 (7.9%) holdings from Al Dhahirah governorate (152 cattle, 212 sheep, 232 goat and 135 camels), 90 (7.1%) holdings in Al Buraimi governorate (81 cattle, 267 sheep, 146 goats and 140 camels), 30 (2.4%) holdings from Muscat governorate (69 cattle, 82 sheep, 73 goats and 15 camels) and 25 (1.9%) holdings from the Musandam governorate (15 cattle, 35 sheep, 95 goats and 6 camels).

**Figure 3.2 Distribution of the total livestock population (%) in various wilayats (districts) of Oman (MAF, 2005).**

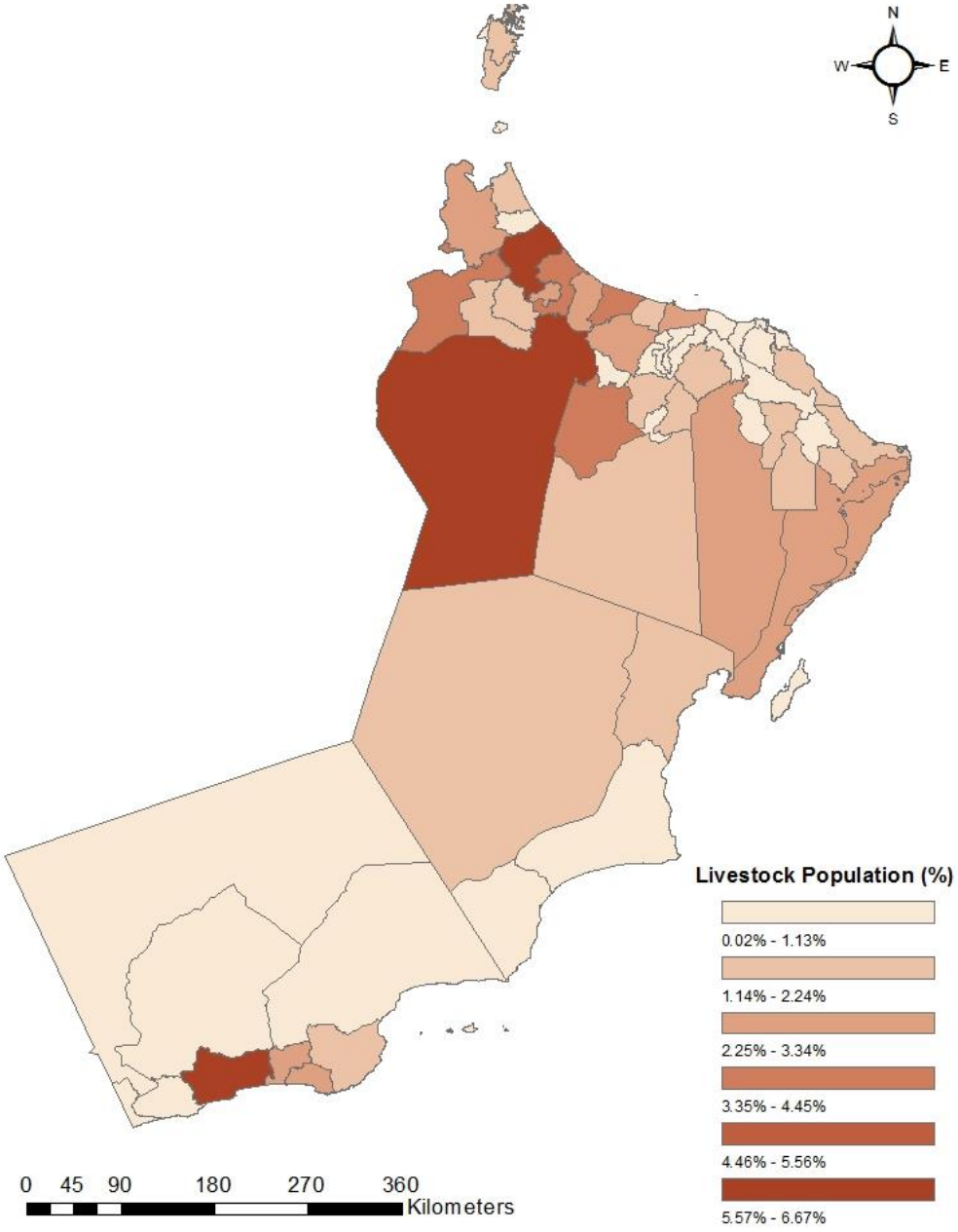


**Figure 3.3 Distribution of the cattle population (%) in various wilayats (districts) of Oman (MAF, 2005).**

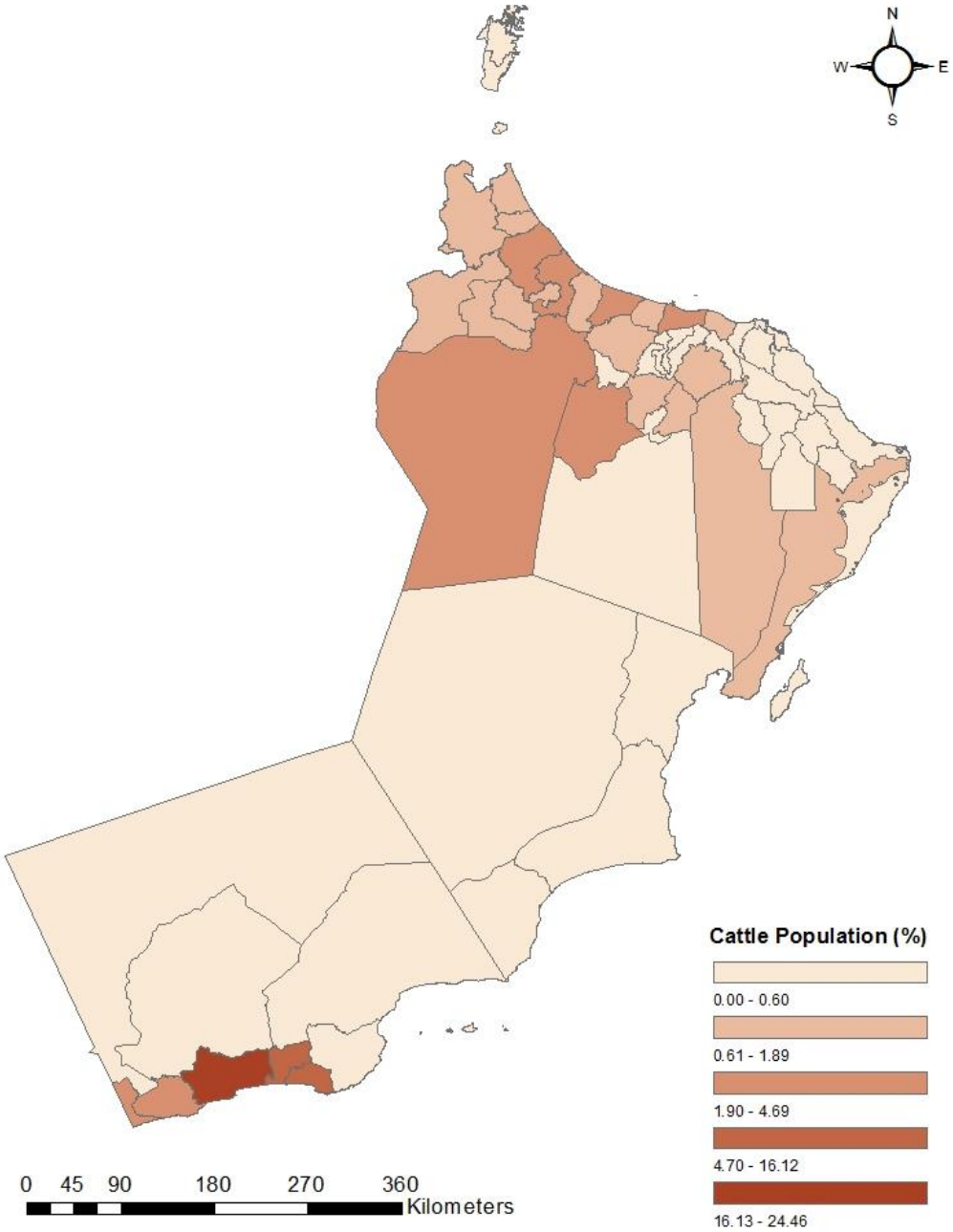


**Figure 3.4 Distribution of the sheep population (%) in various wilayats of Oman (MAF, 2005).Figure 3.5 Distribution of the goat population (%) in various wilayats of Oman (MAF, 2005).**

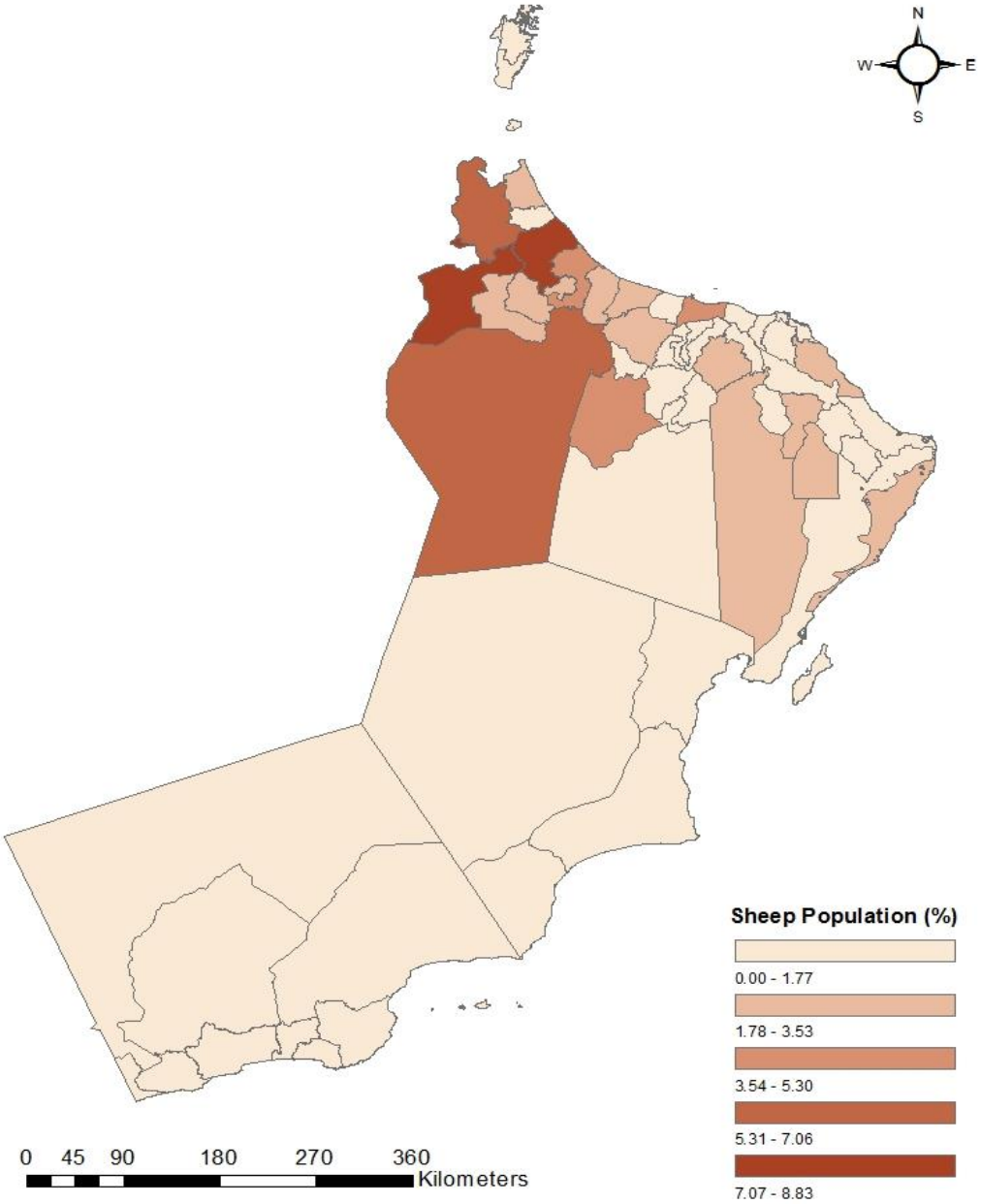


**Figure 3.5 Distribution of the goat population (%) in various wilayats of Oman (MAF, 2005).**

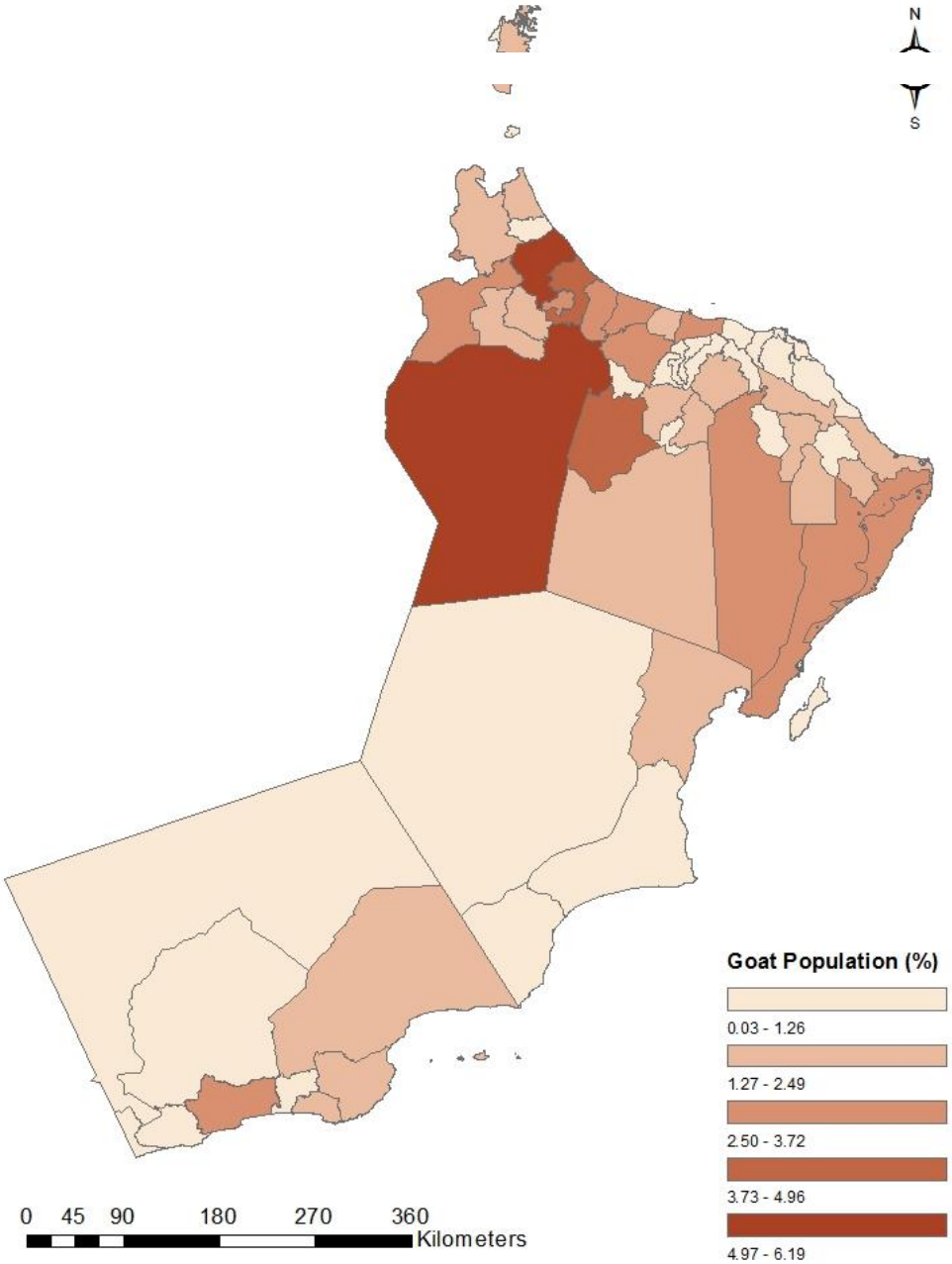


**Figure 3.6 Distribution of the camel population (%) in various wilayats of Oman (MAF, 2005).**

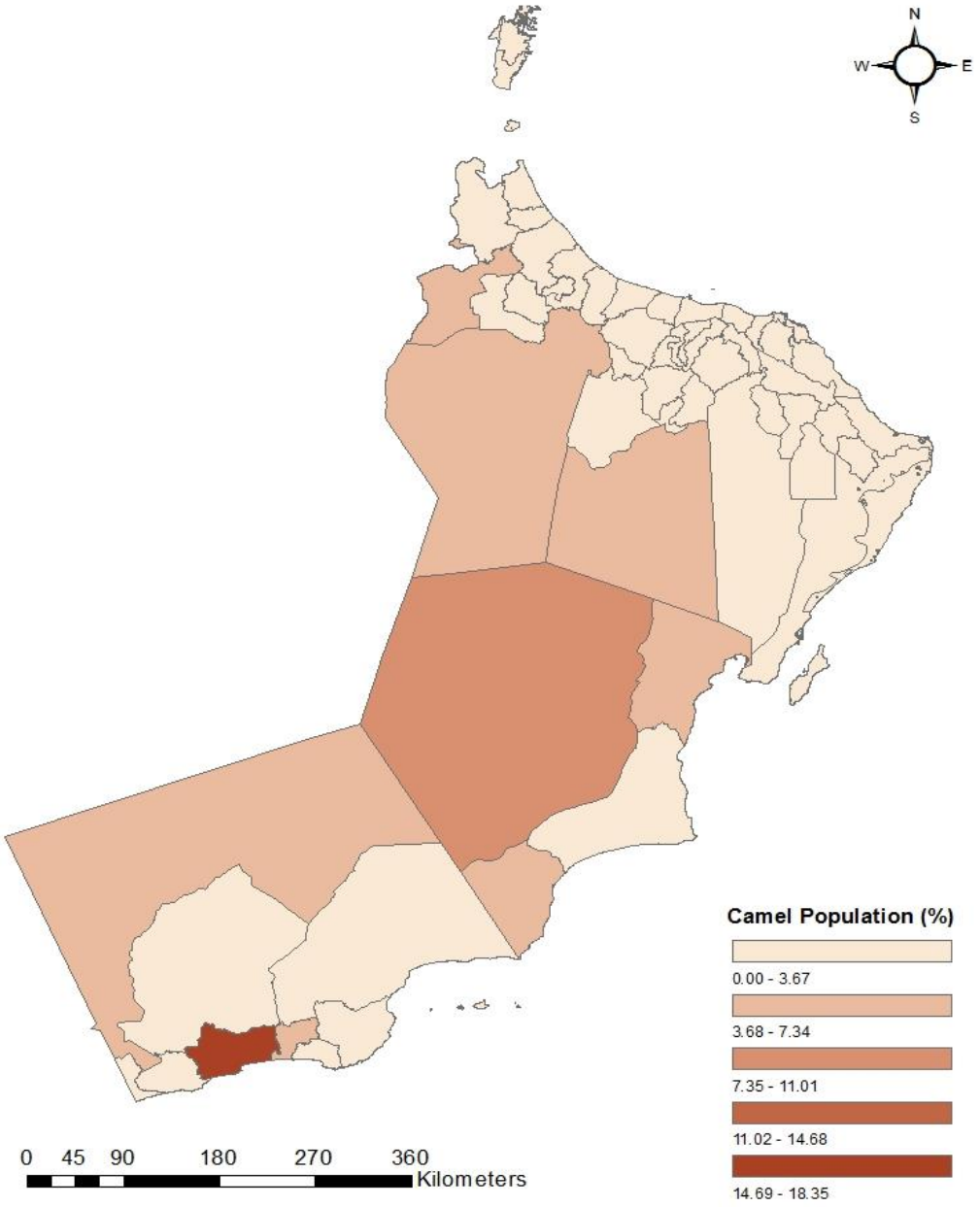


**Figure 3.7 Location of the livestock holdings sampled for mapping of brucellosis in the Sultanate**

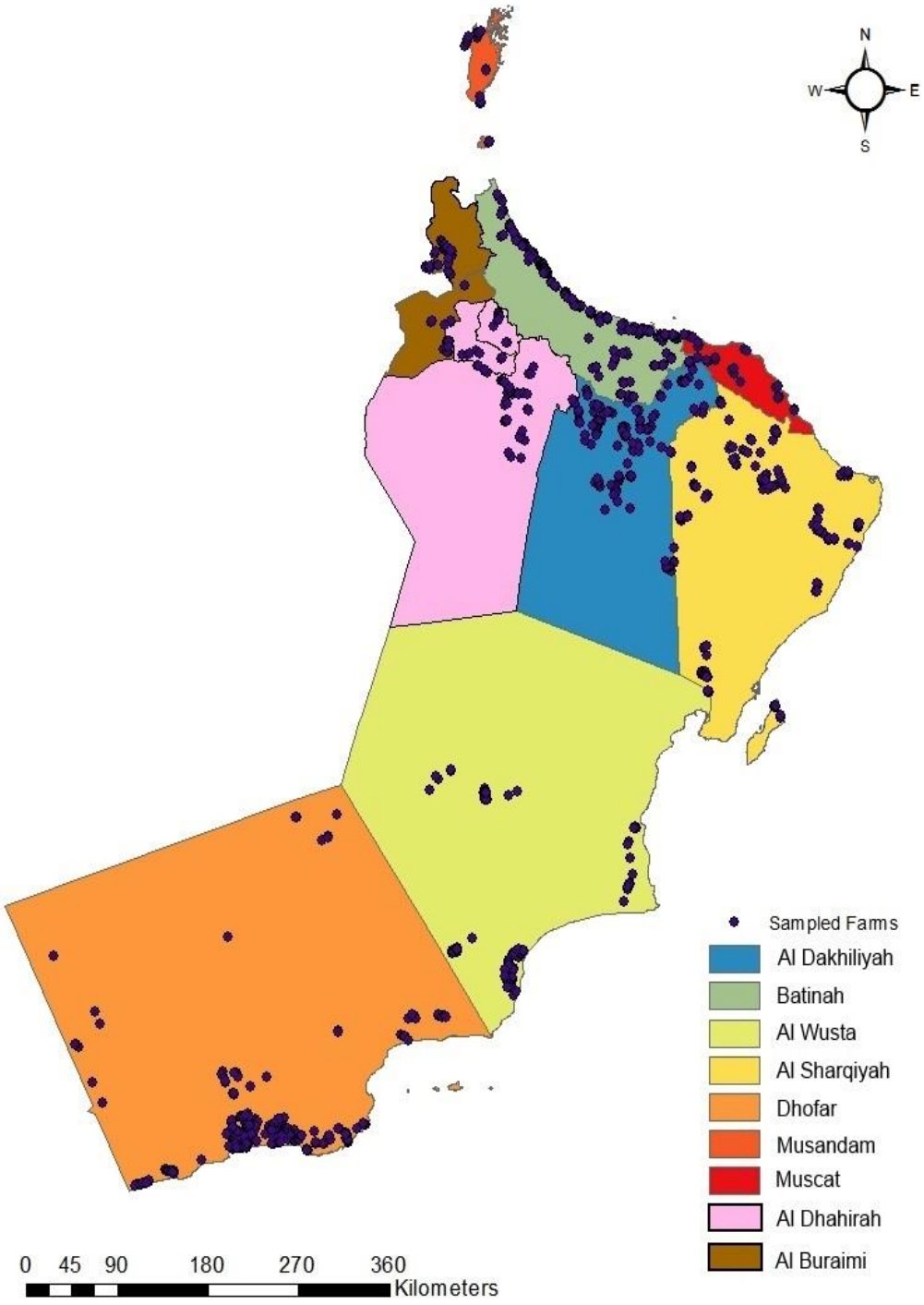


**Table 3.5 Distribution of samples collected for the study on the seroprevalence of brucellosis in Oman**

| Governorate | Cattle | | Sheep | | Goats | | Camels | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Holdings (% of total) | Individual No. (% of total) | Holdings (% of total) | Individual No. (% of total) | Holdings (% of total) | Individual No. (% of total) | Holdings (% of total) | Individual No. (% of total) | Holdings (% of total) | Individual No. (% of total) |
| Batinah | 209 (1.45) | 753 (1.13) | 170 (2.08) | 659 (0.59) | 165 (0.69) | 676 (0.99) | 40 (3.14) | 118 (2.09) | 274 (0.57) | 2206 (0.36) |
| Al Buraimi | 21 (2.56) | 81 (1.36) | 56 (4.7) | 267 (0.49) | 30 (1.85) | 146 (0.15) | 43 (11.05) | 140 (2.01) | 90 (2.24) | 634 (0.39) |
| Al Dakhiliyah | 79 (1.42) | 260 (1.35) | 71 (1.74) | 278 (0.64) | 85 (0.97) | 367 (0.18) | 41 (3.85) | 138 (2.05) | 164 (0.84) | 1043 (0.38) |
| Al Dhahirah | 52 (6.33) | 152 (0.92) | 50 (1.50) | 212 (0.52) | 53 (0.80) | 232 (0.16) | 40 (4.24) | 135 (1.55) | 101 (0.67) | 731 (0.34) |
| Dhofar | 41 (0.48) | 198 (0.11) | 35 (10.2) | 312 (4.10) | 74 (1.59) | 1208 (0.71) | 162 (2.66) | 904 (1.69) | 232 (1.19) | 2622 (0.65) |

| Governorate | Cattle | | Sheep | | Goats | | Camels | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Holdings (% of total)** | **Individual No. (% of total)** | **Holdings (% of total)** | **Individual No. (% of total)** | **Holdings (% of total)** | **Individual No. (% of total)** | **Holdings (% of total)** | **Individual No. (% of total)** | **Holdings (% of total)** | **Individual No. (% of total)** |
| Musandam | 5 (6.58) | 15 (4.62) | 8 (1.70) | 35 (0.62) | 21 (1.02) | 95 (0.14) | 2 (16.67) | 6 (15.79) | 25 (0.96) | 151 (0.20) |
| Muscat | 18 (1.68) | 69 (1.55) | 19 (1.12) | 82 (0.59) | 16 (0.47) | 73 (0.15) | 4 (19.05) | 15 (28.85) | 30 (0.48) | 239 (0.36) |
| Sharqiyah | 55 (0.87) | 172 (1.17) | 75 (0.93) | 272 (0.45) | 93 (0.55) | 406 (0.13) | 75 (2.09) | 247 (1.08) | 179 (0.51) | 1097 (0.27) |
| Al Wusta | 2 (10.00) | 4 (9.31) | 24 (2.31) | 98 (0.66) | 25 (1.22) | 105 (0.15) | 145 (9.22) | 547 (2.39) | 172 (3.68) | 754 (0.69) |
| Total | 482 (1.18) | 1704 (0.57) | 508 (1.79) | 2215 (0.63) | 562 (0.80) | 3308 (0.21) | 552 (3.69) | 2250 (1.92) | 1267 (0.82) | 9477 (0.41) |

### 3.2.1.4. Distribution of samples

#### 3.2.1.4.1 Age related distribution of samples

The sampled livestock were categorized into 4 age groups. For cattle and camels the groups were ≤ 2 years; > 2 to ≤ 5 years; >5 to ≤ 10 years; and > 10 years. For sheep and goats the animals were categorized as ≤ 1 year; > 1 to ≤ 3 years; > 3 to ≤ 5 years; and > 5 years. Data regarding this distribution are presented in Table 3.6 for cattle and camels, and in Table 3.7 for small ruminants (sheep and goats).

Of the 1704 samples from cattle, 28.9% (492) were ≤ 2 years, 41.5% (708) were > 2 to ≤ 5 years, 24.6% (419) were >5 to ≤ 10 years and 4.9% (85) were > 10 years. The age distribution of 2250 camels sampled was: 17.9% (402) ≤ 2 years of age, 22.5% (507) between the age of > 2 to ≤ 5 years, 40.5% (912) between > 5 to ≤ 10 years of age and 19.1% (429) above the age of 10 years.

**Table 3.6 Age related distribution of cattle and camels sampled to determine the seroprevalence of brucellosis**

| Species | Age Groups (years) | | | | Total |
|---|---|---|---|---|---|
| | ≤ 2 | > 2 to ≤ 5 | >5 to ≤ 10 | > 10 | |
| Cattle | 492[2] | 708[1] | 419[2] | 85[3] | 1,704 |
| Camel | 402[b] | 507[b] | 912[a] | 429[b] | 2,250 |

Values with different numerical superscripts are significantly different, $\chi^2$=70.9, 3df, p<0.001

Values with different alphabetic superscripts are significantly different, $\chi^2$=114.5, 3df, p<0.001

**Table 3.7 Age related distribution of sheep and goats sampled to determine the seroprevalence of brucellosis**

| Species | Age Groups (years) | | | | Total |
|---|---|---|---|---|---|
| | ≤ 1 | >1 to ≤ 3 | >3 to ≤ 5 | > 5 | |
| Sheep | 221[3] | 1320[1] | 469[2] | 205[3] | 2,215 |
| Goat | 199[d] | 1315[a] | 1127[b] | 667[c] | 3,308 |

Values with different numerical superscripts are significantly different, $\chi^2$=435.01, 3df, p<0.001

Values with different alphabet superscripts are significantly different, $\chi^2$=142.97, 3df, p<0.001

### 3.2.1.4.2 Sex related distribution of sampled animals

The number of males and females sampled for each of the different species is presented in Table 3.8. As the study was directed towards breeding herds, significantly more (p<0.05) females were sampled than males. When data for all species were combined, 83.8% of the samples were collected from females.

### 3.2.1.4.3 Breed related distribution in the sampled populations

Data regarding the distribution of breed in the different species is presented in

Table 3.9. Samples from local breeds of livestock constituted 79.5% (n=7538) of all samples, with 12.9% (1219) of samples coming from cross-bred animals and 7.6% (720) from imported animals. Most animals sampled were of a local breed with 63.4% (n=1081), 50.2% (n=1112), 94.3% (n=3119) and 98.9% (n=2226) of cattle, sheep, goats and camels, respectively, being local breeds.

A greater proportion of imported animals were sampled in sheep (16.2%) compared with 12.9, 3.7 and 0.8% for cattle, goats and camels, respectively. The highest proportion of crossbred animals (33.6%) was also in sheep followed by cattle (23.7%), goats (2.0%) and camels (0.3%). These differences were statistically significant (P<0.001).

**Table 3.8 Sex related distribution of samples collected to determine the seroprevalence of brucellosis in Oman**

| Animal Species | Male | | Female | | Total | P Value |
|---|---|---|---|---|---|---|
| | No. Tested | % of total species sampled | No. Tested | % of total species sampled | | |
| Camel | 252 | 11.2 | 1998 | 88.8 | 2250 | <0.001 |
| Cattle | 422 | 24.8 | 1282 | 75.2 | 1704 | <0.001 |
| Goat | 429 | 13.0 | 2879 | 87.0 | 3308 | <0.001 |
| Sheep | 431 | 19.5 | 1784 | 80.5 | 2215 | <0.001 |
| Total | 1534 | 16.2 | 7943 | 83.8 | 9477 | <0.001 |

**Table 3.9 The distribution of samples collected from different breeds**

| Animal Species | Breed | | | Total | P Value |
|---|---|---|---|---|---|
| | Local (%) | Imported (%) | Cross (%) | | |
| Camel | 2226 (98.9) | 18 (0.8) | 6 (0.3) | 2250 | p<0.001 |
| Cattle | 1081 (63.4) | 220 (12.9) | 403 (23.7) | 1704 | p<0.001 |
| Goat | 3119 (94.3) | 124 (3.7) | 65 (2.0) | 3308 | p<0.001 |
| Sheep | 1112 (50.2) | 358 (16.2) | 745 (33.6) | 2215 | p<0.001 |
| Total | 7538 (79.5) | 720 (7.6) | 1219 (12.9) | 9477 | p<0.001 |

## 3.3 Results

### 3.3.1 General Seroprevalence of Brucellosis in Oman

#### 3.3.1.1 Herd based seroprevalence of brucellosis

For the purpose of calculating herd based prevalence of brucellosis, only unvaccinated holdings were considered. This resulted in samples from only 1267 holdings being included in this aspect of the seroprevalence study. Samples were first screened with the RBPT test and then the cELISA was used to confirm seropositive animals. In total 30 (2.4%, 95% CI 1.6, 3.4) holdings contained one or more seropositive animals. The geographical distribution of the positive holdings is presented in Table 3.10. The highest

herd prevalence was in the Dhofar governorate (8.6%, 95% CI 5.3, 13). No seropositive animals were found in Al Buraimi, Al Wusta and Musandam governorates. The difference between the herd prevalence between localities was significant ($\chi^2$ (8df) =50.26, p<0.001) (Table 3.10). The overall herd seroprevalence in the northern governorate was 0.97% (95% CI 0.5, 1.8).

When the seroprevalence was calculated for individual species, 2.3% of goat holdings (95% CI 1.2, 3.9), 1.4% of camel holdings (95% CI 0.6, 2.8), 1.2% of cattle holdings (95% CI 0.5, 2.7), and 0.6% of sheep holdings (95% CI 0.1, 1.7) contained some seropositive animals. These differences between species were not significantly different ($\chi^2$ (3df) =5.78, p=0.122).

There was no significant difference in the herd prevalence for cattle between localities (p=0.319). The highest herd prevalence for cattle was observed in Muscat (5.6%) followed by Dhofar (4.9%).

Only three holdings (0.59%, 95% CI 0.1, 1.7) contained seropositive sheep, with one each in Batinah (0.6%, 95% CI 0.0, 3.2), Al Dakhiliyah (1.4%, 95% CI 0.0, 7.6) and Al Dhahirah Governorates (2.0%, 95% CI 0.1, 10.6). There was no significant difference in the herd prevalence for sheep between the sampled localities ($\chi^2$ (8df) =3.97, p=0.876).

There was a significant difference in the herd prevalence for goats between governorates ($\chi^2$ (1df) =24.19, p<0.001). Of 562 goat holdings, 13 (2.3%, 95% CI 1.2, 3.9) contained positive animals and these holdings were found only in Batinah (0.6%, 95% CI 0.0, 3.3) and Dhofar governorates (16.2%, 95% CI 8.7, 26.6).

The highest percentage of camel holdings containing some seropositive animals was in Dhofar governorate (3.7%, 95% CI 1.4, 7.9) followed by one herd in each of Al Sharqiyah (1.3%, 95% CI 0.0, 7.2) and Batinah governorates (2.5%, 95% CI 0.1, 13.2). These differences were, however, not significant ($\chi^2$ (2df) =1.05, p=0.59).

### 3.3.1.2 Individual animal seroprevalence

In total 33 (0.4%; 95% CI 0.2, 0.5) animals were seropositive to brucellosis (Table 3.11). When data for all species were combined the highest animal level prevalence was in the Dhofar governorate (0.9%, 95% CI 0.6, 1.3). In this governorate 23 animals belonging to 20 unvaccinated herds were seropositive. Other positive animals were detected in Muscat (0.4%, n=1), Al Dakhiliyah (0.19%, n=2), Batinah (0.18%, n=1), Al Sharqiyah (0.18%, n=2), and Al Dhahirah (0.14%, n=1). The animal level seroprevalence was significantly different between governorates ($\chi^2$ (5df) =20.69, p<0.001).

**Table 3.10 Herd level seroprevalence of brucellosis in the sampled holdings**

| Governorate | Cattle | | Sheep | | Goat | | Camel | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pos./Tested (Prev. %) | 95% CI | Pos./Tested (Prev. %) | 95% CI | Pos./Tested (Prev. %) | 95% CI | Pos./Tested (Prev. %) | 95% CI | Pos./Tested (Prev. %) | 95% CI |
| Batinah | 1/209 (0.48) | 0.0-2.6 | 1/170 (0.59) | 0-3.2 | 1/165 (0.61) | 0.0-3.3 | 1/40 (2.5) | 0.1-13.2 | 4/274 (1.46)c | 0.4-3.7 |
| Al Buraimi | 0/21 (0.0) | 0.0-16.1 | 0/56 (0.0) | 0-6.4 | 0/30 (0) | 0.0-11.6 | 0/43 (0) | 0.0-8.2 | 0/90 (0) | 0.0-4.0 |
| Al Dakhiliyah | 1/79 (1.27) | 0.0-6.9 | 1/71 (1.41) | 0-7.6 | 0/85 (0) | 0.0-4.2 | 0/41 (0) | 0.0-8.6 | 2/164 (1.22)c | 0.1-4.3 |
| Al Dhahirah | 0/52 (0.0) | 0.0-6.8 | 1/50 (2.0) | 0.1-10.6 | 0/53 (0) | 0.0-6.7 | 0/40 (0) | 0.0-8.8 | 1/101 (0.99)c | 0.0-5.4 |
| Dhofar | 2/41 (4.88) | 0.6-16.5 | 0/35(0) | 0-10 | 12/74 (16.2)* | 8.7-26.6 | 6/162 (3.7) | 1.4-7.9 | 20/232 (8.62)a | 5.3-13.0 |
| Musandam | 0/5 (0.0) | 0.0-52.2 | 0/8(0) | 0-36.9 | 0/21 (0) | 0.0-16.1 | 0/2 (0) | 0.0-84.2 | 0/25 (0) | 0.0-13.7 |
| Muscat | 1/18 (5.56) | 0.1-27.3 | 0/19(0) | 0-17.6 | 0/16 (0) | 0.0-20.6 | 0/4 (0) | 0.0-60.2 | 1/30 (3.3)a,c | 0.1-17.2 |
| Sharqiyah | 1/55 (1.82) | 0-9.7 | 0/75(0) | 0-4.8 | 0/93 (0) | 0.0-3.9 | 1/75(1.33) | 0.0-7.2 | 2/179 (1.12)c | 0.1-4.0 |
| Al Wusta | 0/2 (0.0) | 0-84.2 | 0/24(0) | 0-14.2 | 0/25 (0) | 0.0-13.7 | 0/145(0) | 0.0-2.5 | 0/172 (0) | 0.0-2.1 |
| Total | 6/482 (1.24) | 0.5-2.7 | 3/508(0.59) | 0.1-1.7 | 13/562(2.31) | 1.2-3.9 | 8/552(1.45) | 0.6-2.8 | 30/1267 (2.37) | 1.6-3.4 |

*Values with different superscripts are significantly different, $\chi^2$=73.05, 8df, p<0.001

Values with different alphabetic superscripts in the total column are significantly different, $\chi^2$=50.26 8df, p<0.001

In cattle, 0.4% (n=7, 95% CI 0.2, 0.8) were seropositive. Three of these originated from the Dhofar governorate (1.5%) and there was one seropositive cattle from Muscat (1.4%), Al Sharqiyah (0.6%), Al Dakhiliyah (0.4%) and Batinah (0.1%) governorates. These differences were not significantly different ($\chi^2$ (4df) =7.86, p=0.096) (Table ).

Only 3 sheep were seropositive (0.1%, 95% CI 0.0, 0.4) and one each came from Batinah, Al Dakhiliyah and Al Dhahirah governorates. These geographical differences in the seroprevalence for sheep were not significant ($\chi^2$ (2) =0.78, p=0.678).

In contrast the seroprevalence for goats varied significantly between governorates ($\chi^2$ (1df) =5.06, p=0.02). More goats were positive than any other species with 13 from the Dhofar governorate (1.08%, 95% CI 0.6, 1.8) and 1 from Batinah (0.1%, 95% CI 0.0, 0.8) being positive.

There were 9 seropositive camels (0.4%, 95% CI 0.2, 0.8). There was no significant difference in the seroprevalence in camels between Governorates ($\chi^2$ (2df) =0.42, p=0.811). The highest seroprevalence was observed in camels from Dhofar and Batinah governorates (0.8% each) followed by Al Sharqiyah (0.4%).

**Table 3.11 Individual animal seroprevalence to brucellosis in sampled livestock species**

| Governorate | Cattle | | Sheep | | Goat | | Camel | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pos./Tested (Prev%) | 95% CI | Pos./Tested (Prev%) | 95% CI | Pos./Tested * (Prev%) | 95% CI | Pos./Tested (Prev%) | 95% CI | Pos./Tested (Prev%) | 95% CI |
| **Batinah** | 1/753 (0.13) | 0.0-0.7 | 1/659 (0.15) | 0.0-0.8 | 1/676 (0.15) | 0.0-0.8 | 1/118 (0.85) | 0.0-4.6 | 4/2206 (0.18)[b] | 0.0-0.5 |
| **Al Buraimi** | 0/81 (0) | 0.0-4.5 | 0/267 (0) | 0.0-1.4 | 0/146 (0) | 0.0-2.5 | 0/140 (0) | 0.0-2.6 | 0/634 (0) | 0.0-0.6 |
| **Al Dakhiliyah** | 1/260 (0.38) | 0.0-2.1 | 1/278 (0.36) | 0.0-2.0 | 0/367 (0) | 0.0-1.0 | 0/138 (0) | 0.0-2.6 | 2/1043 (0.19)[b] | 0.0-0.7 |
| **Al Dhahirah** | 0/152 (0) | 0.0-2.4 | 1/212 (0.47) | 0.0-2.6 | 0/232 (0) | 0.0-1.6 | 0/135 (0) | 0.0-2.7 | 1/731 (0.14)[b] | 0.0-0.8 |
| **Dhofar** | 3/198 (1.52) | 0.3-4.4 | 0/312 (0) | 0.3-1.2 | 13/1208 (1.08) | 0.6-1.8 | 7/904 (0.77) | 0.3-1.6 | 23/2622 (0.88)[a] | 0.6-1.3 |
| **Musandam** | 0/15 (0) | 0.0-21.8 | 0/35 (0) | 0.0-10.0 | 0/95 (0) | 0.0-3.8 | 0/6 (0) | 0.0-45.9 | 0/151 (0) | 0.0-2.4 |
| **Muscat** | 1/69 (1.45) | 0.0-7.8 | 0/82 (0) | 0.0-4.4 | 0/73 (0) | 0.0-4.9 | 0/15 (0) | 0.0-21.8 | 1/239 (0.42)[b] | 0.0-2.3 |
| **Sharqiyah** | 1/172 (0.58) | 0.0-3.2 | 0/272 (0) | 0.0-1.3 | 0/406 (0) | 0.0-0.9 | 1/247 (0.4) | 0.0-2.2 | 2/1097 (0.18)[b] | 0.0-0.7 |
| **Al Wusta** | 0/2 (0) | 0.0-60.2 | 0/98 (0) | 0.0-3.7 | 0/105 (0) | 0.0-3.5 | 0/547 (0) | 0.0-0.7 | 0/754 (0) | 0.0-0.5 |
| **Total** | 7/1704 (0.41) | 0.2-0.8 | 3/2215 (0.14) | 0.0-0.4 | 14/3308 (0.42) | 0.2-0.7 | 9/2250 (0.4) | 0.2-0.8 | 33/9477 (0.35) | 0.2-0.5 |

*Values in the column are significantly different, $\chi^2$=19.53, 8df, p=0.01

Values with different alphabetic superscripts in a column are significantly different, $\chi^2$=31.11 8df, p<0.001

### 3.3.2 Specific seroprevalence for brucellosis

#### 3.3.2.1 Sex specific seroprevalence for brucellosis

Data regarding the seroprevalence in the different sex categories are summarised in Table 3.12. There was no significant difference in the seroprevalence between the genders when all species data were combined ($\chi^2$ (1df) =1.19, p=0.275).

A comparison between the sexes was only possible for cattle where 3 of 7 positive animals were males (0.7%; 95% CI 0.1, 2.1) and 4 (0.03%; 95% CI 0.1, 0.8) were females. These differences were not significant ($\chi^2$ (1df) =1.09, p=0.297) (Table 3.12). All of the seropositive sheep, goats and camels were female.

#### 3.3.2.2 Age related prevalence of brucellosis

There was no difference in the seroprevalence of brucellosis in different age groups of cattle ($\chi^2$ (3df) = 2.282, p=0.516) and camels ($\chi^2$=0.18, 2df, p=0.915) (Table 3.13).The highest seroprevalence in cattle was observed in the >10 year old animals (1.18%, 95% CI 0.0, 6.4) followed by 0.61% (95% CI 0.1, 1.8) in cattle up to the age of 2 years, 0.28% (95% CI 0.0, 1.0) in cattle from 2 to 5 years of age and 0.24% (95% CI 0.0, 1.3) in animals 5 to 10 years of age.

The highest seroprevalence observed in different age groups of camels was as follows: 0.5% in the > 10 years old camels (95% CI 0.2, 1.3), 0.5% in the 5.1 to 10 years old camels (95% CI 0.1, 1.7) and 0.4% in the 2.1 to 5 years old camels (95% CI 0.0, 1.4). No camels belonging to the ≤ 2 years age group (95% CI 0.0, 0.9) were seropositive for brucellosis. The differences observed for the prevalence among various age groups were not significant ($\chi^2$ (2df) =0.18, p=0.915).

No valid comparison was possible regarding the observed prevalence of brucellosis in different age groups for sheep as all positive animals (n=3, 0.2%) belonged to the sheep between > 1 to ≤ 3 years of age (Table 3.14). In goats the highest seroprevalence was observed in the <5 years of age (0.6%, 95% CI 0.2, 1.5), however there were no significant differences between age groups for goats ($\chi^2$ (2df) =0.48, p=0.785).

### 3.3.2.3 Influence of breed on prevalence of brucellosis

When data for all species were combined the highest seroprevalence was observed in imported breeds, where 6 (0.83%, 95% CI 0.3, 1.8) animals were found to be seropositive. This seroprevalence was higher than that for local (0.33%, 95% CI 0.2, 0.5) and cross bred animals (0.16%, 95% CI 0.0, 0.6) ($\chi^2$ (2df) =6.17, p=0.045).

Similarly for cattle, the seroprevalence in imported breeds (1.82%, 95% CI 0.5, 4.6) was significantly higher than that of crossbreds (0.5%, 95% CI 0.1, 1.8) and local cattle (0.09%, 95% CI 0.0, 0.5) ($\chi^2$ (2df) =13.49, p=0.001). Although the seroprevalence in imported breeds (0.56%, 95% CI 0.1, 2.0) of sheep was higher than for local breeds (0.09%, 0.0, 1.5), this difference was not significant ($\chi^2$ (1df) =2.93, p=0.086). All seropositive goats (0.45%, 95% CI 0.2, 0.8) and camels (0.4%, 95% CI 0.2, 0.8) were of local breeds (Table 3.15).

**Table 3.12 Sex related seroprevalence of brucellosis in the sampled animals**

| Animal Species | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Tested | Positive | Prevalence (95% CI) | Tested | Positive | Prevalence (95% CI) |
| Camel | 252 | 0 | 0 (0.0, 1.5) | 1998 | 9 | 0.45 (0.2, 0.9) |
| Cattle | 422 | 3 | 0.68 (0.1, 0.1) | 1282 | 4 | 0.31 (0.1, 0.8) |
| Goat | 429 | 0 | 0 (0.0, 0.9) | 2879 | 14 | 0.49 (0.3, 0.8) |
| Sheep | 431 | 0 | 0 (0.0, 0.9) | 1784 | 3 | 0.17 (0.0, 0.5) |
| Total | 1534 | 3 | $0.2^{1}$ (0.0, 0.6) | 7943 | 30 | $0.38^{1}$(0.3, 0.5) |

Values with similar superscripts are not significantly different, $\chi^2$ (1df) =1.19, p=0.275

**Table 3.13 Age related seroprevalence of brucellosis in sampled cattle and camels**

| Age Groups (years) | Camel | | Cattle | |
|---|---|---|---|---|
| | Positive / Tested | Prevalence 95%CI | Positive / Tested | Prevalence 95%CI |
| ≤2 | 0/402 | 0 (0.0, 0.9) | 3/492 | $0.61^{a}$ (0.1, 1.8) |
| 2.1 to ≤5 | 2/507 | $0.39^{1}$ (0.0, 1.4) | 2/708 | $0.28^{a}$ (0.0, 1.0) |
| 5.1 to ≤10 | 5/912 | $0.55^{1}$ (0.2, 1.3) | 1/419 | $0.24^{a}$ (0.0, 1.3) |
| >10 | 2/429 | $0.47^{1}$ (0.1, 1.7) | 1/85 | $1.18^{a}$ (0.0, 6.4) |

Values with the same alphabetic superscripts are not significantly different, $\chi^2$ (3df) =2.282, p=0.516

Values with the same numerical superscripts are not significantly different, $\chi^2$ (2df) =0.18, p=0.915

**Table 3.14 Age related seroprevalence of brucellosis in sampled sheep and goats**

| Age Groups (years) | Goat | | Sheep | |
|---|---|---|---|---|
| | Positive / Tested | Prevalence 95%CI* | Positive / Tested | Prevalence 95%CI |
| ≤ 1 year | 0/199 | 0 (0.0, 1.8) | 0/221 | 0 (0.0, 1.7) |
| >1 to ≤ 3 | 5/1315 | 0.38 (0.1, 0.9) | 3/1320 | 0.23 (0.0, 0.7) |
| >3 to ≤ 5 | 5/1127 | 0.44 (0.1, 1.0) | 0/469 | 0 (0.0, 0.8) |
| > 5 | 4/667 | 0.6 (0.2, 1.5) | 0/205 | 0 (0.0, 1.8) |

*Not significantly different, $\chi^2$ (2df) =0.48, p=0.78

**Table 3.15 Breed related seroprevalence of brucellosis in sampled animal species**

| Species | Local | | Imported | | Crossbred animals | |
|---|---|---|---|---|---|---|
| | Positive / Tested | Prev. (%) 95 % CI | Positive / Tested | Prev. (%) 95 % CI | Positive / Tested | Prev. (%) 95 % CI |
| Camel | 9/2226 | 0.4 (0.2, 0.8) | 0/18 | 0 (0.0, 18.5) | 0/6 | 0 (0.0, 45.9) |
| Cattle | 1/1081 | 0.09 (0.0, 0.5) | 4/220 | $1.82^{1}$ (0.5, 4.6 ) | 2/403 | $0.5^{2}$ (0.1, 1.8) |
| Goat | 14/3119 | 0.45 (0.2, 0.8) | 0/124 | 0 (0.0, 2.9) | 0/65 | 0 (0.0, 5.5) |
| Sheep | 1/1112 | 0.09 (0.0, 0.5) | 2/358 | 0.56 (0.1, 2.0 ) | 0/745 | 0 (0.0, 0.5) |
| Total | 25/7538 | 0.33 (0.2, 0.5) | 6/720 | $0.83^{a}$ (0.3, 1.8 ) | 2/1219 | $0.16^{b}$ (0.0, 0.6) |

Values with different alphabetic superscripts are significantly different, $\chi^2$ (2df) =6.17, p=0.045

Values with different numerical superscripts are significantly different, $\chi^2$ (2df) =13.49, p=0.001

### 3.3.3 Within-herd seroprevalence of brucellosis in affected herds

Within-herd seroprevalence of all positive cattle, sheep, goat and camel holdings, along with their geographical locations, are presented in

Table 3.15 to 3.19 and Figures 3.8 to 3.11.

In 6 herds where at least one head of cattle was found seropositive for brucellosis, the within-herd prevalence varied from 1.4 to 100% (

Table 3.16). The herds containing a seropositive animal in Al Dakhiliyah (50%), Al Sharqiyah (100%) and Muscat (25%) governorates were all small (comprising only 1 to 4 cattle) and all seropositive animals were imported and consequently the vaccination status of the animals could not be confirmed. These animals were purchased from local markets for sacrificial purposes, although the duration they had been on the farms was not ascertained. The seropositive herds in Batinah and Dhofar governorates contained a sufficient number of cattle and the within-herd seroprevalence varied from 1.4 to 4.4%. The differences in the within-herd prevalence were not significant between localities ($\chi^2$ =1.24, 2df, p=0.536).

In sheep herds containing seropositive animals the prevalence varied from 5 to 20% (

Table 3.17), however these differences were not significant ($\chi^2$=1.61 2df, p=0.447). The seroprevalence varied from 0.7 to 16.7% in positive goat herds. These positive herds were found only in 7 wilayats of Batinah and Dhofar governorates (Table 3.18). There was also no significant difference in the seroprevalence between infected goat herds ($\chi^2$=17.42, 12df, p=0.134). The within-herd seroprevalence in the 8 infected camel herds varied from 1.7 to 20%. The within herd seroprevalence for camels was also not significantly different between infected herds ($\chi^2$ =7.42, 7df, p=0.386) (Table 3.19).

### 3.3.4 Prevalence of brucellosis in different wilayats

The seroprevalence of brucellosis in cattle, sheep, goat and camel herds located in different wilayats in Oman is presented in Table 3.20 to 3.23. Maps based upon this distribution were built to identify the wilayats of higher risk in Oman (Figures 3.8 to 3.12). In total herds from 15 wilayats (24.6%) were found to be seropositive for brucellosis.

Positive cattle herds were located in the wilayats of Barka (Batinah), Sumail (Al Dakhiliyah), Dima wa Taaiyin (Al Sharqiyah) and A'seeb (Muscat) of northern Oman (Table 3.20 & Figure 3.8). From the southern Dhofar governorate an infected herd was found in both Salalah and Taqah. The herd prevalence varied from 3.7 to 16.7%, however these differences were not significant ($\chi^2$=2.45, 5df, p=0.783). The within-herd prevalence in these wilayats varied from 0.99 to 4.35% and these were also not significantly different ($\chi^2$ =3.37, 5df, p=0.642).

Positive sheep holdings were found only in wilayats located in the northern governorates i.e. Saham (Batinah), Sumail (Al Dakhiliyah) and Yunqul (Al Dhahirah). The herd level prevalence varied from 4.76 to 10% ($\chi^2$=0.33, 2df, p=0.846). There was no significant difference in the within herd seroprevalence between the wilayats (range 1.1 to 2.4%) ($\chi^2$=0.37, 2df, p=0.832) (Table 3.21 and Figure 3.9).

Only one goat farm was found positive in Batinah governorate (wilayat Saham) from the north of Oman while 12 herds from 7 wilayats (Al Mizyounah, Muqshin, Sadah, Salalah, Shaleem, Taqah and Thumrayt) in the southern Dhofar governorate were positive (Table 3.22 and Figure 3.10). The herd prevalence varied from 4.76 to 44.4% in positive goat herds, although these were not significantly different ($\chi^2$=10.48, 7df, p=0.162). The within herd prevalence varied from 0.4 to 16.7% and these differences

were also not significant ($\chi^2$=5.48, 7df, p=0.601).

From the north of Oman, two camel herds were positive in the wilayats of Shinas (Batinah) and Ja'alan Bani Bu Hassan (Al Sharqiyah). In Dhofar, 6 camel herds were positive for brucellosis in three wilayats (Al Mizyounah, Salalah and Taqah). The herd level prevalence was similar between wilayats ($\chi^2$=11.51, 4df, p=0.021) and ranged from 6.4 to 100%. The individual animal prevalence was significantly different between wilayats ($\chi^2$=16.59, 4df, p=0.002) varying from 1.4 to 33.3% (Table 3.23 and Figure 3.11).

Overall the herd level prevalence varied from 2.6 to 14.3% in the 15 wilayats of Oman where seropositive animals were detected ($\chi^2$=7.49, 14df, p=0.914) (Table 3.24 and Figure 3.12). The highest herd level prevalence was observed in the wilayat of Mazyounah where three (14.3%) herds contained at least one seropositive animal. Other wilayats with seropositive animals included Salalah (n=9, 12.9%), Sumail (n=2, 11.8%), Seeb (n=1, 10%), Taqah (n=3, 10%), Sadah (n=1, 9.1%), Shinas (n=1, 8.3%), Dima wa Altaaiyin (n=1, 7.7%), Shaleem (n=2, 7.4%), Saham (n=2, 6.3%), Yunqal (n=1, 6.3%), Muqshin (n=1, 4.2%), Thumrayt (n=1, 3.8%), Ja'alan bani bu Hasan (n=1, 3.7%) and Barka (n=1, 2.6%).

The individual level prevalence in seropositive herds (n=30) ranged from 0.23% in Thumrayt (n=1) to 2.39% in Salalah (n=12). The individual prevalence observed in decreasing order in other wilayats was 1.96% in Sumail, 1.45% in Seeb and Dima wa Taaiyin, 1.40% in Mazyounah, 1.22% in Taqah, 0.82% in Shinas, 0.76% in Yunqal, 0.74% in Ja'alan bani bu Hasan, 0.68% in Saham, 0.56% in Sadah, 0.49% in Muqshin, and 0.38% in Barka and Shaleem. However, these differences were not significant ($\chi^2$=19.43, 14df, p=0.1491).

**Table 3.16 The within-herd seroprevalence of brucellosis in infected cattle holdings**

| Governorate | Wilayat | Town / Village | No. positive animals (No. tested) | Prevalence % (95% CI) |
|---|---|---|---|---|
| Batinah | Barka | Al Naa'man | 1 (70) | 1.4 (0.0-7.7) |
| Al Dakhiliyah | Sumail | Al Hayl | 1 (2)* | 50.0 (1.3-98.7) |
| Al Sharqiyah | Dima wa Taaiyin | Miss | 1 (2)* | 50.0 (1.3-98.7) |
| Muscat | A'Seeb | Mabellah North | 1 (4)* | 25.0 (0.6-80.6) |
| Dhofar | Salalah | Zaik | 2 (45) | 4.4 (0.5-15.1) |
| Dhofar | Taqah | Damr | 1 (60) | 1.7 (0.0-8.9) |
| Total | 6 Wilayats | 6 Towns / Villages | 7 (183) | 3.8 (1.6-7.7) |

*Positive cattle were males of imported breeds and were bought from local markets for sacrificial purposes. The vaccination status against brucellosis in these animals could not be ascertained.

**Table 3.17 Within-herd seroprevalence of brucellosis in positive sheep holdings**

| Governorate | Wilayat | Town/Village | No. positive animals (No. tested) | Prevalence % (95% CI)* |
|---|---|---|---|---|
| Al Batinah | Saham | Al Mantaifa | 1 (20) | 5.0 (0.1-24.9) |
| Al Dakhiliyah | Al Somrah | Sumrah | 1 (5) | 20.0 (0.5-71.6) |
| Al Dhahirah | Yunqal | Alwuqba | 1 (20) | 5.0 (0.1-24.9) |
| Total | 3 Wilayats | 3 Towns / Villages | 3 (45) | 6.7 (1.4-18.3) |

* Not significantly different, $\chi^2$ (2df) =1.61, p=0.4477

**Table 3.18 Within-herd seroprevalence of brucellosis in positive goat holdings**

| Governorate | Wilayat | Town / Village | No. positive animals (No. tested) | Prevalence % (95% CI)* |
|---|---|---|---|---|
| Batinah | Saham | Mjez Sughra | 1 (33) | 3.0 (0.1-15.8) |
| Dhofar | Muqshin | City Center | 1 (25) | 4.0 (0.1-20.4) |
| Dhofar | Mazyounah | Mitan | 1 (40) | 2.5 (0.1-13.2) |
| Dhofar | Mazyounah | Mitan | 1 (30) | 3.3 (0.1-17.2) |
| Dhofar | Sadah | Soob | 1 (150) | 0.7 (0.0-3.7) |
| Dhofar | Shaleem | Shaleem | 1 (70) | 1.4 (0.0-7.7) |
| Dhofar | Shaleem | Showmiyah | 1 (100) | 1.0 (0.0-5.4) |
| Dhofar | Thumrayt | Rawiyah | 1 (50) | 2.0 (0.1-10.6) |
| Dhofar | Salalah | Alsan | 1 (30) | 3.3 (0.1-17.2) |
| Dhofar | Salalah | Alsan | 1 (12) | 8.3 (0.2-38.5) |
| Dhofar | Salalah | Salalah | 1 (50) | 2.0 (0.1-10.6) |
| Dhofar | Salalah | Salalah | 2 (12) | 16.7 (2.1-48.4) |
| Dhofar | Taqah | Jibjat | 1 (20) | 5.0 (0.1-24.9) |
| Total | 8 Wilayats | 10 Town / Villages | 14 (622) | 2.3 (1.2-3.7) |

* Not significantly different, $\chi^2$ (12df) =17.42, p=0.1343

**Table 3.19 Within-herd seroprevalence of brucellosis in positive camel holdings**

| Governorate | Wilayat | Town / Village | No. Positive animals (No. Tested) | Prevalence % (95% CI)* |
|---|---|---|---|---|
| Batinah | Shinas | Alfarfarah | 1 (6) | 16.7 (0.4-64.1) |
| Al Sharqiyah | Ja'alan Bani Bu Hassan | Falaj Al Mashaikh | 1 (5) | 20.0 (0.5-71.4) |
| Dhofar | Mazyounah | Mizyounah | 1 (20) | 5.0 (0.1-24.9) |
| Dhofar | Salalah | Ghado | 2 (40) | 5.0 (0.6-16.9) |
| Dhofar | Salalah | Hajeef | 1 (60) | 1.7 (0.0-8.9) |
| Dhofar | Salalah | Hajeef | 1 (50) | 2.0 (0.1-10.6) |
| Dhofar | Salalah | Salalah | 1 (25) | 4.0 (0.1-20.4) |
| Dhofar | Taqah | Jibjat | 1 (20) | 5.0 (0.1-24.9) |
| Total | 5 Wilayats | 7 Town / Village | 9 (226) | 3.9 (1.8-7.4) |

* Not significantly different, $\chi^2$ (7df) =7.42, p=0. 3861

**Table 3.20 Herd based and individual seroprevalence of brucellosis in cattle originating from different wilayats of Oman**

| Governorate | Wilayat | Cattle Herds | | Individual Cattle | |
|---|---|---|---|---|---|
| | | Positive (Tested) | Prevalence % (95% CI)# | Positive (Tested) | Prevalence % (95% CI)* |
| Batinah | Barka | 1 (27) | 3.7 (0.1-19.0) | 1 (101) | 0.99 (0.0-5.4) |
| Al Dakhiliyah | Sumail | 1 (6) | 16.7 (0.4-64.1) | 1 (15) | 6.67 (0.2-31.9) |
| Al Sharqiyah | Dima wa Taaiyin | 1 (6) | 16.7 (0.4-64.1) | 1 (23) | 4.35 (0.1-21.9) |
| Muscat | A'Seeb | 1 (8) | 12.5 (0.3-52.7) | 1 (33) | 3.03 (0.1-15.8) |
| Dhofar | Salalah | 1 (12) | 8.3 (0.2-38.5) | 2 (72) | 2.78 (0.3-9.7) |
| Dhofar | Taqah | 1 (20) | 5.0 (0.1-24.9) | 1 (88) | 1.14 (0.0-6.2) |

* Not significantly different, $\chi^2$ (5df) =2.45, p=0.7833

# Not significantly different, $\chi^2$ (5df) =3.37, p=0.0.6424

**Table 3.21 Herd based and individual seroprevalence of brucellosis in sheep originating from different wilayats of Oman**

| Governorate | Wilayat | Sheep Herds | | Individual Sheep | |
|---|---|---|---|---|---|
| | | Positive (Tested) | Prevalence % (95% CI)* | Positive (Tested) | Prevalence % (95% CI)# |
| Batinah | Saham | 1 (21) | 4.76 (0.1-23.8) | 1 (88) | 1.14 (0.0-6.2) |
| Al Dakhiliyah | Sumail | 1 (12) | 8.33 (0.2-38.5) | 1 (41) | 2.44 (0.1-12.9) |
| Al Dhahirah | Yunqul | 1 (10) | 10.0 (0.3-44.5) | 1 (45) | 2.22 (0.1-11.8) |

# Not significantly different, $\chi^2$ (2df) =0.33, p=0.8463

* Not significantly different, $\chi^2$ (2df) =0.37, p=0.8326

**Table 3.22 Herd based and individual seroprevalence of brucellosis in goats originating from different wilayats of Oman**

| Govt. | Wilayat | Goat Herds | | Individual Goats | |
|---|---|---|---|---|---|
| | | **Positive (Tested)** | **Prevalence % [a] (95% CI)** | **Positive (Tested)** | **Prevalence % [b] (95% CI)** |
| Batinah | Saham | 1 (21) | 4.76 (0.1-23.8) | 1 (88) | 1.14 (0.0-6.2) |
| Dhofar | Muqshin | 1 (3) | 33.3 (0.8-90.6) | 1 (161) | 0.62 (0.0-3.4) |
| Dhofar | Mazyounah | 2 (9) | 22.2 (2.8-60.0) | 2 (124) | 1.61 (0.2-5.7) |
| Dhofar | Sadah | 1 (10) | 10.0 (0.3-44.5) | 1 (242) | 0.41 (0.0-2.3) |
| Dhofar | Shaleem & The Hallanitat Isl. | 2 (19) | 10.5 (1.3-33.10 | 2 (359) | 0.56 (0.1-2.0) |
| Dhofar | Thumrayt | 1 (13) | 7.6 (0.2-36.0) | 1 (267) | 0.37 (0.0-2.1) |
| Dhofar | Salalah | 4 (9) | 44.4 (13.7-78.8) | 5 (283) | 1.77 (0.6-4.1) |
| Dhofar | Taqah | 1 (4) | 25.0 (0.6-80.6) | 1 (91) | 1.1 (0.0-6.0) |

[a] not significantly different, $\chi^2$ (7df) =10.48, p=0.163

[b] not significantly different, $\chi^2$ (7df) =5.48, p=0.602

**Table 3.23 Herd based and individual seroprevalence of brucellosis in camels originating from different wilayats of Oman**

| Govt. | Wilayat | Camel Herds | | Individual Camels | |
|---|---|---|---|---|---|
| | | Positive (Tested) | Prevalence % (95% CI)* | Positive (Tested) | Prevalence % (95% CI)# |
| Batinah | Shinas | 1 (1) | 100 (2.5-100.0) | 1 (3) | 33.3 (0.8-90.6) |
| Al Sharqiyah | Ja'alan Bani Bu Hassan | 1 (8) | 12.5 (0.3-52.70 | 1 (18) | 5.6 (0.1-27.3) |
| Dhofar | Al Mizyounah | 1 (11) | 9.1 (0.2-41.3) | 1 (52) | 1.9 (0.0-10.3) |
| Dhofar | Salalah | 4 (62) | 6.4 (1.8-15.7) | 5 (308) | 1.6 (0.5-3.7) |
| Dhofar | Taqah | 1 (14) | 7.1 (0.2-33.9) | 1 (70) | 1.4 (0.0-7.7) |

#Significantly different, $\chi^2$ (4df) =11.51, p=0.0214

* Not Significantly different, $\chi^2$ (3df) =0.45, p=0.930

F Value=3.097, P=0.019 for all 5 wilayats

**Table 3.24 Herd based and individual seroprevalence of brucellosis in different wilayats of Oman**

| Govt. | Wilayat | Herd | | Individual | |
|---|---|---|---|---|---|
| | | Pos. (Tested) | Prev. [a] (95% CI) | Pos. (Tested) | Prev. [b] (95% CI) |
| Batinah | Barka | 1 (39) | 2.6% (0.1-13.5) | 1 (266) | 0.38% (0.0-2.1) |
| | Saham | 2 (32) | 6.3% (0.8-20.8) | 2 (293) | 0.68% (0.1-2.4) |
| | Shinas | 1 (12) | 8.3% (0.2-38.5) | 1 (122) | 0.82% (0.0-4.5) |
| Al Dakhiliyah | Samail | 2 (17) | 11.8% (1.5-36.4) | 2 (102) | 1.96% (0.2-6.9) |
| Al Sharqiyah | Dima wa Taaiyin | 1 (13) | 7.7% (0.2-36.0) | 1 (69) | 1.45% (0.0-7.8) |
| | Ja'alan Bani Bu Hassan | 1 (27) | 3.7% (0.1-19.0) | 1 (135) | 0.74% (0.0-4.1) |
| Al Dhahirah | Yunqal | 1 (16) | 6.3% (0.2-30.2) | 1 (131) | 0.76% (0.0-4.2) |
| Muscat | Seeb | 1 (10) | 10.0% (0.3-44.5) | 1 (69) | 1.45% (0.0-7.8) |
| Dhofar | Salalah | 9 (70) | 12.9% (6.1-23.0) | 12 (503) | 2.39% (1.2-4.1) |
| | Taqah | 3 (30) | 10.0% (2.1-26.5) | 3 (245) | 1.22% (0.3-3.5) |
| | Muqshin | 1 (24) | 4.2% (0.1-21.1) | 1 (205) | 0.49% (0-2.7) |
| | Mazyounah | 3 (21) | 14.3% (3-36.3) | 3 (214) | 1.40% (0.3-4) |
| | Sadah | 1 (11) | 9.1% (0.2-41.3) | 1 (180) | 0.56% (0-3.1) |
| | Shaleem & The Hallanitat Island | 2 (27) | 7.4% (0.9-24.3) | 2 (528) | 0.38% (0-1.4) |
| | Thumrayt | 1 (26) | 3.8% (0.1-19.6) | 1 (430) | 0.23% (0-1.3) |

a - not significantly different, $\chi^2$ (14df) =7.49, p=0.9141

b - not significantly different, $\chi^2$ (14df) =19.43, p=0.1491)

**Figure 3.8 Herd based prevalence of brucellosis in cattle from different wilayats of Oman**

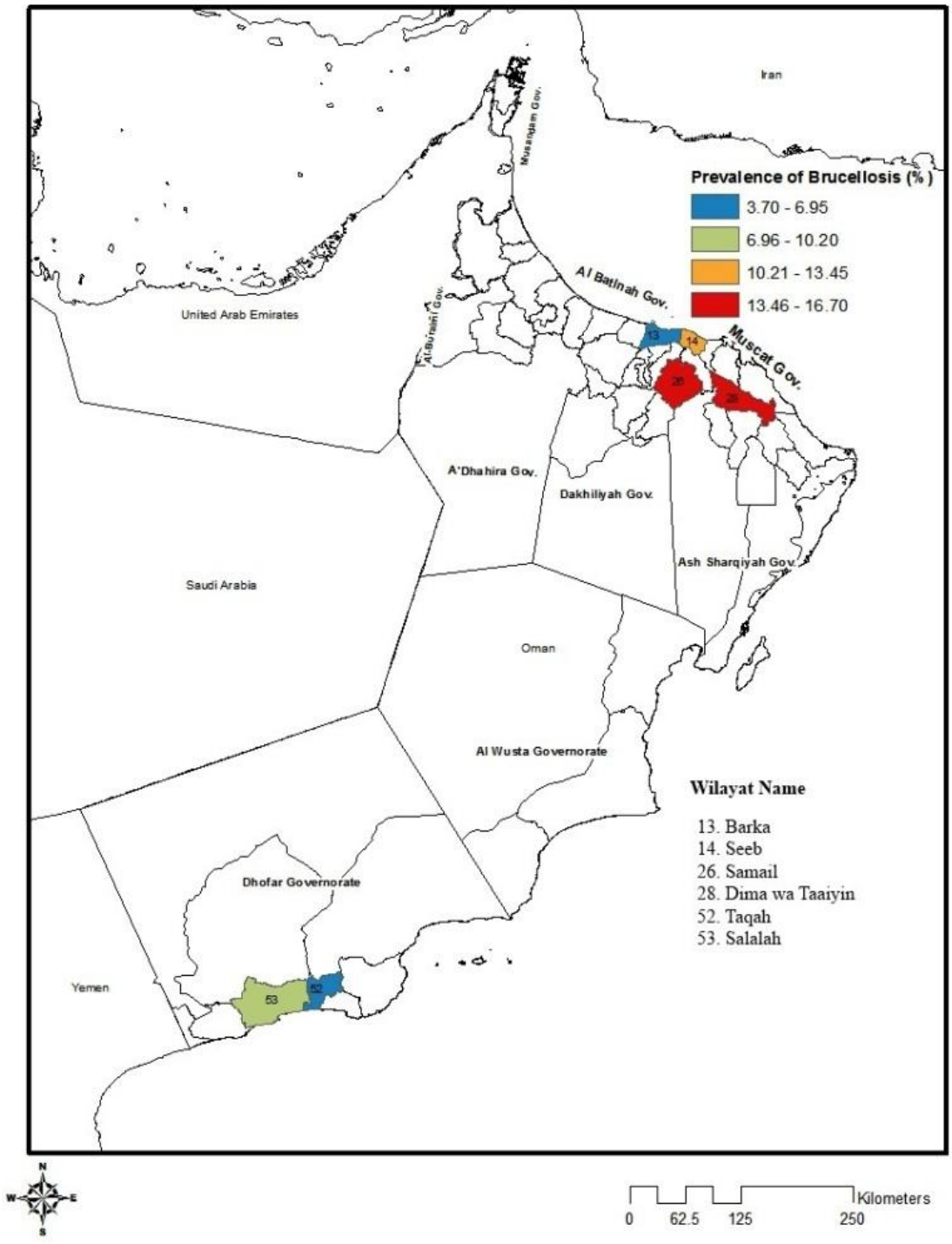


**Figure 3.9 Herd based prevalence of brucellosis in sheep from different wilayats of Oman**

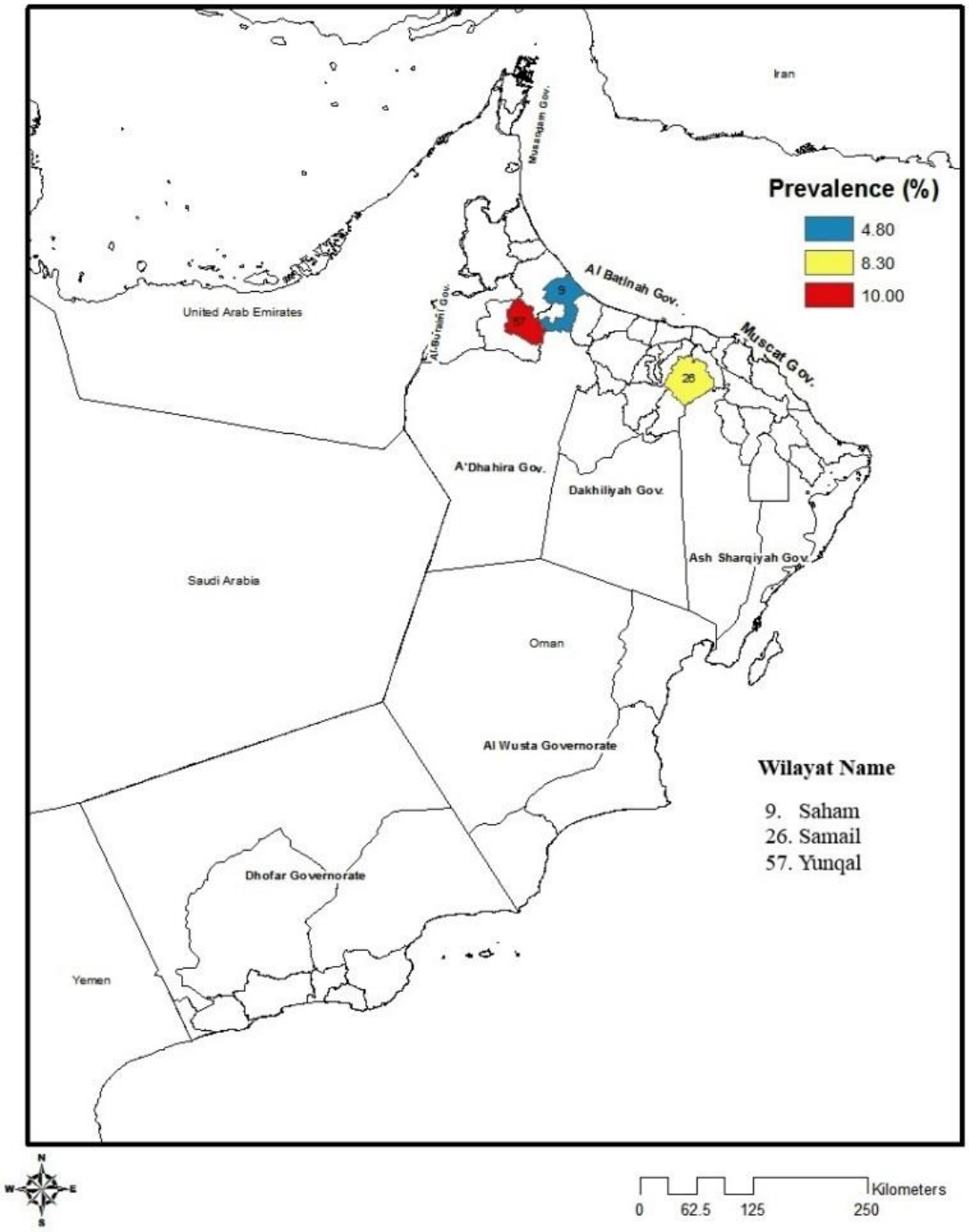


**Figure 3.10 Herd based prevalence of brucellosis in goats from different wilayats of Oman**

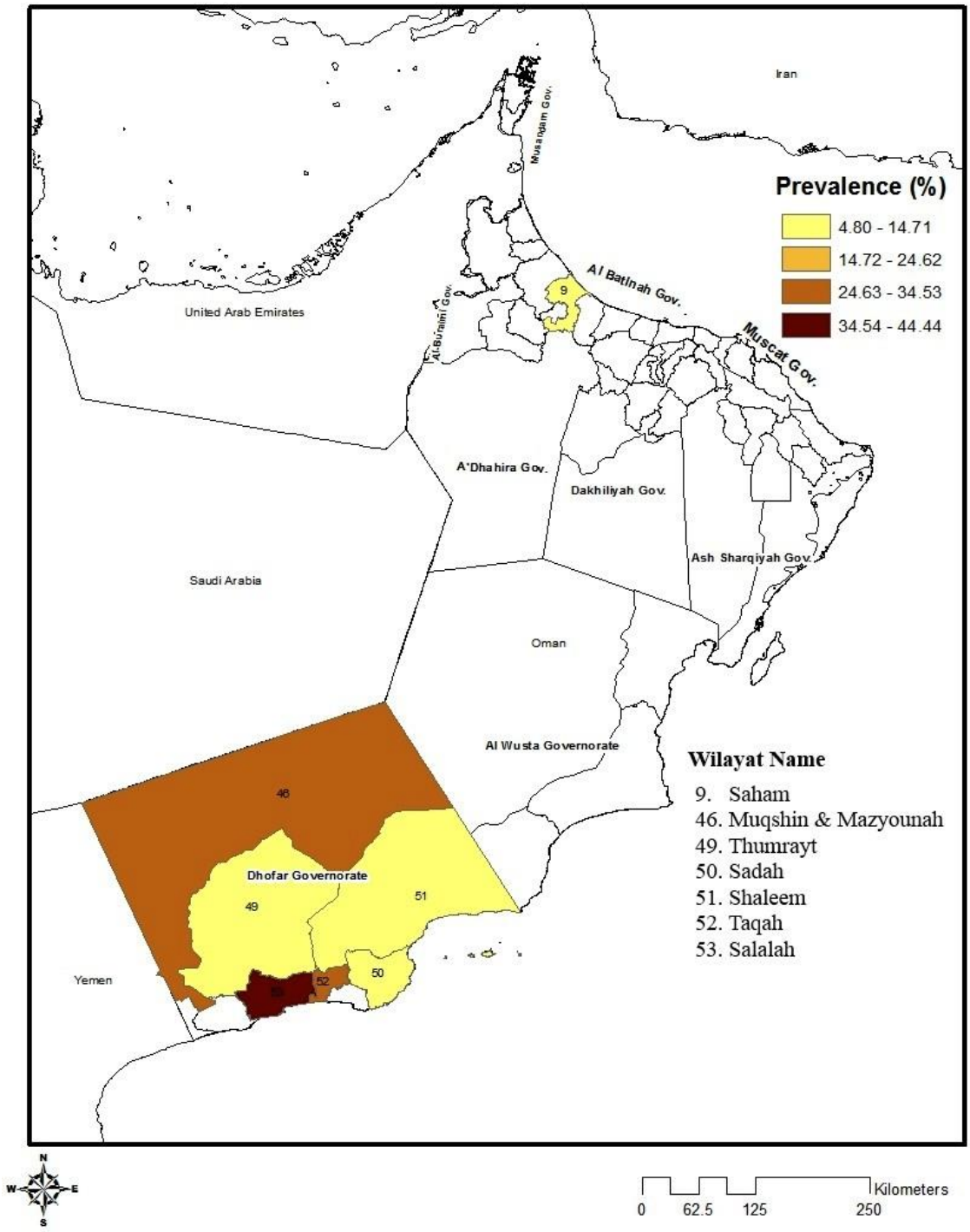


**Figure 3.11 Herd based prevalence of brucellosis in camels from different wilayats of Oman**

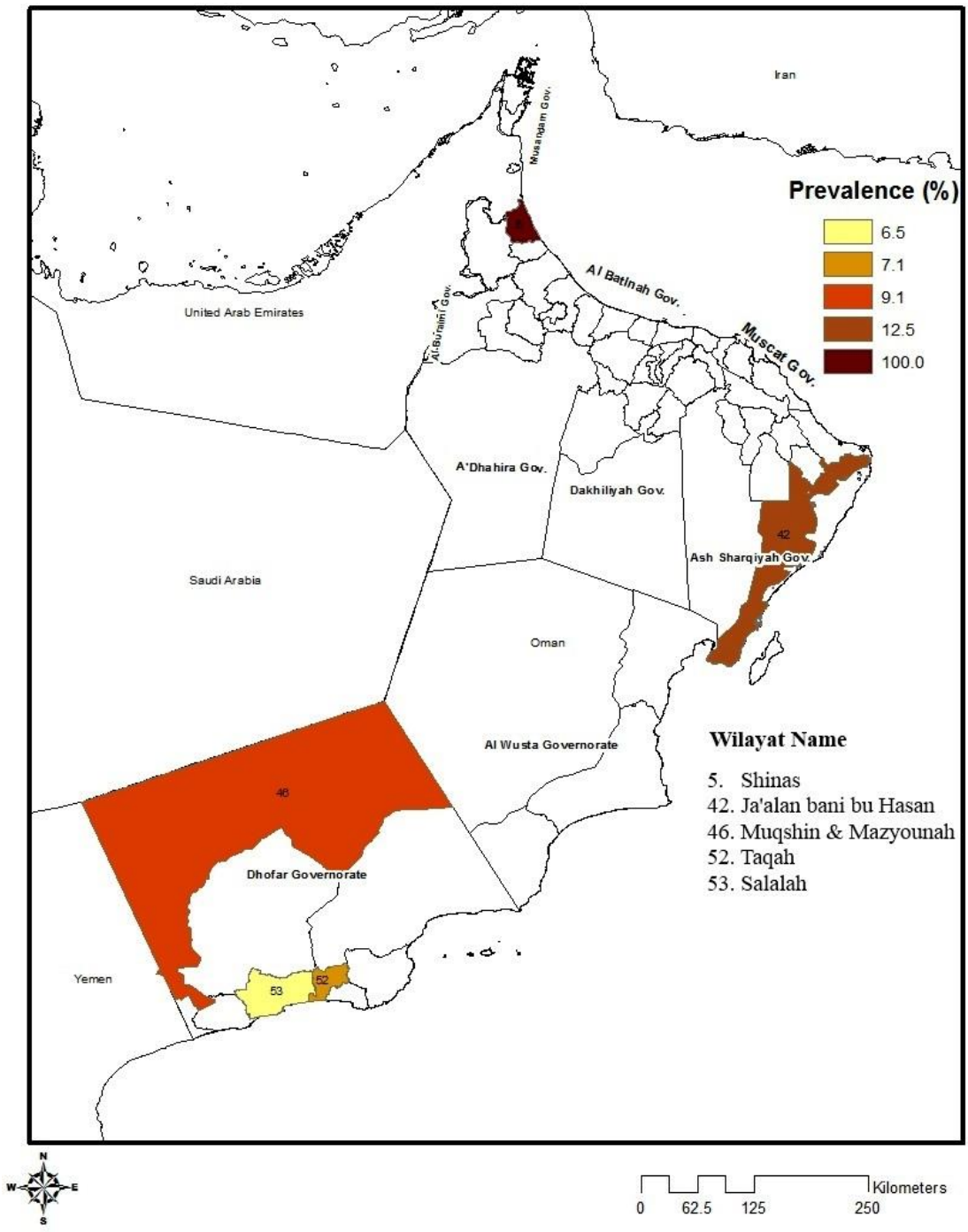
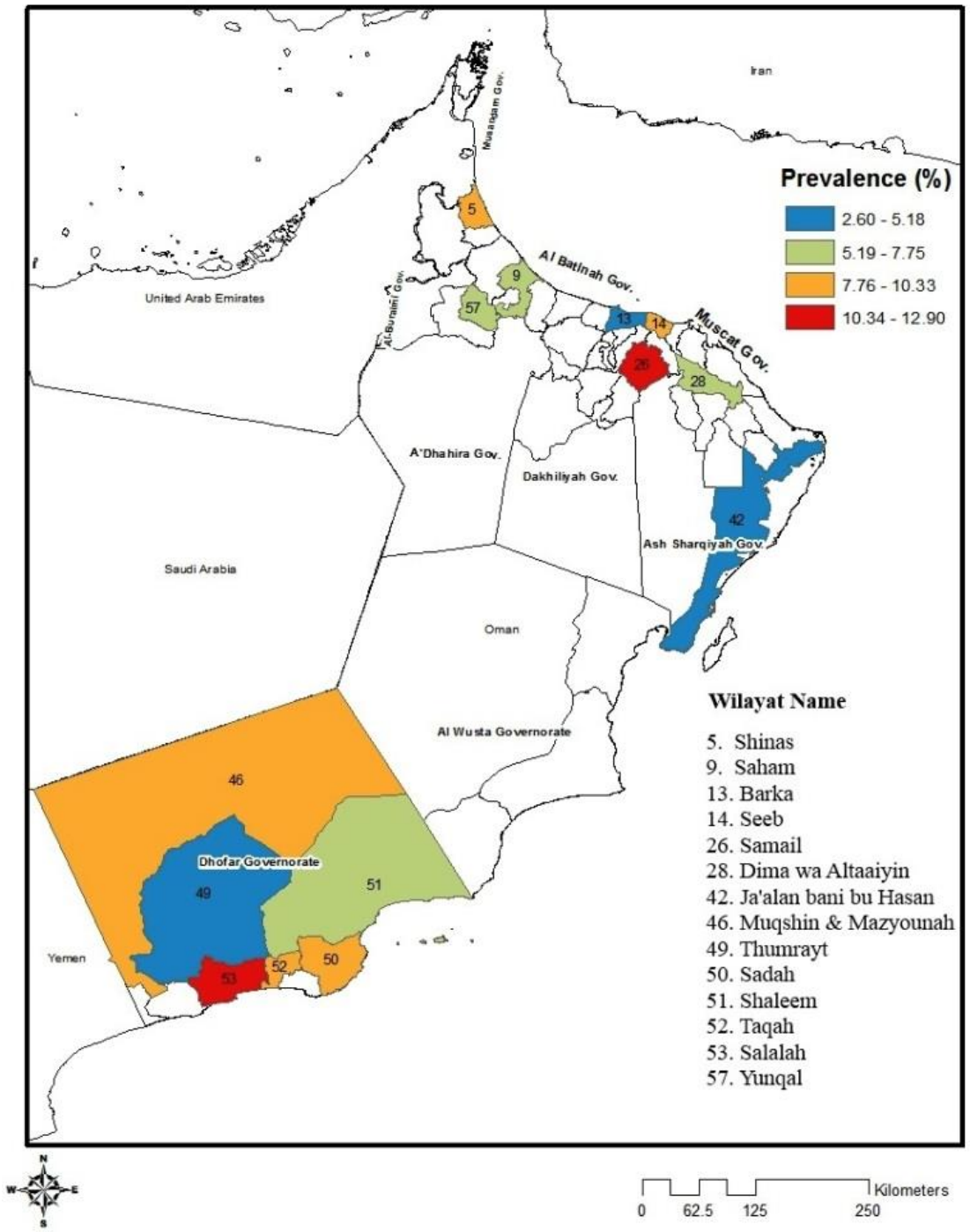


**Figure 3.12 Herd based prevalence of brucellosis in all species from different wilayats of Oman**



### 3.4 Discussion

One of the main objectives of the current study was to determine the seroprevalence of brucellosis in farm animals (cattle, sheep, goats and camels) of Oman and map its distribution by using Geographic Information System (GIS) tools. The study was also important from a public health perspective as current knowledge on disease distribution can help in the development of efficient control methods for this zoonotic disease in the Sultanate.

There has been an emergence or re-emergence of many zoonoses worldwide (Beran and Steel, 1994; Godfroid *et al*, 2005). One step towards their eradication is the identification and elimination of infected reservoir animals (Aldomy *et al*, 1992). The infection of animals or contamination of their products may result in the direct or indirect exposure of humans to these zoonotic agents and subsequent infection. It has been recommended that the control of human and animal brucellosis should be a major international priority for the medical and veterinary professions (Beran and Steel, 1994).

Brucellosis, caused by *Brucella melitensis*, remains a widespread public health problem. In Oman, *B. melitensis* is considered a public health problem, especially in the Dhofar Governorate where people of all ages are likely to consume raw milk and milk products. A comprehensive study of brucellosis in Oman has not previously been undertaken, and the real prevalence of brucellosis in all governorates and wilayats of the country has previously not been determined. This study is the first comprehensive seroprevalence study of brucellosis in farm animals (camel, cattle, goat and sheep) in Oman and provides valuable information in developing suitable control programs for the disease in this country.

### 3.4.1 Prevalence of brucellosis in Oman

#### 3.4.1.1 Seroprevalence of brucellosis in Oman on a herd basis

Although many countries have developed strategic plans and control programs to eradicate *B. melitensis* from farm animals, and many others have significantly reduced the prevalence of infection among their animal populations, *B. melitensis* is still widely distributed in the world (Corbel, 1989). In many less developed and developing countries, *B. melitensis* continues to cause major losses in livestock and poses a serious threat to people (Crawford *et al*, 1990).

According to the results of this study, 30 (2.8%) of 1267 holdings contained seropositive animals for brucellosis. The distribution of positive holdings was significantly different between southern and northern governorates. In the southern governorate (Dhofar) significantly more holdings (n = 20, 8.6%) contained seropositive animals compared to the northern governorates (n = 10, 0.97%) ($p < 0.001$). The high herd prevalence in Dhofar can be credited to: the active importation of live animals from countries in the Horn of Africa without prior monitoring of their brucellosis status; communal grazing of livestock; and the tropical weather, including monsoon rains and suitable temperatures, which favor the spread of the disease. The restriction of the exportation and movement of female animals from Dhofar to other regions in Oman and the approved importation of only castrated males to other regions may explain the containment of the disease to Dhofar and the low prevalence in the northern region.

In 2003, at the beginning of the control program, the prevalence of disease among cattle herds in Dhofar was determined by the *Brucella* Diagnostic Unit (BDU) at 6.5%. In the current study the herd prevalence in cattle in Dhofar was slightly lower at 4.9%. In contrast a study undertaken in 2010 by the same scientist who conducted the initial

serological study in 2003 in Dhofar reported an increase in the prevalence to 24% in cattle herds. Although the decrease in seroprevalence in the current study compared to 2003 could be a result of the ongoing control program and better education of the owners, the drastic increase in 2010 study was unexpected. This may have been due to a real increase in the prevalence due to the improper adoption of the control program over the years or it may have reflected misclassification of vaccinated and non-vaccinated animals as a consequence of a failure to use animal identification.

Although, there was no significant differences in the seroprevalence between infected cattle herds, the highest percentage was observed in Muscat (5.6%) followed by Dhofar (4.9%). The result of Dhofar can be explained due to the endemic nature of the disease and the size of the cattle herds in this region. However in Muscat, the capital of Oman, there are only small numbers of animals, and quarantine facilities and slaughter houses are present, consequently the significantly higher prevalence ($P<0.001$) as compared to other northern districts was surprising. This higher seroprevalence could be due to the limited number of samples (95%CI 0.1-27.3) collected in this governorate, and also the seropositive case was an imported bull and consequently a history of vaccination could not be excluded. Furthermore the majority of the positive reactors found in cattle in the northern area were males of imported breeds kept for sacrificial purposes, consequently a prior history of vaccination could not be excluded and this may have resulted in seropositive reactions.

Seropositive sheep were detected in only three flocks (0.6%) and there was no significant difference between districts. In contrast 2.3% of goat herds were seropositive and infection was significantly higher in Dhofar (16.2%) than in the other districts. In Oman, small ruminants (sheep and goats) are usually treated as one group and the

results of most previous studies did not separate out the prevalence for the two species. The prevalence of disease among small ruminants in Dhofar was determined by the BDU at 16.9 and 7.9% in 2003 and 2010, respectively. However, in the current study the prevalence was 16.2% in the goat herds, while no sheep herd was found positive in Dhofar. This consistent prevalence observed in goats could be related to the fact that during the current study herds from all wilayats of the northern region of Oman were sampled compared with previous studies where samples were only collected from wilayats where a vaccination program was being undertaken. Thus the differences observed could be due to the low prevalence of infection in the BDU studies as a result of vaccination or could be due to differences in the study design, animal selection or diagnostic tests used.

Only 8 herds contained seropositive camels (1.4%) with most seropositive cases and herds located in Dhofar (6 cases). However, there was no significant difference between the herd prevalence in camels belonging to the northern and southern regions of Oman. Dhofar, the endemic area, contains approximately 50% of the total camel and cattle population in Oman and as a consequence most of the human cases of brucellosis have also been recorded here. In 2009, the BDU conducted a study to determine the herd prevalence among camel holdings (4.3%); however the study was limited to one area of Dhofar (Salalah).

In conclusion, this study documented the overall seroprevalence of brucellosis in animal holdings to be 2.8% with 30 infected holdings. A significantly higher prevalence was observed in the Dhofar governorate than in any other governorates/regions. A possible reason for this might be the already endemic nature of disease in Dhofar, more inter-species contact, sharing of common pasture, large herd size and unhygienic husbandry

conditions in this governorate as supported by findings reported by other researchers (Alton *et al,* 1988; Grilló *et al,* 1997; European Commission, 2001; Corbel *et al,* 2006). Overall the seroprevalence in the Northern governorates was lower than in Dhofar governorate where the disease would appear to be endemic. A comparatively higher number of goat holdings were positive for brucellosis when compared to the other animal species tested. This could be due to the reported higher susceptibility of all goat breeds to brucellosis, as compared to sheep, and the apparent higher susceptibility of milking breeds compared to those kept for meat purposes (Corbel & Brinley-Morgan, 1984). Furthermore the vaginal excretions of goats are more copious and last for at least 2-3 months post-partum and usually two thirds of the naturally acquired infections result in an infection of the udder with excretion of *Brucella* in the milk during subsequent lactations (Alton, 1990a). This study found serological evidence of infection in camels with these animals having had contact with infected small ruminants. Similar observations have been reported in various other studies (European Commission, 2001; Musa and Shigidi, 2001; Abbas and Aqab, 2002). However, clinical disease/signs (including abortions) were rarely reported by the owners of positive camel holdings. Others have reported that the exhibition of clinical disease is very rare in camels infected with *B. melitensis*, and the current findings concur with their research (Abbas and Aqab, 2002; Corbel *et al,* 2006). Although clinical disease is seldom seen in camels, possible shedding of the organism in milk may lead to transmission of infection to humans and subsequent disease.

#### 3.4.1.2 Individual level seroprevalence of brucellosis in Oman

The individual animal seroprevalence to brucellosis was generally low (< 1%) in the sampled cattle holdings. Even in the brucellosis endemic Dhofar governorate, the

individual animal prevalence was manageable (< 2%). These findings may be a result of the ongoing control programs through mass vaccination. However, a lack of cooperation from some local farmers did not allow the collection of samples from the desired number of unvaccinated herds, thus the real prevalence could be different to that reported in this study which may have introduced selection bias by not being a true cross-section of susceptible herds. Moreover, the seroprevalence in the northern regions could also differ from that reported here as most of the positive animals (3 of 4) were imported and consequently may have been vaccinated which have a greater probability of inducing false positive serological reactions. Since the scientific community is still looking for a reliable test to distinguish between naturally infected and vaccinated animals (European Commission, 2001; Corbel *et al*, 2006), the true prevalence of brucellosis might differ from past and future studies.

In 2003, the individual seroprevalence of cattle in Dhofar was reported to be 3.7% and this increased to 8% in 2010 (BDU), however in the current study the individual seroprevalence of cattle in Dhofar was only 1.5%. Cases in sheep and goats were also reported in Dhofar with the individual seroprevalence reported by the BDU dropping from 4.5% in 2003 to 1.3% in 2010. However in the current study the individual seroprevalence in small ruminants from Dhofar was only 1% and was significantly different from that found in other governorates. In contrast the individual seroprevalence of brucellosis in camels in this study was only 0.8% and there was no significant difference between the seroprevalence in camels from the Southern and Northern regions. In Dhofar, the individual seroprevalence in camels was 0.77% which was slightly lower than the 1% reported in 2009 (MAF, 2009).

A higher seroprevalence was found in imported animals in this study and this may be

due to their susceptibility to infection if they were imported from disease-free-areas or the result of false positive reactions if they had previously been vaccinated against brucellosis in their country of origin (Thrusfield, 2005). A higher prevalence of brucellosis was also reported by Seboxa (1982) and Tschopp *et al.* (2013) in Ethiopia, and Ali *et al.* (2014) in Pakistan in crossbred cattle. This could be linked to the open herd management structure where bulls were purchased from markets without knowledge on their disease or vaccine status, malnutrition, poor husbandry or tropical environmental stress.

The seroprevalence of brucellosis was not affected by the gender of the sampled animals. This is not unexpected due to the similar management of males and females. However, males are usually kept for a shorter period of time than females which may reduce the chance of interaction with infected females or other herds and hence the probability of infection (Teklue *et al*, 2008; Tolosa *et al*, 2008; Dinka and Chala, 2009).

Generally the prevalence of brucellosis increased with the age of the animals, possibly because of the higher risk of contracting the disease after puberty due to increased contacts with potentially infected animals as described by others (Tolosa *et al*, 2008; Dinka and Chala, 2009; Teklue *et al*, 2013).

The individual animal seroprevalence was compared with studies from other countries in the region. However, other countries in the Middle East are not isolated as trade in animals and animal products are significant. The southern governorate of Dhofar shares a border with the Republic of Yemen while the northern region shares borders with the United Arab Emirates (UAE) and Saudi Arabia. The individual animal prevalence in cattle (0.4%) was higher than that found in Yemen (0.06%), whilst lower than that reported in UAE (1.3%), Saudi Arabia (18.7%), Iraq (3%) and Iran (0.8%) (Gul and

Khan, 2007). This finding is probably associated with the lower number of goats and cattle imported from the Horn of Africa into Oman for consumption and breeding purposes when compared with Saudi Arabia and the UAE. However the numbers imported into Oman is higher than that into Yemen which may account for the higher prevalence in Oman than Yemen.

The seroprevalence in sheep sampled in this study (0.1%) was also slightly lower than that reported in Yemen (0.6%) (AlShamahy *et al,* 2000) and significantly lower than that reported in the UAE, Saudi Arabia and Iran (3.4%, 9.7% and 10.8%, respectively) (Gul and Khan, 2007). This may partly be explained by the overall lower number of sheep in Oman, compared to these other countries. Unlike goats, the limited numbers of imported sheep mainly go to slaughter houses and very few are kept for breeding. Generally Omanis prefer the meat of goat and cattle to that of sheep.

Although goats comprise approximately 70% of the total livestock in Oman (MAF, 2005), the seroprevalence of brucellosis in goats was lower than in neighbouring Middle Eastern countries. For instance, the prevalence of disease among goats in Jordon was reported at 27% (AlMajali, 2005) and 9.7% in Saudi Arabia. These values are markedly higher than that of Oman (0.4%) and Yemen (1.3%) (Gul and Khan, 2007). Good quarantine measures with the limited importation of goats into Oman, when compared with Saudi Arabia, may have played an important role in these differences.

In camels, the individual prevalence found in this study (0.4 %) was much less than that reported in Iraq, Saudi Arabia, and UAE (17.2, 8 and 2% respectively) (Gul and Khan, 2007). In Oman, the importation of camels has been banned for a long time. The main purpose for which camels are reared in the southern region is for meat production and there is limited transfer of live camels to the northern region where camels are mostly

kept for racing.

However, most of the other studies mentioned did not calculate herd level prevalence, and this may also account for differences between the current study and the previously conducted studies.

# CHAPTER FOUR

# RISK FACTORS ANALYSIS

## 4.1 Introduction

The environmental conditions and type of husbandry, including lambing in enclosures and overcrowding, greatly influence the spread of brucellosis (Al-Majali *et al,* 2009). The greatest risk for the entry of the disease is through the introduction of infected animals (Kellar *et al,* 1976; Reviriego *et al,* 2000; Crawford *et al,* 1990). The practice of transhumance has been reported to have a strong correlation with the presence of brucellosis (Alton, 1990a; Kabagambe *et al,* 2001; Refai, 2002). The disease is also enhanced in village flocks where animals graze communal pastures (Nicoletti *et al,* 1987; Crawford *et al,* 1990). Several authors have highlighted the correlation of disease with several factors including age, breed and a history of abortion in the herd (Silva *et al,* 2000; Luna-Martínez and Mejía-Terán, 2002; Amin *et al,* 2005).

This study was designed to investigate the role of several potential risk factors that can affect the prevalence of brucellosis in Oman. The variables at the individual animal level included age, sex, breed, and animal's history of abortion and at the herd level variables were herd size, cohorts, sharing of pasture, history of abortion in a herd and the location of the herd.

## 4.2 Materials and Methods

Information regarding different individual and herd level variables was collected on predesigned and pretested proformas as described in Chapter 3. All the data was inserted in the Excel sheets and variables were categorized and later coded to facilitate final analysis.

The Chi-square test for independence was used to determine if the prevalence varied between different genders, age groups and locations. The Fisher's exact test was used if any of the cells were less than 5 in a 2 x 2 Table. Confidence intervals for seroprevalence were calculated using the Exact Binomial Method. Odds ratios (OR) and their 95% confidence intervals were calculated to determine the association between factors and the presence of antibodies to brucellosis. The associations between the outcome response variables (seropositivity) and explanatory variables (information recorded through the proforma) were determined using binary logistic regression (IBM SPSS Statistics 17.0 for Windows®, IBM Corporation, Route 100 Somers, New York, USA). The outcome variable was dichotomized (0=negative and 1=positive) and the response variables were dichotomized or categorized wherever applicable. Bivariable screening was conducted and variables yielding significant associations at $P \leq 0.20$ (based on the Wald statistic) were offered to a binary logistic regression model. A backward stepwise model was constructed. All variables found significant ($P \leq 0.20$) were offered to the initial screening model and then removed based on the likelihood ratio tests. The Hosmer-Lemeshow test, the Nagelkerke R square test and the observed versus predicted values (Residual statistics) to identify outliers at 0.5 cut off point were used to assess the fit of the final models (Urdaz-Rodriguez *et al*, 2009).

## 4.3 Results

### 4.3.1 Univariable analyses for the seropositivity of brucellosis in cattle

Univariable analysis of the risk factors for individual animals revealed that older cattle (> 10 years of age) were more likely to test positive for brucellosis than cattle aged >5 to ≤10 years (OR: 4.98, 95%CI = 0.31 - 79.56), >2 to ≤5 (OR: 4.20, 95%CI = 0.38 - 46.37) and ≤ 2 (OR: 1.94, 95%CI = 0.20 - 18.68). Age was further categorized to

construct two groups (≤ 5 years and > 5 years of age) and analysis revealed that odds for testing positive were almost identical in cattle ≤ 5 years (OR: 1.05, 95%CI = 0.20, 5.43) and those > 5 years (P=0.953).

Males were more likely to be seropositive than females (OR: 2.29, 95%CI 0.51, 10.26), however this difference was not significant (p=0.266). Imported cattle were more likely to be seropositive than crossbred cattle (OR: 3.71, 95%CI 0.68, 20.43) although this was also not significant. However imported cattle were significantly more likely to be seropositive than local cattle (OR: 20.0, 95%CI 2.22, 179.82). Individual cattle with a previous history of abortion were more likely to be seropositive than cattle without a history of abortion (OR: 22.19, 95%CI 2.43, 202.35).

Analysis of data at the herd level indicated that cattle belonging to larger herds (more than 30 animals) were more likely to test positive than those kept in smaller herds (up to 5 head), although this difference was not significant (OR: 4.56, 95%CI 0.88, 23.50). Cattle reared separately without having interaction with other ruminants on the farm were significantly more likely to be seropositive than those managed with small ruminants (OR: 19.70, 95%CI 3.13, 123.86), or both small ruminants and camels (OR: 18.26, 95% CI 1.82, 183.19). The sharing of pasture between cattle and other species of livestock was found not to influence the likelihood of seropositivity in cattle (OR: 1.03, 95%CI 0.19, 5.66). Cattle belonging to herds with a history of abortions were more likely to test positive (OR: 9.02, 95%CI 1.58, 51.62) than those from herds with no history of abortions. Cattle belonging to herds located in the south of Oman (Dhofar governorate) were slightly more likely to test seropositive to brucellosis compared with cattle from the north of Oman (OR: 5.45, 95%CI 0.97, 30.68).

### 4.3.2 Multivariable analysis to identify factors influencing the seroprevalence of brucellosis in cattle

Of the 9 variables analyzed in the initial univariable analyses (Table 4.1) sex, breed (P=0.001), abortion history (P<0.001), herd size (P=0.001), cohort animals (P<0.001), history of abortions in herd (P=0.003) and governorate (P=0.031)] were significantly associated with the presence of antibodies to brucellosis (Wald P < 0.2). All other variables [age (P=0.516), sex (P=0.266) and sharing of pasture (P=0.977)] were not associated with seropositivity (Wald P > 0.2).

For infection in individual animals variables with a P < 0.2 (breed and history of abortion) were entered into a multivariable logistic regression model. Breed was removed at the first step (

Table 4.2) and only a prior history of abortion was found to be significantly associated with brucellosis (P=0.006). Consequently no multivariable model could be presented for the seroprevalence of brucellosis at the individual level in cattle.

All herd level variables yielding a P < 0.2 were entered into a second multivariable regression model. Governorate, herd size and abortion history of the herd were removed on subsequent steps and only the variable cohort animals (0.007) was found significantly associated with prevalence (Table 4.3). Consequently again no suitable multivariable model could be developed to determine risk factors for herd infectivity.

**Table 4.1 Univariable risk factors for seropositivity to brucellosis in Cattle**

| Variable Name | Variable | Percent seropositive | Odds ratio (95% CI) | P |
|---|---|---|---|---|
| **Age (years)** | <= 2 | 3/492 (0.61) | 0.52 (0.05, 5.0) | 0.516 |
| | 2.1 to 5 | 2/708 (0.28) | 0.24 (0.02, 2.7) | |
| | 5.1 to 10 | 1/419 (0.24) | 0.2 (0.01, 3.3) | |
| | >10 | 1/85 (1.18) | 1.0 | |
| Combined Age | ≤ 5 years | 5/1200 (0.42) | 1.05 (0.20, 5.43) | 0.953 |
| | > 5 years | 2/504 (0.39) | 1 | |
| **Breed** | Local | 1/1081 (0.09) | 0.19 (0.02, 2.05) | 0.001 |
| | Imported | 4/220 (1.82) | 3.71 (0.68, 20.43) | |
| | Crossbred | 2/403 (0.5) | 1.0 | |
| **Sex** | Male | 3/422 (0.68) | 2.29 (0.51, 10.26) | 0.266 |
| | Female | 4/1282 (0.31) | 1.0 | |
| **Abortion History** | Yes | 1/15 (6.67) | 22.19 (2.43, 202.35) | < 0.001 |
| | No | 6/1689 (0.35) | 1.0 | |
| **Herd Size** | ≤ 5 | 3/167 (1.79) | 0.22 (0.04, 1.13) | 0.001 |

| Variable Name | Variable | Percent seropositive | Odds ratio (95% CI) | P |
|---|---|---|---|---|
| | 6 to 15 | 0/207 (0.00) | - | |
| | 16 to 30 | 0/69 (0.00) | - | |
| | > 30 | 3/39 (7.69) | 1.0 | |
| **Cohorts** | Small ruminants | 2/304 (0.66) | 0.51 (0.01, 0.32) | < 0.001 |
| | Camels | 0/11 (0.00) | - | |
| | Small ruminants & Camels | 1/141 (0.71) | 0.06 (0.01, 0.55) | |
| | Cattle | 3/26 (11.54) | 1.0 | |
| **Pasture sharing** | Yes | 2/158 (1.27) | 1.03 (0.19, 5.66) | 0.977 |
| | No | 4/324 (1.23) | 1.0 | |
| **Abortion history in herd** | Yes | 2/27 (7.41) | 9.02 (1.58, 51.62) | 0.003 |
| | No | 4/455 (0.88) | 1.0 | |
| **Governorate** | South (Dhofar) | 2/42 (4.76) | 5.60 (0.99, 33.56) | 0.085 |
| | North (Rest of Oman) | 4/440 (0.91) | 1.0 | |

**Table 4.2 Final binary logistic regression model for predicting brucellosis at individual level in cattle surveyed for the prevalence of brucellosis in Oman**

| Variable Name | Variables | B | S.E. | Wald | Sig. | Odds Ratio | 95% C.I. | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Abortion | | | | | | | | |
| | History of Abortion | 3.099 | 1.128 | 7.552 | 0.006 | 22.186 | 2.432 | 202.354 |
| | Constant | -5.739 | 0.448 | 164.124 | 0 | 0.003 | | |

**Table 4.3 Final logistic regression model for predicting brucellosis at herd level in cattle surveyed for the prevalence of brucellosis in Oman**

| Variable Name | Variables | B | S.E. | Wald | Sig. | Odds Ratio | 95% C.I. | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Cohorts | | | | 12.262 | 0.007 | | | |
| | Cattle only | 2.905 | 1.176 | 6.097 | 0.014 | 18.261 | 1.82 | 183.18 |
| | Cattle with small ruminants | -0.076 | 1.229 | 0.004 | 0.951 | 0.927 | 0.083 | 10.311 |
| | Cattle with Camels | -16.261 | 12118.636 | 0 | 0.999 | 0 | 0 | . |
| | Constant | -4.942 | 1.004 | 24.247 | 0 | 0.007 | | |

Variable(s) entered on final step: Cohort animals

### 4.3.3 Univariable analysis to identify factors influencing the seropositivity to brucellosis in sheep

The influence of age, sex, breed, and individual animal history of abortion on seropositivity to the brucellosis were analysed at the individual sheep level. At the flock level, the variables flock size, cohorts, sharing of pasture, history of abortion in a flock and location of flock were also tested for association with the presence of infection (Table 4.4).

Imported sheep were more likely, although not significantly, to be seropositive than local breeds (OR: 6.24, 95% CI 0.56, 69.04). No other comparison was possible with respect to age, sex and history of abortion as all seropositive sheep belonged to only one age group (>3 to ≤5), were females and had no prior history of abortion.

Flock level analysis was only possible for flock size and sheep belonging to flocks containing 10 to 25 individuals were more likely, although not significantly, to test positive than those from flocks containing less than 10 sheep (OR: 1.87, 95% CI 0.17, 20.78). No other comparison was possible as all sheep found positive were kept with goats on mixed managed farms, did not have access to common pasture, originated from flocks with no recent history of abortions and all were located in governorates in the north of Oman.

### 4.3.4 Multivariable analysis to identify factors influencing the seroprevalence of brucellosis in sheep

The results for the 9 variables analysed are displayed in Table 4.4. Of these variables, only breed ($P = 0.052$) was found to be associated with brucellosis (Wald $P < 0.2$). Thus no multivariable analysis was possible regarding the seroprevalence of brucellosis in sheep.

**Table 4.4 Univariable risk factors for seropositivity to brucellosis in sheep**

| Variable Name | Variable | Percent seropositive | Odds Ratio (95% CI) | P Value |
|---|---|---|---|---|
| Age (years) | Age <= 1years | 0/221 (0.0) | - | NA |
| | Age 1.1 to 3 years | 3/1320 (0.23) | - | |
| | Age 3.1 to 5 years | 0/469 (0.0) | - | |
| | Age > 5 years | 0/205 (0.0) | 1 | |
| Combined Age | > 5 years | 0/205 (0.0) | - | NA |
| | ≤ 5 years | 3/2010 (0.15) | 1 | |
| Breed | Cross | 0/745 (0.0) | - | NA |
| | Imported | 2/358 (0.56) | 6.24 (0.56, 69.04) | |
| | Local | 1/1112 (0.09) | 1 | |
| Sex | Male | 0/431 (0.0) | - | NA |
| | Female | 3/1784 (0.17) | NA | |
| Abortion History | Yes | 0/16 (0.0) | | NA |
| | No | 3/2199 (0.14) | | |
| Flock Size | Up to 10 Sheep | 1/169 (0.59) | 1 | 0.629 |

| Variable Name | Variable | Percent seropositive | Odds Ratio (95% CI) | P Value |
|---|---|---|---|---|
| | 10 to 25 Sheep | 2/182 (1.09) | 1.87 (0.17, 20.78) | |
| | 26 to 50 Sheep | 0/110 (0.0) | - | |
| | > 50 Sheep | 0/47 (0.0) | - | |
| Cohorts | Sheep Only | 0/5 (0.0) | - | |
| | Cattle | 0/6 (0.0) | - | |
| | Goats | 0/95 (0.0) | - | NA |
| | Camels | 0/3 (0.0) | - | |
| | With other ruminants | 3/399 (0.75) | - | |
| Pasture Sharing | Yes | 0/252 (0.0) | - | NA |
| | No | 3/252 (1.19) | | |
| Abortion history in herd | Yes | 0/22 (0.0) | - | NA |
| | No | 3/486 (0.62) | - | |
| Governorate | South (Dhofar) | 0/35 (0.0) | - | NA |
| | North (Rest of Oman) | 3/473 (0.63) | - | |

### 4.3.5 Univariable analysis to identify factors influencing the seropositivity to brucellosis in goats

The influence of individual level variables (age, sex, breed, individual animal history of abortion) and herd level variables (herd size, cohorts, sharing of pasture, history of abortion in a herd and location of herd) on the seroprevalence of brucellosis in goats are summarized in Table 4.5.

Analysis of individual level variables indicated that goats older than 5 were slightly more likely to be seropositive than those >1 to ≤3 years (OR: 1.67, 95% CI 0.45 6.22) and >3 to ≤5 (OR: 1.37, 95% CI 0.37, 5.12), although this difference was not significant (P=0.704). Age categories were further combined to construct two groups (≤ 5 years and > 5 years of age) and analysis revealed that goats > 5 years were more likely to test positive (OR: 1.59, 95% CI 0.49, 5.08) as compared to those ≤ 5 years. However, the difference was still statistically not significant (P=0.432). No analyses by odds ratios were possible for sex, breed and individual animal abortion history as all positive goats were female, of local breeds and had no previous history of abortions.

When data were analysed at the herd level the following outcomes were observed. Although goats kept in herds with 26-50 head and 10-25 head were more likely to test positive than those in herds of more than 50 animals (OR: 1.03, 95% CI=0.23, 4.69 and OR: 1.36, 95% CI=0.33, 5.55, respectively), this difference was not significant (P=0.501). Goats in mixed herds with camels (OR: 1.95, 95% CI=0.41, 9.41), with sheep (OR: 10.86, 95% CI 1.16, 101.35) and in mixed herds with all ruminant species (OR: 10.23, 95% CI 2.60, 40.25) were more likely to test seropositive than those kept alone. Goats sharing pasture with other ruminants were found more likely, but not significantly, to test positive for brucellosis (OR: 2.51, 95% CI 0.68, 9.23). Goats

belonging to herds having a history of abortion were significantly more likely to contain seropositive animals (OR: 10.81, 95%CI 3.33, 35.06) than those from flocks with no history of abortions. Similarly goats originating from flocks located in the south of Oman (Dhofar) were more likely to be infected than flocks from the north of Oman (OR: 94.26, 95% CI 12.05, 737.39).

### 4.3.6 Multivariable analysis to identify factors influencing the seroprevalence of brucellosis in goats

Of the 9 variables analysed in the univariable analyses (Table 4.5), sex (P=0.148), cohort animals (P<0.001), pasture sharing (P=0.151), history of abortions in herd (P<0.001) and governorate (P<0.001) were significantly associated with brucellosis (Wald P < 0.2). All other variables [age (P=0.704), breed (P=0.653), individual animal's history of abortion (P=0.59) and herd size (P=0.501)] were not associated with disease (Wald P > 0.2).

Only one variable was found to be significantly associated with brucellosis at the individual level, so no multivariate analysis was possible. All herd level variables found significantly associated with brucellosis in goats were used for the multivariate model building. Cohorts and sharing of pasture were removed from the model (P > 0.05) and only the variables governorate and history of abortions in a herd were found significantly associated with the presence of brucellosis. The results showed that goats belonging to herds located in the Dhofar governorate (OR: 74.88, 95% CI = 9.41, 596.04) and having a previous history of abortion in the herd (OR: 4.43, 95% CI = 1.18, 16.65) were more likely to test positive for brucellosis (Table 4.6). However, the model did not have a good fit (Chi-Square = 0.00 and 0.416 - Hosmer-Lemeshow test and Nagelkerke R Square values respectively).

**Table 4.5 Univariable risk factors for seropositivity to brucellosis in Goats**

| Variable Name | Variable | % seropositive | OR (95% CI) | P Value |
|---|---|---|---|---|
| Age (years) | <= 1 | 0/199 (0.0) | - | 0.704 |
| | Age 1.1 to 3 years | 5/1315 (0.38) | 0.63 (0.17, 2.36) | |
| | Age 3.1 to 5 years | 5/1127 (0.44) | 0.74 (0.19, 2.76) | |
| | Age > 5 years | 4/667 (0.6) | 1 | |
| Combined Age | > 5 years | 4/667 (0.44) | 1.59 (0.49, 5.08) | 0.432 |
| | ≤ 5 years | 10/2641 (0.38) | 1 | |
| Breed | Local | 14/3119 (0.45) | - | NA |
| | Imported | 0/124 (0.0) | - | |
| | Cross | 0/65 (0.0) | - | |
| Sex | Male | 0/429 (0.0) | - | NA |
| | Female | 14/2879 (0.49) | - | |
| Abortion History | Yes | 0/67 (0.0) | - | NA |
| | No | 14/3241 (0.43) | - | |
| Herd Size | Up to 10 Goats | 0/75 (0.0) | - | 0.501 |
| | 10 to 25 Goats | 4/167 (2.39) | 1.03 (0.23, 4.69) | |
| | 26 to 50 Goats | 6/191 (3.14) | 1.36 (0.33, 5.55) | |

| Variable Name | Variable | % seropositive | OR (95% CI) | P Value |
|---|---|---|---|---|
| | > 50 Goats | 3/129 (2.33) | 1 | |
| Cohorts | Cattle | 0/46 (0.0) | - | < 0.001 |
| | Sheep | 1/77 (1.29) | 0.18 (0.02, 1.78) | |
| | Camels | 4/32 (12.5) | 1.95 (0.41, 9.41) | |
| | with ruminants | 5/363 (1.38) | 0.19 (0.04, 0.83) | |
| | Only Goats | 3/44 (6.82) | 1 | |
| Pasture Sharing | Yes | 10/323 (3.09) | 2.51 (0.68, 9.23) | 0.151 |
| | No | 3/239 (1.26) | 1 | |
| Abortion History in Herd | Yes | 5/35 (14.29) | 10.81 (3.33, 35.06) | <0.001 |
| | No | 8/527 (1.52) | 1 | |
| Governorate | South (Dhofar) | 12/74 (16.21) | 94.26 (12.05, 737.36) | < 0.001 |
| | North (Rest of Oman) | 1/488 (0.2) | 1 | |

**Table 4.6 Final binary logistic regression model for predicting brucellosis at the herd level in goats in Oman**

| Variable Name | Variables | B | S.E. | Wald | Sig. | Odds Ratio | 95% C.I | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Region | Southern Region | 4.316 | 1.058 | 16.627 | < 0.001 | 74.876 | 9.406 | 596.041 |
| Abortion History in Herd | Presence of Abortion | 1.489 | 0.675 | 4.863 | 0.027 | 4.434 | 1.18 | 16.656 |
| | Constant | -10.647 | 2.029 | 27.527 | | | | |

### 4.3.7 Univariable analysis to identify factors influencing the seropositivity to brucellosis in camels

The influence of individual level variables (age, sex, breed, individual animal history of abortion) and herd level variables (herd size, cohorts, sharing of pasture, history of abortion in a herd and governorate) on the seroprevalence of brucellosis in camels was analyzed (Table 4.7).

Analysis of individual level variables indicated that the camels which were in the age group of >5 to ≤10 years were more likely, although not significantly, to test positive than camels >2 to ≤5 years of age (OR: 1.39, 95% CI 0.27, 7.20) and >10 years old (OR: 1.18, 95% CI 0.23, 6.09). Furthermore, age categories were combined to construct two groups (≤ 5 years and > 5 years of age) and analysis was performed again. It was revealed that camels > 5 years were more likely to test positive (OR: 2.38, 95% CI 0.49, 11.48) as compared to those ≤ 5 years. However, no odds ratio analyses were possible for sex, breed and individual animal abortion history as all positive camels were female, local breeds and had no prior history of abortion.

When variables at the herd level were analyzed, camels kept in large sized herds (>30 head) were found more likely to contain seropositive animals than in herds with ≤ 5 head (OR: 5.01, 95% CI 0.52, 48.55), 6-15 heads (OR: 7.54, 95%CI 0.78, 73.02) or 16-30 camels (OR: 1.99, 95% CI 0.39, 10.00). Camels kept with cattle in mixed herds were more likely to test positive for brucellosis than those kept alone (OR: 4.60, 95% CI 0.73, 29.18). However, camels kept with only small ruminants were less likely to test positive than those kept with only camels (OR: 0.22, 95% CI 0.02, 2.15), and with cattle and small ruminants (OR: 0.60, 95% CI 0.10, 3.67). Camels belonging to herds having a history of abortions were significantly more likely to contain seropositive

animals than those from herds with no history of abortion (OR: 8.71, 95% CI 2.12, 35.81). Camels originating from herds located in the south of Oman (Dhofar) were more likely to be seropositive for brucellosis than those from the north of Oman (OR: 7.46, 95% CI 1.49, 37.37). As all seropositive camels shared pasture with other ruminants no comparison by odds ratios was possible for this variable.

### 4.3.8 Multivariable analysis to identify factors influencing the seroprevalence of brucellosis in camels

Of the 9 variables analysed with univariable analyses (Table 4.7), abortion history in herd (P<0.001), pasture sharing (P=0.032), herd size (0.182), cohorts (0.016) and governorate (P=0.004) were found to be significantly associated with brucellosis (Wald P < 0.2). The variables age (P=0.539) and individual animal's history of abortion (P=0.671) were not associated with disease (Wald P > 0.2). No valid comparison was possible regarding the breed and sex of camels as all positive animals were of female sex and local breeds.

No variable was found significantly associated with seroprevalence of brucellosis at the individual level, and consequently no multivariate analysis could be conducted. Herd level variables found significant on the initial univariable analyses were used to construct the initial multivariate model. However, the variables sharing of pasture, cohorts, herd size and governorate were removed at subsequent steps and only previous history of abortion in a herd was found to be significantly associated with the presence of brucellosis. Thus no multivariable model could be produced to explain the presence of brucellosis in camels (Table 4.8).

**Table 4.7 Univariable risk factors for seropositivity to brucellosis in camels**

| Variable Name | Variable | % seropositive | OR (95% CI) | P Value |
|---|---|---|---|---|
| Age | ≤ 2 years | 0/403 (0.0) | - | 0.539 |
| | > 2 to ≤ 5 years | 2/506 (0.39) | 0.85 (0.12, 6.03) | |
| | > 5 to ≤ 10 years | 5/912 (0.55) | 1.18 (0.23, 6.09) | |
| | >10 years | 2/429 (0.47) | 1 | |
| Combined Age Group | > 5 years | 7/1341 (0.52) | 2.38 (0.49, 11.48) | 0.28 |
| | ≤ 5 years | 2/909 (0.22) | 1 | |
| Breed | Local | 9/226 (0.4) | - | NA |
| | Imported | 0/18 (0.0) | - | |
| | Cross | 0/6 (0.0) | - | |
| Sex | Male | 0/252 (0.0) | - | NA |
| | Female | 9/1998 (0.45) | - | |
| Abortion History | Yes | 0/44 (0.0) | - | NA |
| | No | 9/2206 (0.41) | - | |
| Herd Size | ≤ 5 Camels | 1/128 (0.78) | 0.19 (0.02, 1.95) | 0.182 |
| | 6 to 15 Camels | 1/191 (0.53) | 0.13 (0.01, 1.30) | |

| Variable Name | Variable | % seropositive | OR (95% CI) | P Value |
|---|---|---|---|---|
| | 16 to 30 Camels | 3/154 (1.95) | 0.50 (0.09, 2.55) | |
| | > 30 Camels | 3/79 (3.8) | 1 | |
| Cohorts | Small ruminants | 1/219 (0.46) | 0.22 (0.02, 2.15) | 0.016 |
| | Cattle | 2/23 (8.69) | 4.60 (0.73, 29.18) | |
| | Cattle & small ruminants | 2/162 (1.23) | 0.60 (0.10, 3.67) | |
| | Only Camels | 3/148 (2.03) | 1 | |
| Pasture Sharing | Yes | 8/353 (2.27) | - | NA |
| | No | 0/199 (0.0) | - | |
| Abortion History in Herd | Yes | 4/60 (6.67) | 8.71 (2.12, 35.81) | <0.001 |
| | No | 4/492 (0.81) | 1 | |
| Governorate | South (Dhofar) | 6/162 (3.7) | 7.46 (1.49, 37.37) | 0.004 |
| | North (Rest of Oman) | 2/390 (0.51) | 1 | |

**Table 4.8 Final binary logistic regression model for predicting brucellosis at herd level in camels in Oman**

| Variable Name | Variable | B | S.E. | Wald | Sig. | Odds Ratio | 95% C.I. | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Abortion History | History of abortion | 2.165 | 0.721 | 9.015 | 0.003 | 8.714 | 2.121 | 35.809 |
| | Constant | -4.804 | 0.502 | 91.564 | 0 | 0.008 | | |

## 4.4 Discussion

Many factors, including host, agent and environmental factors, directly or indirectly influence the prevalence, distribution and transmission of a disease (Burridge, 1981). A large herd size, a high stocking density, older animals, frequent introduction of untested livestock, unrestricted grazing and grazing of communal pastures can all be associated with a high seroprevalence of brucellosis (Nicoletti, 1976; Breitmeyer *et al*, 1992; Kadohira *et al*, 1997).

In order to estimate the effects of risk factors on the seroprevalence of disease in the sultanate, several parameters were structured in form of a questionnaire (Appendix 1) and the results presented in Tables (4.1 to 4.8). Information on disease is often collected from three sources: the owner; direct observation of herds/flocks; and from neighbours of the owner's livestock holdings. However in Oman, the quality of information collected from these sources may be questionable. In the current study, most private livestock owners had no systematic herd records or an animal identification system. Consequently no reliable data were available regarding the number of births, early mortalities, the birth of weak young or stillbirths or the number of abortions occurring each year in the flocks/herds. Most of the sampled herds were managed by illiterate expatriate workers who were not familiar with the origin of the animals if they had been purchased. Owners were usually reluctant to provide exact financial details regarding the annual purchase and selling of animals.

Several factors were analyzed as potential risk factors at both the individual and herd/flock level. In this study the individual animal factors analyzed included age, sex, breed and individual animal history of abortion and the herd/flock level factors included herd size, cohorts, sharing of pasture, history of abortion in a herd and

location of the herd.

Questionnaire based information (Appendix 1) collected during this study indicated that several factors could be considered as potential risk factors for the disease increasing the risk of an animal or herd/flock being infected with brucellosis. However, the risk factors associated with seropositivity varied between species at both the individual and herd/flock levels in the univariable and multivariable analyses.

Individual animal level analysis revealed that breed ($P = 0.01$) and history of abortion ($P < 0.001$) were significantly associated with brucellosis in cattle (Table 4.1). Similarly, in sheep, breed ($P = 0.05$) and a history of abortion ($P = 0.02$) were also significantly associated with seropositivity. However these variables were not associated with seropositivity in goats and camels.

The finding of a significantly higher seroprevalence in imported cattle (1.8%) and sheep (0.6%) was in agreement with other studies. Chantal and Thomas (1976) and Akakpo (1987) indicated that crossbred cattle (*B. taurus* and *B. indicus*) were more susceptible to *Brucella* than were purebred *B. indicus* cattle. This may have been explained by the emergence of resistance in local breeds to the endemic diseases as compared to imported and cross-bred animals. However it is likely that management of imported and cross-bred animals is different to that of local bred animals. These animals may be housed more intensively resulting in a greater opportunity in transmission of bacteria between the species. Others have highlighted the importance of management and husbandry practices adopted on the transmission of *Brucella* (Crawford *et al,* 1990; Reviriego *et al,* 2000).

The seroprevalence of disease in cattle with a history of abortion (6.7%) was

significantly higher than those without an abortion history (OR=22). This was expected, as the disease usually manifests itself as abortions (Jones, 1982; Mahajan and Kulshreshtha, 1986; Arda *et al,* 1987; Kenar *et al,* 1990). Fensterbank (1977) reported that one infected cow at parturition could shed enough bacteria to infect up to 600,000 animals and the antibodies induced by infection are likely to last for the duration of the animal's life (Alton, 1990a; Durán-Ferrer, 1998). However, a history of abortion was not associated with brucellosis in sheep where no sheep with a history of abortion were seropositive compared to 0.14% in those without a history of abortion. In Oman, it is well accepted that abortion in sheep from brucellosis is rare compared to cattle and goats (MAF, 2013). This may be due to either more tolerance by the local sheep to disease or it may reflect the findings of other researchers where sheep have been shown to be very resistant to re-infection compared to other ruminants (Alton, 1990a; Durán-Ferrer, 1998).The higher susceptibility of goats compared to sheep has also been recorded by Crespo (1994) and Reviriego *et al,* (2000). It also could be related to the latent carrier status of goats which have been identified as an important source of infection in other regions of the world (Plommet *et al,* 1973; Lapraik *et al,* 1975; Fensterbank, 1978; Dolan, 1980).

Age is known as one of the intrinsic factors influencing brucellosis seropositivity (Megersa *et al,* 2011)**.** The influence of age on seroprevalence has already been mentioned in previous brucellosis studies (Kadohira *et al,* 1997; Kubuafor *et al,* 2000; Faye *et al,* 2005; Muma *et al,* 2006; Chimana *et al,* 2010). In the current study, although the prevalence of disease was not significantly affected by age, it is obvious that the older cattle, goats and camels had higher seroprevalences than did the younger animals. Similar findings were reported by Akakpo (1987) and Kadohira *et al.* (1997). Clinical disease mainly affects the actively producing animals that are

allowed to graze freely on contaminated pasture as compared to young animals which have not reached reproductive productive age. Furthermore older animals have more opportunities to have contact with infected animals than do other animals. Similar observations have been reported by Botha and Williamson, (1989), Silva *et al,* (2000) and Amin *et al,* (2005). It is also possible that the high prevalence of brucellosis among the older animals might be related to maturity and therefore, the organism propagates and produces either a latent infection or overt clinical manifestations.

As observed by Ocholi *et al.* (1996), this study also failed to find a significant difference between the prevalence in male and female ($P$ =0.266) cattle, although a higher seropositivity was observed in female small ruminants and camels compared with males. The equal susceptibility of male and female has been reported previously (European Commission, 2001). It was expected that females may have a higher prevalence as the disease is mainly manifested in adult reproductive animals. The similar prevalence in adult males and females may be explained by the similar likelihood of contacting animals when grazing communal pasture.

In the univariable analysis of infection in cattle herds, herd size, cohorts (animals that were grazed with cattle), a history of abortions in the herd and location were associated with seroprevalence. Similarly in sheep, cohorts, history of abortions in the flock and location were significant. Again in camels similar findings were reported with pasture sharing, cohorts, history of abortion in the herd and location significantly associated with evidence of infection.

Crawford (1990) and Enright and Boca (1990) reported that the risk factors associated with the spread of the disease within a herd included herd size, population density and the method of housing. The effect of herd size and mixed farming of

multiple species on the risk of infections with contagious diseases has been well documented (Salman and Meyer 1987). The larger herds may provide more chances for contact between animals and in particular contact with an infected animal. Mixed farming, and especially raising sheep and/or goats along with cattle, has been reported by many researchers to be a risk factor for transmission of *Brucella* between different animal species ( Omer *et al,* 2000; Abbas and Agab, 2002; Al-Majali *et al,* 2007). In this study, animals raised in smaller herds (5 animals or less) were less likely to be seropositive than those from larger herds/flocks (30 or more animals). Abbas and Agab (2002) and Al-Majali (2005) and Al-Majali *et al,* (2008) reported similar findings in camels and goats, respectively. In larger herds/flocks there is a greater opportunity of contact between animals and a greater probability that some animals will be calving/lambing/kidding at any one point in time which would facilitate the spread of infection (Camus, 1980; Akakpo, 1987).

Mixed farming of multiple species of animals was significantly associated with seropositivity ($P < 0.001$) for goats and camels. This may be due to increased opportunity of contact between species and the sharing of potentially contaminated pastures. This is particularly important in Dhofar governorate where sharing of pasture is a common feature of livestock rearing. In contrast higher chances for being seropositive were observed in cattle which were kept separate from other species (11.5%) than those kept with small ruminants (0.66%) or with both camels and small ruminants (0.71%). A possible explanation might be the sharing of communal pastures, confined contaminated farm space and communal water sources that provided more chances of contracting the disease. The role of small ruminants in the epidemiology of *B. melitensis* is well documented, with these animals acting as the main host for this organism. Omer *et al.* (2000) and Abbas and Agab (2002) have

reported the impact of mixing small ruminants with cattle on the seroprevalence of brucellosis, and consequently the finding of the lower prevalence in cattle mixed with small ruminants in this study is surprising. It is possible that other managerial factors may be affecting the spread of *Brucella* within cattle herds. For example farmers removed small ruminant manure more frequently than cattle manure. *Brucella* can survive for up to 60 days in damp soil and 30 days in urine (Bercovich, 1998). Farming cattle alone may also give more opportunities for these animals to be in close contact than with other species as they are usually kept together for a longer period before culling or slaughtering.

The use of communal pastures allows the frequent contact between animals providing increased opportunity of exposure of susceptible animals to infectious materials arising from parturition. Similar observations have been reported by Reviriego *et al*, (2000) who highlighted that contacts between goats and sheep at the flock level was one of most important risk factors for infection. However, the report of European Commission (2001) has reported a low risk for sheep in Latin America, even when raised in contact with infected goats. Furthermore contamination of pasture may also occur through other animals which may remove and distribute placental material such as dogs, cats and other carnivores such as foxes (European Commission, 2001).

A history of abortions in herds/flocks was significantly (P= 0.003) associated with infection in camels (7.1%, OR=8.7), cattle (7.4%, OR=9.02) and goats (14.3%, OR=10.8). This is expected as the major sign of brucellosis is abortion. Abortion facilitates the release of an enormous number of microorganisms which can contaminate the environment and subsequently be ingested by at-risk healthy animals in the infected herd/flock (Alton, 1982).

More camel (3.7%, OR=7.5), cattle (4.9%, OR=5.5) and goat (16.2%, OR=94.3) herds located in the southern Dhofar governorate were seropositive than those located in the northern governorates (0.5%, 0.9%, 0.2%, respectively). This finding was expected as the disease has primarily been localized in Dhofar during the past two decades (Ismaily *et al,* 1988). The high humidity, moderate temperature and shorter periods of direct sunlight for several months in Dhofar may have allowed the bacteria to survive in the environment for a longer period in this southern region compared to the northern regions where high temperatures and strong sunlight are predominant. This finding is in agreement with those reported by Nicoletti (1980), Alton (1985) and Al-Talafha *et al.* (2003). Moreover, introduction of infected animals shipped from the endemic areas in the Horn of Africa directly to the Salalah Port may have introduced brucellosis to this governorate and contributed towards the maintenance of brucellosis in the governorate. Crawford *et al.* (1990) described the introduction of new animals from endemic regions as an important risk factor for the spread of the disease. Therefore, in a country where importation of animals takes place, it is necessary to monitor the population and confirm the biotype present so that an effective control programme can be implemented. In the following chapter a study to identify the biotypes of *Brucella* present in Oman is summarised.

# CHAPTER FIVE

# BIOTYPING, ANTIBIOGRAM AND GENETIC ANALYSIS

## 5.1 Introduction

*Brucellae* are Gram negative, cocco-bacilli, facultative intracellular pathogens which can be transmitted to a susceptible host mostly by direct contact, ingestion or via aerosol. On the basis of pathogenicity, host preference and phenotypic characteristics six species of *Brucella* have been commonly listed (Osterman, 2006) with four new species being recently recognised (Ewalt *et al*, 1994; Foster *et al*, 1996; Clavareau *et al*, 1998; Scholz *et al*, 2010; Tiller *et al*, 2010; Banai and Corbel, 2010; Nymo *et al*, 2011). The phylogenetic tree of the *Brucella* spp. is presented in Figure 5.1. Based on their cultural morphology, serotyping and biochemical characteristics, these species may be further sub-divided into sub-types; also known as biovars, or biotypes (Alton *et al*, 1988; OIE, 2009).

The importance of biotyping of *Brucella* species is to provide epidemiological information, establish the characteristics of the agent(s), to handle outbreaks and to facilitate control/eradication strategies (Unver *et al*, 2006; Ica *et al*, 2012). *Brucella abortus* contains 7 biovars (1 to 6 and 9) and *B. melitensis* contains 3 biovars (OIE, 2008). Each biovar may have many strain types. Biovar 1 of *B. abortus* is considered the most prevalent among cattle, however it is also found worldwide in many animal species including sheep and goats, buffalo, horses, camels, and humans. Besides biovar 1, biovar 2 has also been isolated from infected cattle in New South Wales, Australia (Hornitzky and Searson, 1986).

Several authors have described the two common ways for biotyping isolates: firstly the phage typing method which involves lysis by phages; and secondly by examining

oxidative metabolic profiles on selected amino acid and carbohydrate substrates. However, the latter method has disadvantages in that it is hazardous, time consuming and needs adequate laboratory facilities. Biovars of *B. abortus* can also be differentiated by their utilization of $CO_2$, production of $H_2S$, growth on media with dyes (thionin and basic fuchsin), and utilization of monospecific antiserum (OIE, 2009).

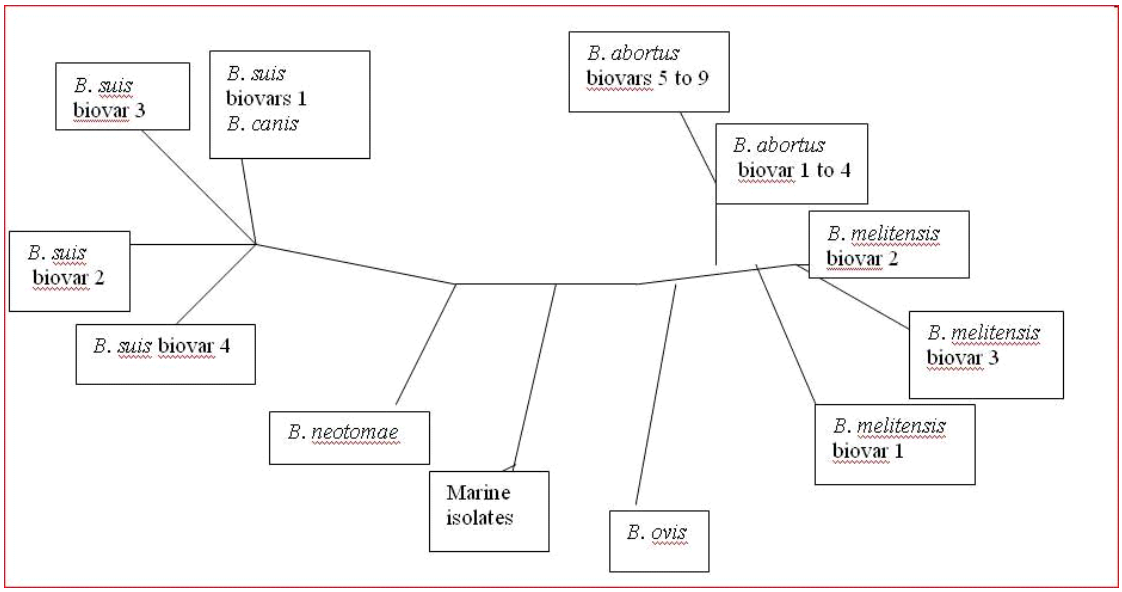
Corbel and Brinley-Morgan (1984) identified significant DNA homology of the species within the genus and similar polynucleotide sequences were detected. As a result, the usual biotyping tests may not always reveal the full extent of differences between biovars, especially where the differences may rely upon a single characteristic. Aldrick (1968) stressed the importance of performing biotyping tests as soon as possible after culturing as the colonies are unstable and may not be visible after repeated subculturing.

Isolation and identification of *Brucella* species are based on culture and phenotypic analysis (biotyping). Although undoubtedly providing valuable information, biotyping was, and remains, a highly specialized and time-consuming approach requiring experienced staff and well-optimized non-commercial reagents ideally used under secure biological containment.

As outlined in Chapter 2 a range of molecular typing techniques are available to differentiate between strains. In Oman, *B. melitensis* biovar-1 is the only type that has been isolated from cattle, camels, sheep and goats in the southern region (Adam and El-Rashied, 2013). However a comprehensive epidemiological study on the strains and types of *Brucella* infecting animal and humans in the Sultanate has not yet been undertaken. In this chapter the findings from the examination of isolates from Oman to

determine their genetic relatedness are reported.

**Figure 5.1 Phylogenetic tree of the *Brucella* spp. (Garritty *et al,* 2005).**




## 5.2 Materials and Methods

### 5.2.1. Collection of samples for bacterial isolation and identification

A total of 14 blood samples were collected (July 2012) from serologically positive (RBPT and cELISA) brucellosis goats originating from a flock (n = 67) in Sohar, which had experienced a series of abortions. The blood samples were collected in sterile test tubes (25mL) containing sterile acid-citrate-dextrose as anticoagulant. The samples were transported to the laboratory in ice packs for bacterial isolation and identification. All microbiological work was conducted in the class II, type A2 biosafety cabinet. The whole facility was restricted to other laboratory staff except for the investigators and all precautions were taken to avoid the spill over infection. The facility was disinfected routinely.

The samples (10mL) were inoculated in duplicate into culture broth vials (Oxoid SIGNAL$^{TM}$ Blood Culture System). The growth indicator device was fixed on each

culture vial aseptically by inserting the needle through the center of the rubber stopper and the cap on the body of the indicator device tightened by turning it clockwise. Following inoculation, the culture systems were incubated at 36 ± 1 °C on a shaker (150 orbits/minute) for the first 24 hours. Thereafter, the system was removed from the shaking apparatus and placed on a shelf of the incubator for the next 6 days. Similarly each of the inoculated vials was incubated in a microaerophilic (5-8% $CO_2$) environment using a $CO_2$ generation system (Oxoid) in a 2.5L gas jar. During incubation, the system was examined twice daily for positive signals of microbial growth (when a small portion of the blood-broth mixture from the culture vials was displaced into the sleeve of indicator system as a result positive pressure developed by microbial growth). Vials with positive signals were sub-cultured as follows. Indicator devices with growth signals were gently mixed, unscrewed and the chamber's contents were cultured on sheep blood agar plates (BAP). The growth signals (blood broth mixture) were also examined by microscopy after applying a Gram stain. The growth on the BAP were examined for colony morphology, hemolytic pattern and processed for catalase and oxidase tests and for urease activity. The catalase test was carried out on a slide by emulsifying a suspected colony in a drop of 3% hydrogen peroxide. The oxidase test was performed using an oxidase test strip (Oxoid, U.K.) and the urease activity of isolates was determined in Christensen's medium (Quinn *et al,* 1984).

### 5.2.2. Procurement of *Brucella* Strains

Previously identified, 21 *Brucellae* were also obtained from the Brucellosis Diagnostic Unit, for bio-typing, molecular identification/typing and antimicrobial susceptibility. These isolates were recovered on microbiological investigation of milk samples (n=186), aborted fetuses (n = 3), placental tissues (n = 2) and uterine swabs (n = 9) of

different animal species (including cattle, sheep, goats and camels) from the Southern region of Oman (Dhofar) from 1997-2007. The sample identification (VRC lab ID), animal species, sample type and year of isolation identification are shown in Table 5.6.

### 5.2.3. Biotyping

For conventional bio-typing at the biovar level, presumptively identified *Brucella* spp. were investigated for their $CO_2$ requirements, $H_2S$ production, sensitivity to dyes (thionin and basic fuchsin) and agglutination with A and M mono-specific sera (Quinn *et al,* 1984; OIE, 2009). For testing sensitivity to thionin and fuchsin, the test was carried out by incorporating the dyes separately in trypticase soy agar at a concentration of 20 µg/ml (1:50,000) or 40 µg/ml (1:25,000). The medium was prepared by heating a 0.1 per cent solution of either dye in a boiling water bath for 20 minutes and then adding it to the required amount of autoclaved agar. The dye was mixed with the agar and poured into Petri dishes. A sterile swab was used to inoculate the dye media with a suspension of the test strain. The inoculated plates were incubated at 37ºC for 3-4 days and then examined for growth. The differential characteristics of biovars of *B. melitensis* and *B. abortus* species Table 5.1) were followed to reach the results.

**Table 5.1 Differential characteristics of the biovars of *Brucella* species**

| **Species** | Biovar | $CO_2$ Requirement | $H_2$ Production | **Growth on Dyes (20microgram/mL)** | | **Agglutination with mono-specific sera** | |
|---|---|---|---|---|---|---|---|
| | | | | Thionin | Basic fuchsin | A | B |
| *B. melitensis* | 1 | - | - | + | + | - | + |
| | 2 | - | - | + | + | + | - |
| | 3 | - | - | + | + | + | + |
| *B. abortus* | 1 | +[a] | + | - | + | + | - |
| | 2 | +[a] | + | - | - | + | - |
| | 3 | +[a] | + | + | + | + | - |
| | 4 | +[a] | + | - | +[b] | - | + |
| | 5 | - | - | + | + | - | + |
| | 6 | - | - | + | + | + | - |
| | 9 | +or _ | + | + | + | - | + |

a strains positive on primary isolation

b Some basic fuchsin-sensitive strains were isolated

### 5.2.4. Determination of antimicrobial susceptibility profiles (antibiogram) of *Brucella melitensis*

*In vitro* antibiotic susceptibility profiles of *B. melitensis* (n = 15) collected over a decade (1998-2008) were assessed to 18 antibiotics/antimicrobials [amoxicillin (AC), ampicillin (AM), ampicillin-sulbactam (AB), amoxicillin clavulanic acid (XL), trimethoprim-sulphamethoxazole (TS), cefotaxime (CT), cefuroxime (XM), ceftriaxone (TX), ceftazidime (TZ), chloramphenicol (CL), enrofloxacin (EF), norfloxacin (NX), gatifloxacin (GA), levofloxacin (LE), gemifloxacin (GEM), ciprofloxacin (CI), moxifloxacin (MX) and ofloxacin (OF)] using the E test following the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2008). *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 (American Type Culture Collection, Rockville, Maryland, USA) were used as the quality control organisms. The details of the procedures used were as follows.

#### 5.2.4.1 Preparation of susceptibility testing medium

The susceptibility testing was performed on Mueller-Hinton agar plates supplemented with 5% sheep blood. The medium was prepared by dissolving 38 grams of dehydrated Mueller-Hinton agar (Oxoid, U.K) in 1 litre of distilled water and this was sterilized by autoclaving at 121˚C for 15 minutes. Immediately after autoclaving, the medium was allowed to cool in a thermostatically controlled water bath set at 50˚C and 5% sheep blood was added to the media and gently mixed to avoid bubbling. The media were then poured into flat-bottomed Petri dishes (150mm) to give a uniform depth of approximately 4 mm. The agar medium was then allowed to cool at room temperature and then was stored at 4˚C in a refrigerator until used.

### 5.2.4.2 Preparation of McFarland turbidity standard and inoculation of plates.

An inoculum equal to 0.5 McFarland turbidity standard (BioMerieux, France) was prepared for each *Brucella* strain by placing bacterial colonies into sterile water. The colonies were diluted to give a final inoculum of $10^5$ to $10^6$ CFU/mL.

After adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of Mueller-Hinton-Blood agar plates was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plates approximately 60˚ each time to ensure an even distribution of inoculum. As a final step the rim of the agar was swabbed. For the determination of antibiotic susceptibility of the *B. melitensis* isolates, antibiotic strips (AB Biodisk, BioMerieux, France) were placed on the surface of the inoculated agar plate using sterile forceps. The plates were inverted and placed in an incubator set at 37˚C. After incubation for 48 hours, the zone of inhibition around each antibiotic strip was read and recorded (Figure 5.2).

### 5.2.5 Molecular identification and typing

This part of the study undertook investigations on 15 isolates of *Brucella* collected between 1998 and 2008 from foetal and milk samples taken from camels originating from the northern region of Oman (Dhofar) and on 14 blood samples isolated from a recently suspected outbreak (2010) of caprine brucellosis in the southern region of Oman (Sohar). All isolates and suspected samples were examined by species specific novel PCR assays (Hinić *et al*, 2008, 2009). All isolates had previously been phage typed and their identities confirmed with standard biochemical procedures (Alton *et al*,

1988; Behroozikhah *et al*, 2005). *In vitro* antimicrobial susceptibility (minimum inhibitory concentrations) (n=15) was determined by the E test as described by CLSI (2008)**.**

### 5.2.5.1 Genomic DNA preparation of *Brucella* isolates and blood samples

The genomic DNA was purified by using a genomic DNA purification kit (Fermentas, Germany) following the manufacturer's instructions.

- 1µl of culture were placed into 1.5ml micro-centrifuge tubes.
- The tubes were centrifuged at 5000 rpm for 10 minutes.
- The supernatant was discarded and the pellet retained.
- The pellet was then suspended in 150µl enzyme buffer (25 mM Tris HCL, 5 mM glucose and 10 mM EDTA) and 500µl Lysostaphin (Sigma) enzyme that had been prepared by dissolving 5mg of the lyophilised content in 100 ml of 0.1 M NaCl.
- The mixture was then incubated at 37°C for 1 hour.
- 400 µl lysis buffer was mixed with 200 µl sample of enzymatic treated mixture.
- The mixture was incubated at 65°C for 30 minutes. This was longer than the 5 minutes recommended by the manufacturer, as this period was not effective.
- Following incubation, 600µl of chloroform was added and the contents gently emulsified by inversion.
- The mixture was then centrifuged at 10,000 rpm for 2 minutes.

- The upper aqueous phase containing DNA was transferred to a fresh 1.5ml eppendorf tube.

- The precipitation solution was prepared by mixing 720µl of nuclease free water with 80µl of 10X concentrated solution supplied in the kit.

- 800µl of the prepared precipitation solution was then added to the mixture.

- The mixture was mixed at room temperature for 1-2 min, and then centrifuged at 10,000 rpm for 2 minutes.

- The supernatant was removed and the DNA pellet dissolved in 100µl of 1.2M NaCl solution.

- 300µl of ethanol was added to the mixture and the DNA precipitated at 20°C for 10 minutes.

- The mixture was then centrifuged at 10,000 rpm for 15 minutes.

- The ethanol was decanted and the pellet washed with 70% pre-chilled ethanol.

- The DNA was resuspended in 100µl of nuclease free water

For the blood samples, individual genomic DNA preparations were made by adding 1.0µl of blood to 20µl of BR-A (Lysis buffer available with BloodReady Multiplex PCR Kit, Genescript, USA). The mixture was mixed well and used directly in the PCR.

### 5.2.5.2 DNA Amplification

The reaction was performed using BactReady$^{TM}$ multiplex PCR system (Genescript, USA). The reaction mixture (20 µl; Table 5.2) was prepared in thin walled, flat cape, DNase-RNase free 0.2 mL tubes (Thermo-Tubes, Thermo-scientific, UK) with 1 µL of template DNA. The genomic target, primer sequence and product size is outlined in Table 5.3. Amplifications were performed using a micro-processed controlled Swift$^{TM}$ Maxi Thermal Cyler Block (ESCO Technologies Inc. France) under the following conditions: activation of Script$^{TM}$ DNA polymerase at 94 for 15 minutes followed by 35 cycles of denaturation (95ºC for 1 minute), annealing (55ºC for 1 minute), extension (72ºC for 1 minute) and a final extension step of 72ºC for 3 minutes. The PCR thermal profile is summarised in Table 5.4. The amplicons were analyzed by agarose gel electrophoresis using a horizontal mini agarose gel electrophoresis system (ENDURO$^{TM}$ Labnet International Inc., Woodbrige, NJ, USA) cell. A mixture of undiluted PCR products (5 µL) and 5X loading dye (1 µL; Fermentas Thermo Fischer Scientific Inc., UK) was loaded onto 1.2% Agarose gel (multipurpose agarose, low EEO, multipurpose, Fischer Scientific Ltd, Loughborough, UK) containing ethidium bromide (0.5 µg/mL; Fischer Scientific Ltd, Loughborough, UK) for DNA staining. The PCR products were loaded using a dye (5x loading dye, Qiagen) to 1.2% agarose gel (TopVision$^{TM}$ Agarose, Fermentas, Germany) containing ethidium bromide (0.5 µG/mL; Fischer Scientific Ltd, Loughborough, UK) in 0.5X TAE buffer. The gels were run in 1X TAE buffer (50X TAE Buffer, Fischer Scientific Ltd, Loughborough, UK) at 80 volts (80mA) for 1 hour. The amplicons were visualised on a transilluminator (Vilbert Lourmart, Cedex France) and saved using a gel documentation systems (DP-CF-011, France). The size of the products was measured

using a ready to use 100 bp molecular marker (O gene-Ruler 100bp DNA ladder, Fermentas, Thermo-scientific, UK).

**Table 5.2 Composition of PCR reaction mixture used for amplification of Brucellae specific genes**

| **Reagents** | **Volume (µl)** | **Final concentration** |
|---|---|---|
| PCR grade (DNAse free) water | 7 | |
| Forward primer | 1 | 50nM |
| Reverse primer | 1 | 50nM |
| DNA solution | 1 | |
| PCR Premix | 10 | |
| **Total volume** | **20** | |

**Table 5.3 DNA target sequence of primers used to detect *Brucella* and PCR product size**

| **Target** | **Sequence** | **Product Size** | **Reference** |
|---|---|---|---|
| *IS711* | ’5GCTTGAAGCTTGCGGACAGT 3’<br>5’ GGCCTACCGCTGCGAAT 3’ | 63 | Hinić *et al*, 2008 |
| *BMEII0466* | ’5GCTTGAAGCTTGCGGACAGT 3’<br>5’GGCCTACCGCTGCGAAT 3’ | 67 | Hinić *et al*, 2008 |
| *BruAb2_0168* | ’5TCGCATCGGCAGTTTCAA3’<br>5’ CCAGCTTTTGGCCTTTTCC3’ | 81 | Hinić *et al*, 2008 |

**Table 5.4 Thermal profiles for PCR of Brucellae amplicons with the set of primers at concentration of 50 nM.**

| Steps | Temperature | Time |
|---|---|---|
| Activation of ScriptTM DNA Polymerase | 94˚C | 15 minutes |
| Denaturation | 94˚C | 40 seconds |
| Annealing | 55˚C | 2 minutes |
| Extension | 65˚C | 5 minutes |
| Final Extension | 72˚C | 3 minutes |
| Total CYCLES = 35 | | |

### 5.2.5.3 Optimization of randomly amplified polymorphic DNA (RAPD) conditions

Since the reproducibility of the RAPD technique is influenced by the reaction components and the machine (Williams *et al,* 1990), the PCR conditions were initially optimised for the concentration of genomic DNA, 10 X PCR buffer, $MgCl_2$, dNTPs, primer and Taq DNA polymerase to obtain reproducible results. The following quantities were used in this reaction.

Thermal Cycler : Eppendorf, Germany

DNA Template : 1.5 µl

Taq DNA polymerase : 0.2 U (MBI, Fermentas, Vilnius, Lithuania)

| | | |
|---|---|---|
| $d_3H_2O$ | : | 9.3 µl |
| 10XPCR Buffer | : | 2.5 µl |
| Gelatin | : | 2.5 µl |
| $MgCl_2$ | : | 3.0 µl |
| $dNTP_S$ | : | 4.0 µl |
| 10 mer Primers | : | 2.0 µl |

The total reaction volume was 25 µl.

The PCR temperature profile was: hot start at 95˚C for 5 minutes; denaturation at 95˚C for 1minute; primer annealing at 34˚C for 1 minute; extension at 72˚C for 2 minutes followed by a final extension at 72˚C for 10 minutes.

**5.2.5.4 RAPD primers**

The oligo decamer primers were synthesized by Gene Link Company (UK). Out of 50 random decamer primers, four were selected on the basis of their pre-tested polymorphic nature. The list of the primers, along with their sequences, is displayed in Table 5.5.

**Table 5.5 List of oligonucleotide RAPD primers along with their sequences used for RAPD-PCR analysis of *B. melitensis***

| S. No. | Primer Name | Sequence (5′3--′) |
|---|---|---|
| 1 | GL DecamerA-09 | GGGTAACGCC |
| 2 | GL Decamer J-05 | CTCCATGGGG |
| 3 | GL Decamer K-01 | CATTCGAGCC |
| 4 | GL Decamer K-19 | CACAGGCGGA |

Fifteen isolates were explored for RAPD analysis by means of 4 oligonucleotide polymorphic RAPD primers. Each primer-template yielded distinct, easily detectable bands of variable intensities. Two of the 15 isolates failed to yield any band and were excluded from the analysis (RAPD data of 13 isolates is shown in figure 5.5). Considering all the primers and isolates, a total of 111 bands were obtained. The genetic similarity matrix of RAPD data for 13 isolates was constructed based on Nei and Li's (1979) coefficient of similarity.

## 5.3 Results

### 5.3.1 Isolation and identification

Of the 14 serologically positive (RBPT and cELISA) caprine blood samples, 7 yielded positive signals for growth between 48-60 hours of incubation in an aerobic environment. On sub-culturing, all samples yielded translucent, smooth, convex, non-hemolytic colonies of approximately 1mm diameter on blood agar plates after 36 hours of incubation. Upon prolonged incubation (56 hours), colonies became yellowish grey, however remained smooth and convex. All cultures did not require supplementary $CO_2$ for growth. Gram-negative cocco-bacilli were observed by microscopy (100×) after Gram staining of the colonies. All isolates (n = 7) were catalase, oxidase and urease positive and inhibited by thionin (1:50000) dye on serum dextrose agar. All isolates also showed agglutination reaction with monospecific antiserum M and were negative for antiserum A on the slide agglutination test. Similarly, *Brucella* isolates obtained from the repository of Oman and originating from the Dhofar region had the same biochemical reactions and had agglutination with mono-specific antiserum M. These results confirmed the isolates as *B. melitensis* belonging to biovar 1 (Table 5.7).

**Table 5.6 Details of previous and recent isolates of *Brucella melitensis* strains (n = 28).**

| Strain ID | Animal | Location | Year of Isolation | Source of isolation |
|---|---|---|---|---|
| B_mel VRC1 | Cow | Dhofar | 1997 | Milk |
| B_mel VRC2 | Cow | Dhofar | 1999 | Milk |
| B_mel VRC3 | Cow | Dhofar | 1999 | Milk |
| B_mel VRC4 | Cow | Dhofar | 1999 | Milk |
| B_mel VRC5 | Cow | Dhofar | 2003 | Milk |
| B_mel VRC6 | Cow | Dhofar | 2004 | Milk |
| B_mel VRC7 | Cow | Dhofar | 2004 | Milk |
| B_mel VRC8 | Goat | Dhofar | 2004 | Milk |
| B_mel VRC9 | Goat | Dhofar | 2004 | Fetus |
| B_mel VRC10 | Goat | Dhofar | 2004 | Milk |
| B_mel VRC11 | Camel | Dhofar | 2004 | Milk |
| B_mel VRC12 | Camel | Dhofar | 2004 | Milk |
| B_mel VRC13 | Camel | Dhofar | 2005 | Milk |
| B_mel VRC14 | Camel | Dhofar | 2005 | Milk |

| Strain ID | Animal | Location | Year of Isolation | Source of isolation |
|---|---|---|---|---|
| B_mel VRC15 | Cow | Dhofar | 2007 | Milk |
| B_mel VRC16 | cow | Dhofar | 1997 | Placenta |
| B_mel VRC17 | Goat | Dhofar | 1997 | Placenta |
| B_mel VRC18 | Goat | Dhofar | 1997 | Fetus |
| B_mel VRC19 | Goat | Dhofar | 1997 | Milk |
| B_mel VRC20 | Goat | Dhofar | 1997 | Milk |
| B_mel VRC21 | Goat | Dhofar | 1997 | Milk |
| B_mel VRC22 | Goat | Dhofar | 1998 | Fetus |
| B_mel VRC23 | Goat | Saham | 2010 | Blood |
| B_mel VRC24 | Goat | Saham | 2010 | Blood |
| B_mel VRC25 | Goat | Saham | 2010 | Blood |
| B_mel VRC26 | Goat | Saham | 2010 | Blood |
| B_mel VRC27 | Goat | Saham | 2010 | Blood |
| B_mel VRC28 | Goat | Saham | 2010 | Blood |

**Table 5.7 Biochemical characteristics and typing results of *Brucella* species from livestock in Oman**

| Strain ID | Catalase | Oxidase | Urease | $H_2S$ production | $CO_2$ requirement | Growth on dyes | | Agglutination with monospecific antisera | | *Brucella* Species | Biovar |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Basic Fuchsin | Thionin | A | M | | |
| B_mel VRC1 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC2 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC3 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC4 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC5 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC6 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC7 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC8 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |

| Strain ID | Catalase | Oxidase | Urease | $H_2S$ production | $CO_2$ requirement | Growth on dyes | | Agglutination with monospecific antisera | | *Brucella* Species | Biovar |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Basic Fuchsin | Thionin | A | M | | |
| B_mel VRC9 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC10 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC11 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC12 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC13 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC14 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC15 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC16 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC17 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |

| Strain ID | Catalase | Oxidase | Urease | $H_2S$ production | $CO_2$ requirement | Growth on dyes | | Agglutination with monospecific antisera | | *Brucella* Species | Biovar |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Basic Fuchsin | Thionin | A | M | | |
| B_mel VRC18 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC19 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC20 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC21 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC22 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC23 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC24 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC25 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC26 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |

| Strain ID | Catalase | Oxidase | Urease | $H_2S$ production | $CO_2$ requirement | Growth on dyes | | Agglutination with monospecific antisera | | *Brucella* Species | Biovar |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Basic Fuchsin | Thionin | A | M | | |
| B_mel VRC27 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |
| B_mel VRC28 | + | + | + | - | - | + | - | - | + | *B. melitensis* | 1 |

### 5.3.2 Antimicrobial susceptibility of *Brucella melitensis* isolates

According to antibiotic susceptibility using the E-test, all isolates (n=28) were susceptible to antibiotics/antimicrobials of the beta-lactam group (including amoxicillin, ampicillin, ampicillin-sulbactam, amoxicillin clavulanic acid, cefotaxime, ceftriaxone) except for cefuroxime and ceftazidime. These latter antibiotics showed higher $MIC_{90}$ values (16 and 24 µg/mL $MIC_{90}$ respectively) (Table 5.8 and 5.9). Trimethoprim-sulphamethoxazole showed good activity since it had 0.125µg/mL $MIC_{90.}$ Similarly all strains were inhibited by chloramphenicol at 2 µg/mL. Of the quinolones, ciprofloxacin (0.125 µg/mL $MIC_{90}$) showed good anti-*Brucella* activity compared to the other antimicrobials of the same group.

**Table 5.8 Inhibitory concentration of different antimicrobials against *B. melitensis* isolates (n = 28) recovered from livestock in Oman**

| Strain ID | Antimicrobials | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AC | AM | AB | XL | TS | CT | XM | TX | TZ | CL | EF | NX | GA | LE | GEM | CI | MX | OF |
| **B_mel VRC1** | 0.38 | 2 | 4 | 1 | 0.05 | 0.8 | 12 | 1 | 24 | 2 | 0.5 | 0.5 | 0.38 | 0.19 | 1 | 0.13 | 0.38 | 0.5 |
| **B_mel VRC2** | 0.38 | 1.5 | 3 | 0.8 | 0.05 | 1 | 6 | 0.5 | 16 | 1.5 | 0.3 | 0.8 | 0.38 | 0.19 | 1 | 0.13 | 0.25 | 0.5 |
| **B_mel VRC3** | 0.19 | 2 | 1.5 | 0.5 | 0.03 | 0.5 | 8 | 1 | 12 | 2 | 0.2 | 0.5 | 0.38 | 0.13 | 1.5 | 0.13 | 0.38 | 0.5 |
| **B_mel VRC4** | 0.75 | 8 | 6 | 1 | 0.05 | 1 | 6 | 0.8 | 24 | 1.5 | 0.5 | 0.5 | 0.38 | 0.13 | 1 | 0.13 | 0.38 | 0.5 |
| **B_mel VRC5** | 0.38 | 1.5 | 4 | 0.8 | 0.13 | 1.5 | 12 | 4 | 16 | 1.5 | 0.3 | 0.8 | 0.38 | 0.09 | 1 | 0.09 | 0.25 | 0.3 |
| **B_mel VRC6** | 0.25 | 1.5 | 6 | 0.5 | 0.13 | 1 | 16 | 1.5 | 16 | 2 | 0.5 | 0.5 | 0.5 | 0.19 | 1.5 | 0.13 | 0.38 | 0.5 |
| **B_mel VRC7** | 0.75 | 8 | 6 | 1 | 0.06 | 6 | 12 | 6 | 16 | 2 | 0.5 | 1 | 0.5 | 0.19 | 0.75 | 0.13 | 0.25 | 0.5 |
| **B_mel VRC8** | 0.38 | 2 | 3 | 0.8 | 0.06 | 1 | 6 | 1 | 12 | 1.5 | 0.5 | 0.8 | 0.38 | 0.13 | 0.75 | 0.13 | 0.19 | 0.4 |
| **B_mel VRC9** | 0.38 | 1.5 | 4 | 0.8 | 0.05 | 1 | 6 | 0.8 | 12 | 2 | 0.5 | 0.8 | 0.5 | 0.09 | 0.75 | 0.09 | 0.38 | 0.4 |
| **B_mel VRC10** | 0.25 | 1.5 | 6 | 0.8 | 0.02 | 0.5 | 4 | 0.8 | 12 | 1 | 0.4 | 0.8 | 0.09 | 0.09 | 1 | 0.09 | 0.25 | 0.4 |
| **B_mel VRC11** | 0.5 | 2 | 3 | 1 | 0.05 | 1.5 | 12 | 16 | 8 | 1.5 | 0.5 | 1 | 0.38 | 0.13 | 1 | 0.13 | 0.13 | 0.5 |

| Strain ID | Antimicrobials | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **AC** | **AM** | **AB** | **XL** | **TS** | **CT** | **XM** | **TX** | **TZ** | **CL** | **EF** | **NX** | **GA** | **LE** | **GEM** | **CI** | **MX** | **OF** |
| **B_mel VRC12** | 0.38 | 0.8 | 6 | 0.8 | 0.09 | 1.5 | 6 | 1 | 24 | 1.5 | 0.3 | 0.8 | 0.38 | 0.13 | 1 | 0.09 | 0.25 | 0.4 |
| **B_mel VRC13** | 0.13 | 2 | 2 | 0.5 | 0.05 | 0.5 | 4 | 0.5 | 12 | 1 | 0.5 | 0.5 | 0.19 | 0.13 | 0.75 | 0.06 | 0.19 | 0.2 |
| **B_mel VRC14** | 0.19 | 1.5 | 1 | 0.4 | 0.06 | 0.5 | 4 | 0.5 | 8 | 1.5 | 0.2 | 0.4 | 0.13 | 0.06 | 0.75 | 0.06 | 0.19 | 0.3 |
| **B_mel VRC15** | 0.25 | 2 | 1.5 | 0.4 | 0.09 | 0.5 | 6 | 1 | 12 | 1.5 | 0.3 | 0.4 | 0.13 | 0.06 | 0.75 | 0.06 | 0.19 | 0.3 |
| **B_mel VRC16** | 0.19 | 1.5 | 2 | 0.8 | 0.02 | 1 | 8 | 1.5 | 16 | 1 | 0.3 | 0.4 | 0.38 | 0.09 | 0.5 | 0.09 | 0.25 | 0.2 |
| **B_mel VRC17** | 0.75 | 8 | 4 | 1 | 0.13 | 1 | 12 | 1 | 24 | 1.5 | 0.5 | 0.5 | 0.5 | 0.19 | 1 | 0.13 | 0.13 | 0.3 |
| **B_mel VRC18** | 0.25 | 2 | 3 | 0.4 | 0.06 | 0.5 | 6 | 2 | 16 | 1.5 | 0.3 | 0.5 | 0.19 | 0.13 | 1 | 0.09 | 0.38 | 0.3 |
| **B_mel VRC19** | 0.25 | 2 | 8 | 1 | 0.13 | 1.5 | 6 | 4 | 12 | 1 | 0.5 | 1 | 0.5 | 0.19 | 0.5 | 0.06 | 0.13 | 0.4 |
| **B_mel VRC20** | 0.38 | 1.5 | 6 | 1 | 0.13 | 1.5 | 12 | 1.5 | 16 | 1 | 0.3 | 0.5 | 0.13 | 0.06 | 1 | 0.13 | 0.38 | 0.5 |
| **B_mel VRC21** | 0.5 | 8 | 6 | 1 | 0.09 | 3 | 12 | 0.8 | 24 | 2 | 0.4 | 0.8 | 0.13 | 0.13 | 1 | 0.09 | 0.19 | 0.5 |
| **B_mel VRC22** | 0.5 | 4 | 2 | 75 | 0.13 | 0.5 | 16 | 4 | 24 | 2 | 0.2 | 1 | 0.19 | 0.13 | 0.75 | 0.06 | 0.13 | 0.2 |
| **B_mel VRC23** | 0.75 | 8 | 8 | 1 | 0.05 | 1.5 | 8 | 8 | 24 | 1.5 | 0.2 | 0.5 | 0.13 | 0.19 | 1 | 0.09 | 0.38 | 0.4 |
| **B_mel VRC24** | 0.38 | 8 | 8 | 0.8 | 0.09 | 1 | 4 | 1.5 | 12 | 1.5 | 0.5 | 0.5 | 0.38 | 0.13 | 1 | 0.13 | 0.19 | 0.4 |

| Strain ID | Antimicrobials | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **AC** | **AM** | **AB** | **XL** | **TS** | **CT** | **XM** | **TX** | **TZ** | **CL** | **EF** | **NX** | **GA** | **LE** | **GEM** | **CI** | **MX** | **OF** |
| **B_mel VRC25** | 0.75 | 8 | 12 | 1 | 0.13 | 3 | 12 | 2 | 12 | 1 | 0.3 | 0.5 | 0.09 | 0.09 | 1 | 0.13 | 0.13 | 0.5 |
| **B_mel VRC26** | 0.5 | 8 | 8 | 1 | 0.02 | 6 | 16 | 4 | 16 | 1.5 | 0.2 | 0.4 | 0.13 | 0.13 | 1.5 | 0.13 | 0.13 | 0.3 |
| **B_mel VRC27** | 0.75 | 8 | 8 | 1 | 0.13 | 0.5 | 8 | 4 | 12 | 1.5 | 0.2 | 0.4 | 0.19 | 0.19 | 1 | 0.09 | 0.19 | 0.2 |
| **B_mel VRC28** | 0.75 | 4 | 6 | 1 | 0.13 | 1.5 | 12 | 6 | 16 | 1 | 0.4 | 0.5 | 0.38 | 0.06 | 1.5 | 0.06 | 0.38 | 0.3 |

B_mel VRC1 thru B_mel VRC21 = Isolates from Dhofar (South) of Oman

B_mel VRC22 thru B_mel VRC28 = Isolates from Sohar (North) of Oman

AC: amoxicillin; AM: ampicillin; AB: ampicillin-sulbactam; XL: amoxicillin clavulanic acid; TS: trimethoprim-sulphamethoxazole; CT: cefotaxime; XM: cefuroxime; TX: ceftriaxone; TZ: ceftazidime; CL: chloramphenicol; EF: enrofloxacin; NX: norfloxacin; GA: gatifloxacin; LE: levofloxacin; GE: gemifloxacin; CI: ciprofloxacin; MX: moxifloxacin; OF: ofloxacin

**Figure 5.2 Antimicrobial susceptibility testing of *Brucella melitensis*. Inoculated Mueller Hinton Blood agar plates.**

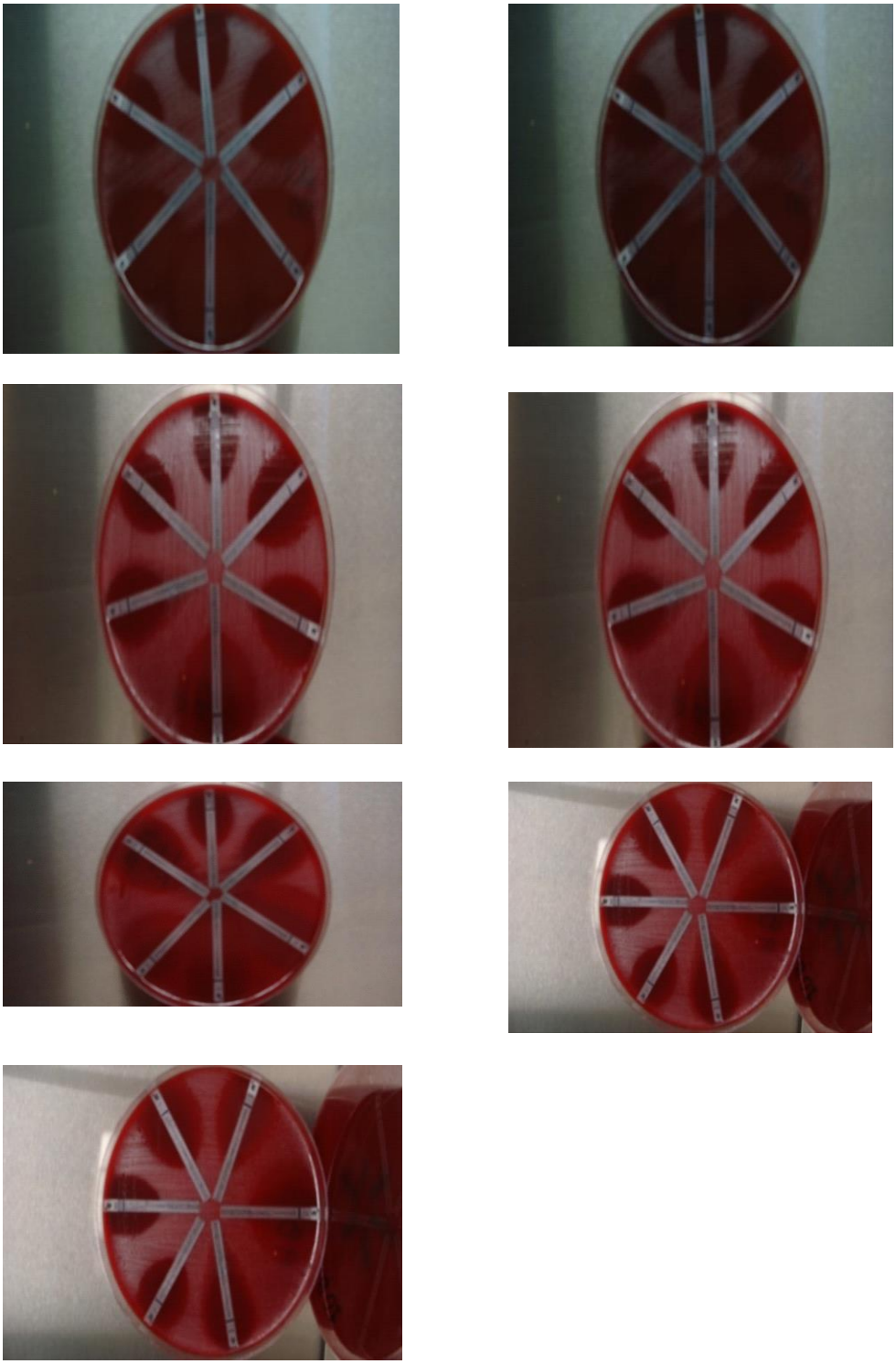


**Table 5.9 Minimum inhibitory Concentration (MICs) of selected Beta-lactam antibiotics against *B. melitensis* (previously and recently isolated)**

| Antimicrobials | Minimum Inhibitory Concentrations (µg/mL) and Number of Isolates | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0.023** | **0.032** | **0.047** | **0.064** | **0.094** | **0.125** | **0.19** | **0.25** | **0.38** | **0.5** | **0.75** | **1** | **1.5** | **2** | **3** | **4** | **6** | **8** | **12** | **16** | **24** |
| Amoxicillin (AC) | - | - | - | - | - | 1 | 3 | 5 | **8*** | 4 | **7**** | - | - | - | - | - | - | - | - | - | - |
| Ampicillin (AM) | -- | - | - | - | - | - | - | - | - | - | 1 | - | 8 | **8*** | | 2 | | **9**** | - | - | - |
| Ampicillin-Sulbactam (AB) | - | - | - | - | - | - | - | - | - | - | - | 1 | 2 | 3 | 4 | **4** | **8*** | **5**** | **1** | - | - |
| Amoxicillin Clavulanic acid (XL) | | | | | | | | | 3 | 3 | **9*** | **13**** | - | - | - | - | - | - | - | - | - |
| Trimethoprim-sulphamethoxazole (TS) | 3 | 1 | 7 | **4*** | 4 | **9**** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Cefotaxime (CT) | - | - | - | - | - | - | - | - | - | 8 | 1 | **8*** | 8 | - | **2**** | | 2 | - | - | - | - |
| Cefuroxime (XM) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 4 | 8 | 4* | 9 | 3** | |
| Ceftriaxone (TX) | | | | | | | | | | 3 | 4 | 6 | **4*** | 2 | - | 5 | **2*** | 1 | 1 | | |
| Ceftazidime (TZ) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 | 10 | 9* | 7** |
| Chloramphenicol (CL) | - | - | - | - | - | - | - | - | - | - | - | 7 | 14* | 7** | - | - | - | - | - | - | - |

| **Antimicrobials** | **Minimum Inhibitory Concentrations (µg/mL) and Number of Isolates** | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0.023** | **0.032** | **0.047** | **0.064** | **0.094** | **0.125** | **0.19** | **0.25** | **0.38** | **0.5** | **0.75** | **1** | **1.5** | **2** | **3** | **4** | **6** | **8** | **12** | **16** | **24** |
| Enrofloxacin (EF) | - | - | - | - | - | - | 6 | 8* | 3 | **11**** | -- | - | - | - | - | - | - | - | - | - | - |
| Norfloxacin (NX) | - | - | - | - | - | - | | | 5 | **12*** | 7 | **4**** | - | - | - | - | - | - | - | - | - |
| Gatifloxacin (GA) | - | - | - | - | 2 | 6 | 4 | | **11*** | **5**** | - | - | - | - | - | - | - | - | - | - | - |
| Levofloxacin (LE) | - | - | - | 4 | 5 | **11*** | **8**** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Gemifloxacin (GEM) | - | - | - | - | - | - | - | - | | 2 | 7 | **15*** | **4**** | - | - | - | - | - | - | - | - |
| Ciprofloxacin (CI) | - | - | - | 6 | **9*** | **13**** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Moxifloxacin (MX) | - | - | - | - | - | 6 | 7 | **6*** | **9**** | - | - | - | - | - | - | - | - | - | - | - | - |
| Ofloxacin (OF) | - | - | - | - | - | - | 4 | 7 | **7*** | **10**** | - | - | - | - | - | - | - | - | - | - | - |

Asterisks (* & **) indicate MIC50 and MIC 90 values, respectively

### 5.3.3 Molecular identification of *Brucellae* in blood samples by conventional PCR

All presumptively identified *Brucella* isolates (n = 21) and 7 of the 14 brucellosis suspect blood samples were correctly identified as *Brucella* by PCR amplification of IS711 target (Figure 5.3). All of these samples were further identified as *B. melitensis* as they produced 67 bp amplicons (Figure 5.5). Based on the analysis of the PCR results, biochemical reactions and agglutination with mono-specific antiserum indicated the existence of *B. melitensis* biovar 1 in the Sultanate of Oman (Table 5.7).

**Figure 5.3 Conventional PCR amplification of *B. melitensis* gDNA prepared from presumptively identified *Brucella* isolates from the southern region of Oman (Dhofar) (# 7-12) and from blood samples from suspected outbreak of brucellosis in the northern region of Oman (Sohar) (#1-6).**

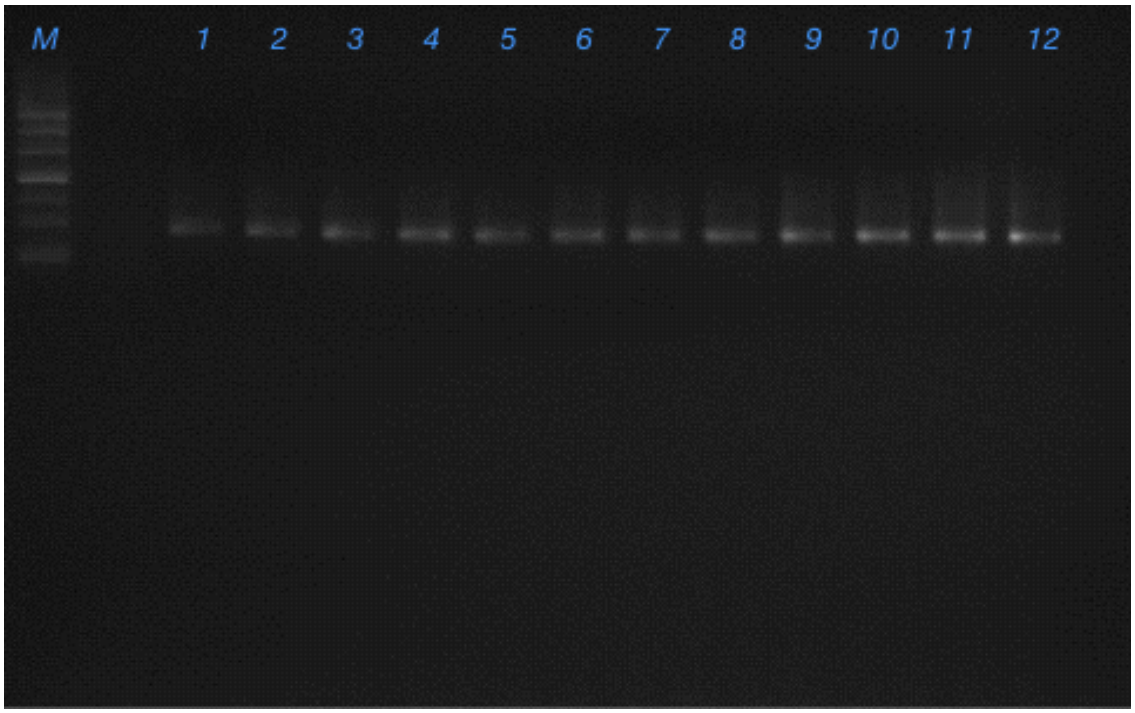
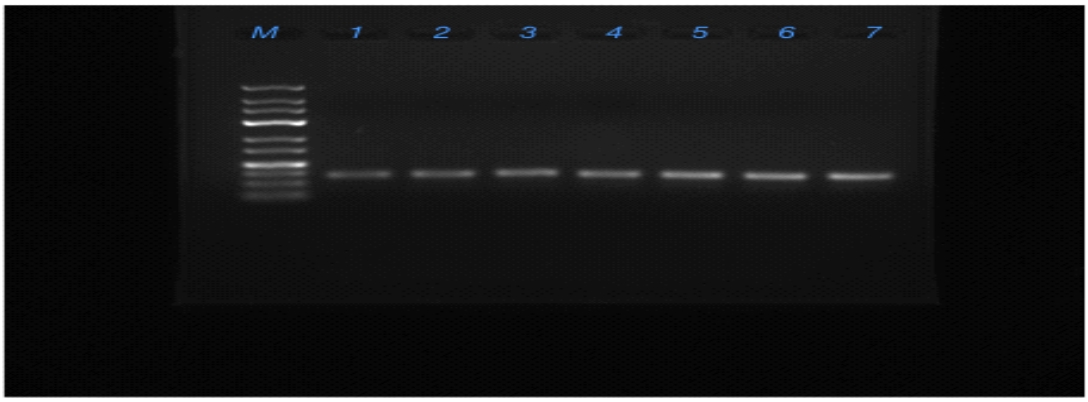


**Figure 5.4 Conventional PCR amplification from blood using the primer set (BMEII0466). Samples of aborted goats from northern region (Sohar) of Oman Lane (1 through 7); M: GelPilot low range (Fermentas)**




Fifteen isolates were explored by RAPD analysis using 4 oligonucleotide polymorphic RAPD primers. Each primer-template yielded distinct, easily detectable bands of variable intensities (Figure 5.5). Considering all of the primers and isolates, a total of 111 bands were obtained. The genetic similarity matrix of RAPD data for 13 isolates was constructed based on Nei and Li's (1979) coefficient of similarity. The similarity matrix (13.3% to 93.7%) indicated a significant difference between isolates (Table 5.10. The UPGMA cluster of the 13 isolates further revealed associations based on the RAPD analysis. The 13 isolates were classified into 4 clusters/clades (Figure 5.6). These results showed that the genetic distances among the isolates might be attributed to time differences between the culture of strains and perpetuation of the organism within the host.

**Figure 5.5 RAPD-PCR amplification profile of B. melitensis (biovar 1) with a single set of primers.**

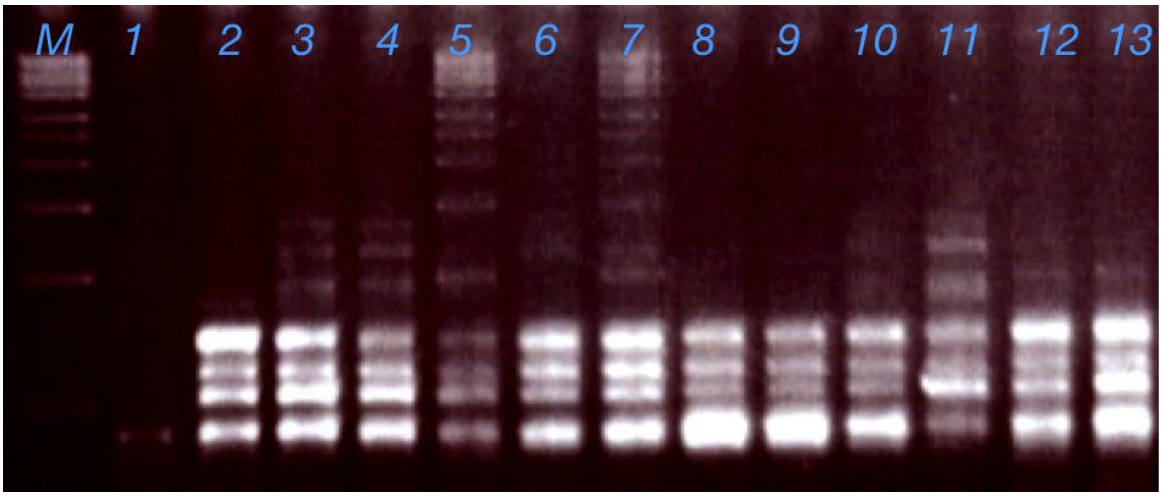



**Figure 5.6 Dendrogram of *B. melitensis* isolates (1 to 13) obtained from similarity matrix based on Nie's UPGMA**

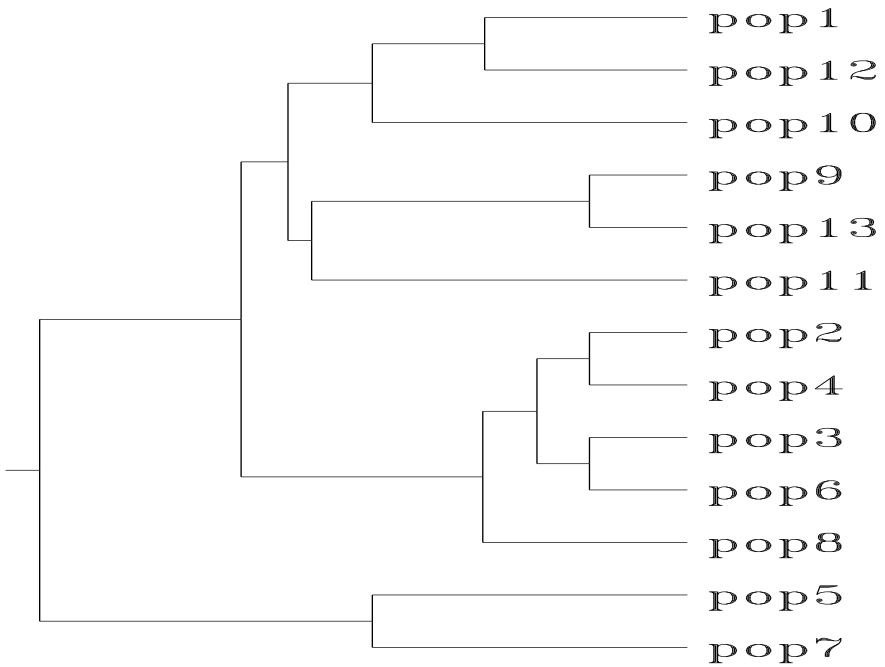


**Table 5.10 Similarity matrix of *B. melitensis* (n = 13) recovered from aborted camel foetus and camel milk. (Nei's genetic identity above the diagonal and genetic distance below the diagonal)**

| Pop ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **** | 0.7500 | 0.7500 | 0.6875 | 0.6875 | 0.8125 | 0.7500 | 0.7500 | 0.7500 | 0.8125 | 0.6875 | 0.8750 | 0.6875 |
| 2 | 0.2877 | **** | 0.8750 | 0.9375 | 0.6875 | 0.9375 | 0.6250 | 0.8750 | 0.7500 | 0.6875 | 0.6875 | 0.7500 | 0.8125 |
| 3 | 0.2877 | 0.1335 | **** | 0.9375 | 0.6875 | 0.9375 | 0.7500 | .08750 | 0.7500 | 0.6875 | .6875 | 0.7500 | 0.6875 |
| 4 | 0.3747 | 0.0645 | 0.0645 | **** | 0.6250 | 0.8750 | 0.6875 | 0.8125 | 0.6875 | 0.6250 | 0.6250 | 0.6875 | 0.7500 |
| 5 | 0.3747 | 0.3747 | 0.3747 | 0.4700 | **** | 0.7500 | 0.8125 | 0.6875 | 0.5625 | 0.6250 | 0.6250 | 0.6875 | 0.5000 |
| 6 | 0.2076 | 0.0645 | 0.0645 | 0.1335 | 0.2877 | **** | 0.6875 | 0.9375 | 0.8125 | 0.7500 | 0.7500 | 0.8125 | 0.7500 |
| 7 | 0.2877 | 0.4700 | 0.2877 | 0.3747 | 0.2076 | 0.3747 | **** | 0.6250 | 0.6250 | 0.5625 | 0.6875 | 0.7500 | 0.5625 |
| 8 | 0.2877 | 0.1335 | 0.1335 | 0.2076 | 0.3747 | 0.0645 | 0.4700 | **** | 0.8750 | 0.8125 | 0.8125 | 0.8750 | 0.8125 |
| 9 | 0.2877 | 0.2877 | 0.2877 | 0.3747 | 0.5754 | 0.2076 | 0.4700 | 0.1335 | **** | 0.8125 | 0.8125 | 0.8750 | 0.9375 |
| 10 | 0.2076 | 0.3747 | 0.3747 | 0.4700 | 0.4700 | 0.2877 | 0.5754 | 0.2076 | 0.2076 | **** | 0.7500 | 0.8125 | 0.7500 |
| 11 | 0.3747 | 0.3747 | 0.3747 | 0.4700 | 0.4700 | 0.2877 | 0.3747 | 0.2076 | 0.2076 | 0.2877 | **** | 0.8125 | 0.7500 |
| 12 | 0.1335 | 0.2877 | 0.2877 | 0.3747 | 0.3747 | 0.2076 | 0.2877 | 0.1335 | 0.1335 | 0.2076 | 0.2076 | **** | 0.8125 |
| 13 | 0.3747 | 0.2076 | 0.3747 | 0.2877 | 0.6931 | 0.2877 | 0.2076 | 0.5754 | 0.2076 | 0.2076 | 0.0645 | 0.2877 | **** |

## 5.4 Discussion

Understanding the epidemiological aspects of brucellosis is pivotal before devising a disease control program. Included in these aspects, is the need to have knowledge of the causation of the disease (type of etiological agent) and the host species involved. Consequently the isolation, typing and subtyping of the organism(s) causing brucellosis in the Sultanate are crucial for planning an effective program to control the disease.

For phenotypic identification of *Brucella* at the biovar level, bacteriological methods in combination with morphological, cultural and biochemical characteristics are followed. Classification of strains into species is based on their natural host preference, sensitivity to *Brucella* phages [Tbilisi (Tb), Weybridge (Wb), Berkeley ($Bk_2$) and Izatnagar (Iz)] and oxidative metabolic profiles (OIE, 2009). Speciation is carried out by determining aerobic requirement for primary isolation, $H_2S$ production, growth in the presence of dyes (thionin and basic fuchsin), and agglutination response to mono-specific antisera (A and M) (Corbel and Brinley-Morgan, 1975; Alton *et al*, 1988; OIE 2009; Godfroid *et al,* 2010). The foregoing protocols are used for typing and enable differentiation of the species and biotypes of *Brucella*.

In the present study, all isolates were identified as *B. melitensis*. These isolates were recovered from various samples (milk, uterine discharges, placenta and fetus) from cows, sheep, goats and camels from the endemic (southern) and "disease free" (northern) regions of Oman. All isolates of *B. melitensis* (n = 28) were classified as biovar 1 and it is likely that this isolate is responsible for most, if not all, outbreaks of brucellosis in Oman.

There are 3 biotypes of *B. melitensis*, which have been isolated in different frequencies from different countries. *Brucella melitensis* biotype 3 is the most prevalent biotype in

countries of the Middle East. Biotypes 1 and 2 are found to a lower extent in these regions, but are more common in south-eastern Europe (OIE, 2009). The results of the present study are in line with those obtained by Nicoletti (1986) who isolated and identified *B. melitensis* from milk samples of goats in Dhofar. Similarly, *B. melitensis* has been isolated from human specimens submitted for diagnosis of brucellosis from patients presented to the major hospitals of the country (Scrimgeour *et al,* 1999).

The route of entry of brucellosis into Dhofar is not known; however, it has been alleged, and is likely, that the disease entered through the importation of animals from other infected countries (including Yemen). Recent genomic analyses (single nucleotide polymorphism; SNP) of 32 isolates of *B. melitensis* from Oman at Northern Arizona University have shown lineages of the local strains with African strains (Nigeria, Chad, Tanzania) (Jeffery Foster, personal communication). This information is also supported by the animal importation policy of Oman, with most small ruminants imported originating from African nations.

The endemicity of the disease in Dhofar is of concern as livestock from this region are a potential source of infection for livestock in the disease free regions (North of Oman). Since inter-strain genomic variability was not observed in the SNP analysis described, the occurrence of the outbreak in Sohar in 2010 would appear to be a result of the transportation of diseased animals from the south to the north of Oman.

Investigations on the role of *B. melitensis* Rev 1 associated abortions in Dhofar have not been investigated, and in this region livestock are widely vaccinated with Rev 1 as part of the control program. Isolation of *B. melitensis* Rev 1 from aborted fetuses and milk of small ruminants has been reported elsewhere (Blasco, 1997, Pishva and Salehi, 2008; Bardenstein *et al*, 2002; European Commission, 2001). In this study, none of the *B.*

*melitensis* isolates showed phenotypic characters similar to those of *B. melitensis* Rev 1 and in this situation it would appear that the abortions were not associated with the vaccinal strain. In conclusion, *B. melitensis* biotype 1 was the only strain isolated from cases of brucellosis in Oman and it is likely that this strain is of importance in the country.

Brucellosis is one of the most common zoonotic infections in the world (Ariza *et al*, 2007; Dean *et al*, 2012) and is considered a reemerging disease in many parts of the world (WHO, 2006a; Russo *et al*, 2009). Nearly half a million new human cases of brucellosis are registered annually (Pappas *et al*, 2006). The disease is transmitted to humans through the consumption of raw milk, unpasteurized dairy products or through direct contact with infected animals, their tissues (primarily placentas) and aborted fetuses. In humans brucellosis causes debility, fever, sweating, fatigue, weight loss, headaches, and joint pain persisting for weeks to months. Neurological complications, endocarditis and multiple abscessation (testicular or bone) can also develop (Corbel, 2006). In addition, brucellosis causes major economic losses through time lost by patients from normal daily activities (Corbel, 2006) and losses through reduced animal production (Roth *et al*, 2003).

Oman has a high incidence of human brucellosis (Idris *et al*, 1993) along with other middle eastern countries including Saudi Arabia, Yemen, United Arab Emirates, Palestine, Syria, Jordan, Iran, Iraq, and Turkey (Refai, 2002). In the Middle East, Bahrain is the only country which has not reported the disease (Refai, 2002). The disease has had a significant impact on the Indian subcontinent where the disease is widespread with a range of prevalences reported (Vaishnavi and Kumar, 2007). Also significant proportions of humans seropositive for brucellosis were reported from India

in samples of high risk individuals (veterinarians and para-veterinarians, shepherds, butchers and animal owners) (Agasthya *et al,* 2007; El Tahir *et al,* 2011).

Brucellosis is an important and ongoing public health problem in the Sultanate, and nearly 95% of cases are seen in the southern region (Dhofar) where semi-nomadic Bedouins live in the mountainous region along with their animal flocks (cows, camels, goats). Early human serological investigations (Idris *et al,* 1993) indicated that nearly 1% of healthy residents (especially children) of this region had been exposed to infection and samples for bacteriological culture invariably yielded *B. melitensis* (Scrimgeour *et al,* 1999). Subsequent investigations of human brucellosis have shown that the ingestion of raw milk, milk products (in 63% of cases) and direct contact with animals (in 87% of cases) were the major routes of infection in children (El-Amin *et al,* 2001).

*Brucella* spp., are intracellular pathogens, that survive within the scavenger cells (macrophages). Therefore, the treatment of brucellosis requires not only combined regimens of antibiotics but also use of antimicrobials that have the ability to attain optimum MICs within the macrophages and retain efficacy even in acidic environments (Pappas *et al,* 2005). The World Health Organization Expert Committee recommends oral combination of doxycycline and rifampicin for 6 weeks for brucellosis (WHO, 2006). Alternatively, doxycycline-streptomycin and doxycycline-sulphamethoxazone-trimethoprim (SXT) combinations are advised (Solera *et al,* 1997b; Pappas *et al,* 2005b). However controlled clinical treatment trials using SXT, newer macrolides and beta-lactams have shown poor results (Falagas and Bliziotis, 2006). Treatment failures and relapses are major problems in the management of brucellosis (WHO, 2006) and are mostly related to pharmacokinetics and pharmacodynamics of antimicrobials rather

than resistance development. The high incidence of relapse, resistance to rifampin (especially in regions with endemic tuberculosis), toxic effects (oto-nephrotoxicity) of streptomycin, and the risk of emergence of resistant *Brucella* strains (Marianelli *et al*, 2004; Pappas *et al*, 2005; Ariza *et al*, 2007) have led to the investigation of new therapeutic options for brucellosis (Mehmet *et al*, 2013). Only a few reports on *in vitro* antimicrobial susceptibilities of *Brucella spp.* using different methods, including broth microdilution (Rubinstein *et al*, 1991; Gur *et al*, 1999), agar dilution (Garcia-Rodriguez, *et al*, 1995; Yamazhan *et al*, 2005), and E-test (Gur *et al*, 1999; Bodur *et al*, 2003; Baykam *et al*, 2004) are available. The uses of *Brucella* agar (Rubinstein *et al*, 1991) and Mueller-Hinton agar supplemented with 5% sheep blood (Gur *et al*, 1999; Baykam *et al*, 2004) have been described for susceptibility testing. E-test is a simple, reliable, reproducible, and less laborious and time consuming method for antimicrobial susceptibility testing and has been reported for the testing of *Brucella* strains (Gur *et al*, 1999; Baykam *et al*, 2004; Yamazhan *et al*, 2005). Non-significant differences in MICs of E-, broth and agar microdilution techniques have been reported (Gur *et al*, 1999) and these differences are believed to be due to different strains and methodologies used (Akova *et al*, 1999; Yamazhan *et al*, 2005). In view of the highly infectious nature of the pathogen, the E-test method was used to investigate *in vitro* susceptibilities of *B. melitensis* strains to 18 antimicrobials.

In the present study a high level of resistance ($MIC_{90} \leq 8$) was noted in ampicillin and its potentiated form (ampicillin-sulbactam), whereas MICs were markedly low for amoxicillin and amoxicillin-clavulanic acid ($MIC_{90} \leq 0.75$ to 1). Among cephalosporins, a high level of resistance was observed against cefuroxime ($MIC_{90} \leq 16$) and ceftazidime ($MIC_{90} \leq 24$). Only 11 strains (39%) were sensitive to ceftriaxone and others were resistant at 6 μg/ml. These results are in agreement with those reported from Turkey,

where variable MICs (0.25 to 8 μg/ml) were found against 50 human isolates (Tanyel *et al,* 2007). In contrast Egyptian workers (Abdel-Maksoud *et al,* 2012) found probable resistance to ceftriaxone among 2% (7/355) of *B. melitensis* isolates. In the results of the current study there is evidence of development of resistance to penicillin antibiotics. These results preclude the use of ceftriaxone as a second line of therapy for brucellosis (Plenque *et al,* 1986). A variety of antimicrobials possess activity *in vitro* against *B. melitensis*, however results of routine susceptibility tests do not always correlate with clinical efficacy. Treatment of humans with brucellosis with beta-lactams (penicillins and cephalosporins) has been associated with a high rate of relapses (WHO, 2006).

Except for norfloxacin and gamifloxacin, all quinolones tested in this study showed low MICs (0.064 to 0.75 μg/ml). Based on available MICs data against *Brucella* spp. (Garcia-Rodriguez *et al,* 1995; Trujillano-Martin *et al,* 1999; Tanyel *et al,* 2004; Turkmani *et al,* 2006; Abdul-Maksoud *et al,* 2012), optimum bioavailability, high tissue and intracellular concentrations (within the macrophages), quinolones are attractive therapeutic alternatives for the treatment of brucellosis in humans. Nevertheless, poor activity of quinolones in an acidic environment (Garcia-Rodriguez *et al,* 1991; Akova *et al,* 1999), would question the effectiveness of these antibiotics (Turkmani *et al,* 2006). Treatment trials with ciprofloxacin in humans (Lang *et al,* 1990, Doganay and Aygen, 1992; López-Merino *et al,* 2004) and levofloxacin alone and in combination with rifampicin in mice (Arda *et al,* 2004) demonstrated the ineffectiveness of these antimicrobials. Moreover, the results of randomized clinical trials of brucellosis have discouraged the use of quinolone based combinations as a first line of therapy (Kalo *et al,* 1996). In contrast 45 days of an oral combination of doxycycline and ofloxacin has been shown to be as effective as a doxycycline-rifampicin combination (Saltoglu *et al,* 2002). However, present data suggests that quinolones might play a role in combination

therapy, particularly where intolerance and resistance preclude the use of recommended combinations for brucellosis (Tanyel *et al,* 2007).

Oral treatment regimens containing sulfas and trimethoprim are considered suitable lower cost combinations than traditional combinations. The SXT combination has mostly been prescribed for children and pregnant women with brucellosis (Young, 1995). The present study revealed low $MIC_{50}$ (0.064 μg/ml) and $MIC_{90}$ (0.125 μg/ml) values for TS and this corresponds with the results of susceptibility reports of *Brucella* spp. from Turkey (Kilic *et al,* 2008 and Bayram *et al,* 2011). Since *in vitro* SXT resistance of *Brucella* spp. has been reported (Kinsara *et al,* 1999; Baykam *et al,* 2004), the selection of this combination for treatment of brucellosis should be based on susceptibility results.

Isolation of *Brucella* is the gold standard diagnostic method for the diagnosis of brucellosis. However, this procedure is laborious and entails a considerable turnover time (~ 1 week). This also requires a biosafety level 3 laboratory and skilled technical personnel. Handling of live *Brucella* cultures involves high risk of laboratory-acquired infections, therefore, very strict biosafety rules must be observed. Molecular diagnostic methods (eg PCR) have considerably reduced this risk and are the most reliable tools in terms of sensitivity and specificity (Leyla *et al,* 2003). More than 400 scientific reports are available in the literature on the PCR-based diagnosis of brucellosis (Yu and Nielsen, 2010).

Both blood and serum samples are often used in PCR-based diagnosis of brucellosis (Leal-Klevezas, 1995, Zarva 2001). However, inhibitors in body fluids frequently affect PCR results (Espy *et al,* 2006). Disappearance of hemoglobin substantially increases PCR sensitivity (Miller *et al,* 1988). Several commercial kits are capable of extracting

low amounts of relatively pure *Brucella* DNA from animal serum (Queipo-Ortuno *et al,* 2008). The use of FTA cards for DNA extraction has been found to be accurate and reproducible (Pizzoli *et al,* 2007). These cards have been used for DNA extraction from body fluids (Yu and Nielsen, 2010). In the present study, gDNA was extracted using a commercially available kit and this yielded a good DNA concentration in the samples tested.

Several types of primer pairs have been used to identify the genus *Brucella*. The primer sequences have been derived from different polymorphic regions of genomes of *Brucella* species and include sequences encoding BCSP 31(B4/B5) (Baily *et al,* 1992), 16SrRNA(F4/R2) (Romero *et al,* 1995), 16s-23S 16S–23S intergenic transcribed spacers (ITS) (Bru ITS-S/Bru ITS-A) (Rijpens *et al,* 1996; Bricker *et al,* 2000), 16S-23S rDNA interspace (ITS66/ITS279) (Keid *et al,* 2007), IS711 (IS313/IS639) (Hénault *et al,* 2000), *per* (*bruc1/bruc5*) (Bogdanovich *et al,* 2004), *omp2* (*JPF/JPR*) (Leal-Kleveza, 1995), outer membrane proteins (*omp 2b*,*omp2a* and *omp31*) (Imaoka *et al,* 2007), proteins of the *omp25/omp31* family of *Brucella spp.* (Vizcaino *et al,* 2004), and arbitrary primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) PCR (Fekete *et al,* 1992). The diagnostic sensitivity and specificity of these sets of primers have been found to be inconsistent. Genus-specific PCR assays targeted at the *Brucella* BCSP31gene and 16S-23S rRNA operon are highly conserved in *Brucella* and are often used for screening of brucellosis in humans, animals and food samples (e.g., milk) (Bricker, 2002). Comparative analyses of three genus-specific PCR assays (*bcsp31*, *omp2* and 16S rRNA gene sequences), revealed a poor diagnostic efficiency of 16S rRNA on bovine blood samples, while bcsp31 was most sensitive and had similar sensitivity to omp2 PCR (Mukherjee *et al,* 2007). A combined use of these two primers (bcsp31 and omp2) significantly augmented the diagnostic sensitivity and specificity of

the assay.

Recently a novel PCR assay for the rapid detection and differentiation of members of the *Brucella* genus and species has been developed (Hinić *et al,* 2009). For the rapid, sensitive and accurate detection of *Brucella* spp., the multiple insertion element IS711, which is stable in both number and position in the *Brucella* chromosomes, was a target. For species differentiation, unique genetic loci of *B. melitensis* have been identified and the BMEII0466 region has been chosen from the open reading frame for the construction of a primer set. This PCR assay is reported to be highly specific and suitable for both conventional and real time PCR formats (Yu and Nielsen, 2010). In the present study, a novel PCR was employed to identify *Brucellae* at the genus and species levels. These tests correctly identified the organisms both in blood and broth medium. These results verify the sensitivity and specificity of PCR primers.

Although PCR tests have high sensitivity and specificity, serological assays are easier to use and more widely adopted in the field. In the following chapter a range of serological assays are compared.

# CHAPTER SIX

# COMPARISON OF SEROLOGICAL TESTS

## 6.1 Introduction

Control programmes for brucellosis depend mainly on diagnosis of infection by serological tests (Nielsen *et al*, 2002). However serological tests may have issues with low specificity and sensitivity. A number of serological tests have been developed to detect brucellosis since the original agglutination test was described (Wright & Smith, 1897). The Rose Bengal test (RBPT), buffered plate agglutination test, the complement fixation test (CFT), and enzyme linked immunosorbent assays (ELISA) have been used for screening both herds and individual animals for brucellosis (OIE, 2009). As none of these tests are suitable for all epidemiological situations (European Commission, 2001), a combination of bacteriological isolation, growth characteristics, serological methods and molecular techniques are critical to identify the bacterium (Nielson *et al*, 2002). Serological tests have been used widely to screen the humoral response in cattle with *B. abortus* infection however false positive and false negative reactions are of concern because the sensitivity and specificity of the tests are not 100%. Modifications have been made to the agglutination test in an attempt to increase its specificity (MacMillan, 1990). The complement fixation test (CFT) was developed to supplement the agglutination tests in cattle (Hill, 1963). However, as the CFT is complex and expensive, it has been used mostly as a confirmatory test (OIE, 2009). Although it is widely assumed that the available serological tests for *B. abortus* infection in cattle are also adequate for diagnosing *B. melitensis* infection in small ruminants (European Commission, 2001), the tests have not been validated as opposed to *B. abortus* (Nielson *et al*, 2002). No serological tests have been developed specifically for *B. melitensis* and

accordingly the RBPT and the CFT are the most widely used tests for the serological diagnosis of brucellosis in sheep and goats (MacMillan, 1990).

Subsequently indirect enzyme-linked immunosorbent assays (iELISA) were developed to enhance the diagnostic sensitivity and specificity. However the specificity can be affected by vaccinal antibodies, as well as antibodies induced by other microorganisms. In these conditions, only the Native Hapten (NH) gel precipitation test appears capable of differentiating immunity induced by natural infection from that arising from vaccination (Díaz *et al,* 1979; OIE, 2008).

The competitive enzyme-linked immunosorbent assay (cELISA) was developed to overcome some of these disadvantages, however the test lacks specificity in vaccinated animals and those infected with *Yersinia enterocolitica* O:9 (Marín *et al,* 1999; Muñoz *et al,* 2005) and is costly to administer (Nielsen *et al,*1989; MacMillan *et al,* 1990; Nielsen *et al,* 1996). This led to the development of the fluorescence polarisation assay (FPA) for detection of antibody to *B. abortus* and *B. suis* (Nielsen *et al,* 1996; Nielsen *et al,* 1999). However, the test was reported to have lower accuracy compared to the ELISAs (Burriel *et al,* 2004). Recently the brucellosis immunochromatography assay (ICA), a simplified version of ELISA, has been developed and this is a convenient, rapid and suitable field test for animal brucellosis (Montasser *et al,* 2012).

This study compared the results of a number of tests in diagnosing brucellosis (*B. melitensis* infection) in different animal species.

### 6.2 Materials and Methods

All samples which tested positive on the RBPT were further tested with a solid phase immunochromatography assay (ICA; Anigen *B. abortus* rapid test, Animal Genetics

Inc., Korea), an indirect ELISA (SVANOVIR® Brucella-AB I-ELISA, Sweden) and a competitive ELISA (COMPLISA, Veterinary Laboratory Agency, UK). An interrater reliability analysis using the Kappa statistic was performed to determine the level of agreement between these tests.

## 6.3 Results

### 6.3.1 Comparison of different tests used for the diagnosis of brucellosis in cattle

In total 78 cattle were found to be positive to the RBPT. These samples were further tested through a rapid test, indirect ELISA (iELISA), and a competitive ELISA (cELISA) (Table 6.1). Of these RBPT positive samples, 30.05% (n=25) and 80.77% (n=63) were positive on the ICA and iELISA, respectively. All 78 animals positive on the RBPT were also positive on the cELISA. Comparison of the Kappa statistic for the ICA and iELISA indicated a "poor" agreement (k = 0.034). No comparison was possible regarding the results of cELISA with all other tests as all RBPT positive animals tested positive to the cELISA (Table 6.2).

**Table 6.1 Results of different tests used for testing against brucellosis in cattle**

| Sample No. | Governorate | Vaccination status for brucellosis | RBPT | ICA | iELISA | cELISA |
|---|---|---|---|---|---|---|
| 1 | Batinah | Not vaccinated | 2+ | -ve | +ve | +ve |
| 2 | Al Dakhiliyah | Not vaccinated | 1+ | -ve | +ve | +ve |
| 3 | Sharqiyah | Not vaccinated | 4+ | +ve | +ve | +ve |
| 4 | Muscat | Not vaccinated | 4+ | +ve | +ve | +ve |
| 5 | Dhofar | Vaccinated | 2+ | +ve | +ve | +ve |
| 6 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 7 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 8 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |

| Sample No. | Governorate | Vaccination status for brucellosis | RBPT | ICA | iELISA | cELISA |
|---|---|---|---|---|---|---|
| 9 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 10 | Dhofar | Vaccinated | 4+ | +ve | -ve | +ve |
| 11 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 12 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 13 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 14 | Dhofar | Vaccinated | 4+ | +ve | -ve | +ve |
| 15 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 16 | Dhofar | Vaccinated | 4+ | -ve | -ve | +ve |
| 17 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 18 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 19 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 20 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 21 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 22 | Dhofar | Vaccinated | 4+ | -ve | -ve | +ve |
| 23 | Dhofar | Vaccinated | 2+ | -ve | +ve | +ve |
| 24 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 25 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 26 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 27 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 28 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 29 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 30 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 31 | Dhofar | Not vaccinated | 4+ | -ve | +ve | +ve |
| 32 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 33 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 34 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 35 | Dhofar | Vaccinated | 2+ | -ve | -ve | +ve |
| 36 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 37 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 38 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |

| Sample No. | Governorate | Vaccination status for brucellosis | RBPT | ICA | iELISA | cELISA |
|---|---|---|---|---|---|---|
| 39 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 40 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 41 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 42 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 43 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 44 | Dhofar | Vaccinated | 4+ | -ve | -ve | +ve |
| 45 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 46 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 47 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 48 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 49 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 50 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 51 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 52 | Dhofar | Vaccinated | 4+ | -ve | +ve | +ve |
| 53 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 54 | Dhofar | Vaccinated | 3+ | +ve | +ve | +ve |
| 55 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 56 | Dhofar | Vaccinated | 4+ | +ve | -ve | +ve |
| 57 | Dhofar | Vaccinated | 4+ | -ve | -ve | +ve |
| 58 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 59 | Dhofar | Vaccinated | 3+ | -ve | -ve | +ve |
| 60 | Dhofar | Vaccinated | 4+ | -ve | -ve | +ve |
| 61 | Dhofar | Not vaccinated | 4+ | +ve | +ve | +ve |
| 62 | Dhofar | Not vaccinated | 4+ | +ve | +ve | +ve |
| 63 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 64 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 65 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 66 | Dhofar | Vaccinated | 3+ | -ve | -ve | +ve |
| 67 | Dhofar | Vaccinated | 4+ | -ve | -ve | +ve |
| 68 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |

| Sample No. | Governorate | Vaccination status for brucellosis | RBPT | ICA | iELISA | cELISA |
|---|---|---|---|---|---|---|
| 69 | Dhofar | Vaccinated | 2+ | -ve | +ve | +ve |
| 70 | Dhofar | Vaccinated | 3+ | -ve | +ve | +ve |
| 71 | Dhofar | Vaccinated | 3+ | -ve | -ve | +ve |
| 72 | Dhofar | Vaccinated | 2+ | -ve | -ve | +ve |
| 73 | Dhofar | Vaccinated | 2+ | -ve | +ve | +ve |
| 74 | Dhofar | Vaccinated | 4+ | +ve | -ve | +ve |
| 75 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 76 | Dhofar | Vaccinated | Trace | -ve | +ve | +ve |
| 77 | Dhofar | Vaccinated | 2+ | +ve | +ve | +ve |
| 78 | Dhofar | Vaccinated | 3+ | +ve | +ve | +ve |

**Table 6.2 Agreement between different Tests used in cattle found positive for brucellosis on the RBPT (n = 78)**

| Comparison | Observed Agreement | 95% CI of Agreement | SE | Kappa Value | 95% CI of Kappa |
|---|---|---|---|---|---|
| ICA vs iELISA | 41.0% | 30-52.7 | 0.066 | 0.034 | -0.095, 0.163 |
| ICA vs cELISA | - | - | - | - | - |
| iELISA vs cELISA | - | - | - | - | - |

### 6.3.2 Comparison of different tests used for the diagnosis of brucellosis in sheep

All sheep samples positive on the RBPT test (n = 29) were tested with the ICA, iELISA, and cELISA (Table 6.3). Of these 12 (41.38%), 14 (48.27%) and 12 (41.37%) were positive on the ICA, iELISA and cELISA, respectively. Agreement between the results of ICA, iELISA and cELISA results were substantial (k = 0.72 each) (Table 6.4). A higher "almost perfect" agreement was found between the iELISA and cELISA results (k = 0.86).

**Table 6.3 Results of different tests used for testing against brucellosis in sheep**

| Sample No. | Govt. | Vaccination status for brucellosis | RBPT | ICA | iELISA | cELISA |
|---|---|---|---|---|---|---|
| 1 | Batinah | Not vaccinated | 1+ | -ve | -ve | -ve |
| 2 | Batinah | Not vaccinated | 2+ | -ve | -ve | -ve |
| 3 | Batinah | Not vaccinated | 1+ | +ve | +ve | +ve |
| 4 | Batinah | Not vaccinated | 1+ | -ve | -ve | -ve |
| 5 | Batinah | Not vaccinated | 2+ | -ve | -ve | -ve |
| 6 | Al Dakhiliyah | Not vaccinated | 2+ | -ve | -ve | -ve |
| 7 | Al Dakhiliyah | Not vaccinated | 3+ | +ve | +ve | +ve |
| 8 | Al Dhahirah | Not vaccinated | 2+ | -ve | +ve | +ve |
| 9 | Dhofar | Vaccinated | 3+ | -ve | -ve | -ve |
| 10 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 11 | Dhofar | Vaccinated | 4+ | -ve | -ve | -ve |

| Sample No. | Govt. | Vaccination status for brucellosis | RBPT | ICA | iELISA | cELISA |
|---|---|---|---|---|---|---|
| 12 | Dhofar | Vaccinated | 4+ | +ve | +ve | -ve |
| 13 | Dhofar | Vaccinated | 3+ | +ve | +ve | +ve |
| 14 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 15 | Dhofar | Vaccinated | 3+ | -ve | -ve | -ve |
| 16 | Dhofar | Vaccinated | 4+ | -ve | -ve | -ve |
| 17 | Dhofar | Vaccinated | 3+ | -ve | -ve | -ve |
| 18 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 19 | Dhofar | Vaccinated | 3+ | -ve | -ve | -ve |
| 20 | Dhofar | Vaccinated | 3+ | +ve | -ve | -ve |
| 21 | Dhofar | Vaccinated | 4+ | -ve | +ve | -ve |
| 22 | Dhofar | Vaccinated | 3+ | +ve | +ve | +ve |
| 23 | Dhofar | Vaccinated | 4+ | +ve | +ve | +ve |
| 24 | Dhofar | Vaccinated | 2+ | -ve | -ve | -ve |
| 25 | Dhofar | Vaccinated | 1+ | -ve | -ve | -ve |
| 26 | Dhofar | Vaccinated | 3+ | +ve | +ve | +ve |
| 27 | Dhofar | Vaccinated | 3+ | +ve | +ve | +ve |
| 28 | Dhofar | Vaccinated | 1+ | -ve | +ve | +ve |
| 29 | Dhofar | Vaccinated | 3+ | -ve | -ve | -ve |

**Table 6.4 Comparison of different tests used for the diagnosis of brucellosis in sheep (n=29) positive to the RBPT**

| Comparison | Observed Agreement (%) | 95% CI of Agreement | SE | Kappa Value | 95% CI of Kappa |
|---|---|---|---|---|---|
| ICA vs iELISA | 86.2 | 68.3-96.1 | 0.128 | 0.722 | 0.472, 0.973 |
| ICA vs cELISA | 86.2 | 68.3-96.1 | 0.132 | 0.716 | 0.457, 0.974 |
| iELISA vs cELISA | 93.1 | 77.2-99.2 | 0.094 | 0.861 | 0.677, 1.045 |

### 6.3.3 Comparison of different Tests used for the diagnosis of brucellosis in goats

In total 57 goat sera were positive on the RBPT and these sera were tested with the ICA, iELISA and cELISA (Table 6.5). Of the 57 sera 57.89% (n=33), 43.86% (n=25) and 35.09% (n=20) were positive on the iELISA, cELISA and ICA, respectively. Agreement between the rapid test, and the iELISA and cELISA was “fair” (k = 0.29 and 0.38, respectively). Agreement between the iELISA and cELISA was “substantial” (k = 0.65) (Table 6.6).

**Table 6.5 Results of different tests used for testing against brucellosis in goats**

| Sample No. | Vaccination status for brucellosis | RBPT* | ICA | iELISA | cELISA |
|---|---|---|---|---|---|
| 1 | Not vaccinated | 1+ | -ve | -ve | -ve |
| 2 | Not vaccinated | 4+ | +ve | +ve | +ve |
| 3 | Not vaccinated | 2+ | -ve | -ve | -ve |
| 4 | Not vaccinated | 3+ | +ve | +ve | +ve |
| 5 | Not vaccinated | 4+ | +ve | +ve | +ve |
| 6 | Not vaccinated | 4+ | +ve | +ve | +ve |
| 7 | Not vaccinated | 2+ | +ve | +ve | +ve |
| 8 | Not vaccinated | 1+ | +ve | +ve | +ve |
| 9 | Not vaccinated | 2+ | +ve | +ve | +ve |
| 10 | Not vaccinated | 4+ | +ve | +ve | +ve |
| 11 | Vaccinated | 2+ | +ve | -ve | -ve |
| 12 | Vaccinated | T | -ve | -ve | -ve |
| 13 | Not vaccinated | 3+ | -ve | -ve | -ve |
| 14 | Not vaccinated | 3+ | -ve | -ve | -ve |
| 15 | Not vaccinated | 2+ | -ve | +ve | +ve |
| 16 | Vaccinated | T | -ve | -ve | -ve |
| 17 | Vaccinated | 4+ | -ve | +ve | -ve |

| Sample No. | Vaccination status for brucellosis | RBPT* | ICA | iELISA | cELISA |
|---|---|---|---|---|---|
| 18 | Vaccinated | 4+ | +ve | +ve | +ve |
| 19 | Vaccinated | T+ | -ve | +ve | -ve |
| 20 | Vaccinated | 2+ | -ve | +ve | +ve |
| 21 | Vaccinated | 4+ | +ve | -ve | -ve |
| 22 | Vaccinated | 1+ | -ve | +ve | +ve |
| 23 | Not vaccinated | 2+ | -ve | -ve | -ve |
| 24 | Vaccinated | 1+ | -ve | -ve | -ve |
| 25 | Not vaccinated | 4+ | -ve | +ve | -ve |
| 26 | Not vaccinated | 3+ | -ve | -ve | -ve |
| 27 | Not vaccinated | 4+ | -ve | +ve | +ve |
| 28 | Vaccinated | 4+ | +ve | +ve | +ve |
| 29 | Vaccinated | 4+ | -ve | +ve | +ve |
| 30 | Not vaccinated | 4+ | +ve | +ve | +ve |
| 31 | Not vaccinated | 3+ | -ve | +ve | +ve |
| 32 | Vaccinated | 4+ | +ve | +ve | +ve |
| 33 | Vaccinated | 4+ | -ve | +ve | -ve |
| 34 | Vaccinated | 4+ | +ve | +ve | +ve |
| 35 | Vaccinated | 3+ | -ve | -ve | -ve |
| 36 | Vaccinated | 4+ | +ve | -ve | -ve |

| Sample No. | Vaccination status for brucellosis | RBPT* | ICA | iELISA | cELISA |
|---|---|---|---|---|---|
| 37 | Vaccinated | 3+ | -ve | -ve | -ve |
| 38 | Vaccinated | 2+ | -ve | -ve | -ve |
| 39 | Vaccinated | 3+ | -ve | -ve | -ve |
| 40 | Vaccinated | 3+ | -ve | +ve | +ve |
| 41 | Vaccinated | 2+ | -ve | +ve | +ve |
| 42 | Vaccinated | 4+ | -ve | -ve | -ve |
| 43 | Not vaccinated | 4+ | -ve | +ve | +ve |
| 44 | Vaccinated | 4+ | -ve | +ve | -ve |
| 45 | Vaccinated | 2+ | -ve | +ve | -ve |
| 46 | Vaccinated | 4+ | +ve | +ve | -ve |
| 47 | Vaccinated | 4+ | +ve | -ve | -ve |
| 48 | Not vaccinated | 4+ | +ve | +ve | +ve |
| 49 | Not vaccinated | 4+ | -ve | +ve | +ve |
| 50 | Vaccinated | 4+ | -ve | -ve | -ve |
| 51 | Vaccinated | 4+ | -ve | -ve | +ve |
| 52 | Vaccinated | 4+ | -ve | -ve | -ve |
| 53 | Vaccinated | 1+ | -ve | +ve | -ve |
| 54 | Vaccinated | 2+ | +ve | +ve | -ve |
| 55 | Vaccinated | 4+ | -ve | -ve | -ve |

| Sample No. | Vaccination status for brucellosis | RBPT* | ICA | iELISA | cELISA |
|---|---|---|---|---|---|
| 56 | Vaccinated | 1+ | -ve | -ve | -ve |
| 57 | Not vaccinated | 2+ | -ve | -ve | -ve |

* Trace

**Table 6.6 Comparison of different tests used in goats (n=57) found positive for brucellosis after RBPT**

| Comparison | Observed Agreement | 95% CI of Agreement | SE | Kappa Value | 95% CI of Kappa |
|---|---|---|---|---|---|
| ICA vs iELISA | 63.2% | 49.3-75.6 | 0.111 | 0.296 | 0.078, 0.515 |
| ICA vs cELISA | 70.2% | 56.6-81.6 | 0.123 | 0.381 | 0.141, 0.621 |
| iELISA vs cELISA | 82.5% | 70.1-91.3 | 0.095 | 0.656 | 0.469, 0.842 |

### 6.3.4 Comparison of different tests used for the diagnosis of brucellosis in camels

Of the 2055 camel sera tested only 10 were found to be positive on the RBPT. All positive sera were tested with ICA rapid test and cELISA (Table 6.7). Upon further testing, only 40% (n=4/10) of samples were positive on the ICA. However, 90% of the RBPT positive samples were positive on the cELISA. A poor agreement was found between ICA and the cELISA results (k = 0.138) (Table 6.8). Due to the unsuitability of

the iELISA for camels, this test was not compared with the other assays (ICA and cELISA).

**Table 6.7 Results of different tests used for testing against brucellosis in goats**

| Sample No. | Governorate | Vaccination status for brucellosis | RBPT | ICA | cELISA |
|---|---|---|---|---|---|
| 1 | Batinah | Not vaccinated | 4+ | +ve | +ve |
| 2 | Al Sharqiyah | Not vaccinated | 2+ | -ve | +ve |
| 3 | Dhofar | Not vaccinated | 3+ | +ve | +ve |
| 4 | Dhofar | Not vaccinated | 4+ | -ve | +ve |
| 5 | Dhofar | Not vaccinated | 4+ | +ve | +ve |
| 6 | Dhofar | Not vaccinated | 4+ | +ve | +ve |
| 7 | Dhofar | Not vaccinated | 3+ | -ve | +ve |
| 8 | Dhofar | Not vaccinated | 4+ | -ve | +ve |
| 9 | Dhofar | Not vaccinated | 1+ | -ve | -ve |
| 10 | Dhofar | Not vaccinated | 3+ | -ve | +ve |

**Table 6.8 Comparison of Different Tests used in camels (n=10) found positive for brucellosis after RBPT**

| Comparison | Observed Agreement | 95% CI of Agreement | SE | Kappa Value | 95% CI of Kappa |
|---|---|---|---|---|---|
| ICA vs cELISA | 50% | 18.7-81.3 | 0.138 | 0.138 | -0.133, 0.409 |

## 6.4 Discussion

A diagnostic test for an infectious disease can be used to demonstrate the presence or absence of a causative agent, or to detect antibodies to a particular infectious agent. Demonstrating the presence of the infecting organism or a surrogate marker of infection is often crucial for effective clinical management and for selecting other appropriate disease control activities such as contact tracing. The diagnostic test(s) must be accurate, user friendly, simple and affordable for the population for which they are intended. They must also provide a timely result for allowing implementation of effective control measures to avoid the spread of disease. For some infections, early diagnosis and treatment can have an important role in preventing the development of long-term complications or in interrupting transmission of the infectious agent. In a broader context, diagnostic tests help in patient management, screening latent infections, disease surveillance, epidemiological investigations, evaluating the effectiveness of interventions including verification of elimination and detecting infections with markers of drug resistance (Banoo *et al*, 2008).

The usefulness of a diagnostic test depends on the sensitivity, specificity and predictive

values and field applicability of the test (Naureen *et al*, 2007). Determining the diagnostic efficiency of the test(s) in absence of a gold standard is, however, common, although not ideal. New tests that are compared with imperfect gold standards will have bias in the error rates of the new test as a result of the lack of a perfect comparative gold standard (Staquent *et al,* 1981; Valenstein 1991). This is especially true for the tests with a higher detection limit than the gold standard. Therefore, evaluation methods for diagnostic tests using maximum likelihood techniques with a latent class model have been described and refined (Enoe *et al,* 2000; Pouillot *et al,* 2002). The agreement between two tests has also been suggested as an evaluation criterion for a diagnostic test (Martin, 1977). The kappa measures the magnitude of agreement between 2 tests and ranges from -1 to 1, where 1 is perfect agreement, 0 is exactly what would be expected by chance and -1 is perfect disagreement (Landis and Koch, 1977; Viera and Garret, 2005). If the kappa value of two tests is high, then any of the tests might be selected for a testing program because the results of both tests provide the same information (Martin, 1977). Kappa values are lower in a low prevalence population and higher in a higher prevalence population. In the disease-free population, agreement is difficult to calculate as little to no variation often occurs in the distribution of results. Different kappa values for different populations are expected, hence, before using and interpreting diagnostic tests, the population of interest must be characterized (Greiner and Gardner, 2000). In the present study, agreements among all combinations of different tests for different animal species (cattle, sheep and goat) except camels were calculated. A very good agreement (k = 0.86) was observed when the two different format of ELISAs for sheep were compared, while ICA and ELISAs indicated a high level of agreement (0.71 to 0.72) for sheep flocks. Substantial agreement (k=0.65) was also noted between ELISAs upon testing goats and a fair agreement (0.29 to 0.38) was estimated between

the ICA and ELISAS in this population. For cattle and camels, the agreement between ICA and cELISA was poor to slight. These results indicate that the ICA carries poor diagnostic efficiency for cattle and camels and seems unsuitable for testing of brucellosis in the field.

As mentioned previously brucellosis is an important zoonotic disease and in the following chapter an investigation into brucellosis in humans in Oman is reported.

# CHAPTER SEVEN

# A RETROSPECTIVE STUDY OF BRUCELLOSIS IN HUMANS IN OMAN

## 7.1 Introduction

Brucellosis in humans is widely distributed all over the world, with regions of high endemicity in areas of the Mediterranean, Middle East, Latin America and parts of Asia (López-Merino, 1989; Corbel, 1997a&b). The World Health Organization (WHO) biosafety manual classifies *B. melitensis* in Risk group III highlighting the importance and impact of this pathogen. Brucellosis is readily transmissible to humans and causes undulant fever which may progress to a chronic form (WHO, 2006b). Several complications have been reported in patients with brucellosis including musculo–skeletal, cardiovascular and central nervous system problems (European Commission, 2001). There is a significant occupational association of brucellosis in humans with veterinarians, abattoir workers, laboratory technicians and farmers who handle infected aborted fetuses and membranes of infected animals being at greater risk of contracting the infection (Corbel *et al,* 2006; Stack and MacMillan, 2006). The main route of infection is orally through ingestion of infected dairy products, alternatively the pathogen can also enter via inhalation or conjunctival routes.

Although the true incidence of brucellosis in humans throughout the world is not known ( European Commission, 2001), it has been reported to vary widely from < 0.01 to > 200 per 100,000 population per year in individual countries (López-Merino, 1989). In 2008, a total of 619 confirmed human brucellosis cases were reported in the European Union (EU) (0.1 cases per 100,000 inhabitants). The highest incidence was recorded in those member states not officially free from bovine and ovine/caprine brucellosis (Greece, Italy, Portugal and Spain). Although overall the incidence in the EU decreased

between 2004 and 2008, cases were reported more frequently in spring and summer (European Food Safety Agency, 2010b).

Childhood brucellosis (*B. melitensis*) in the USA is an imported disease, primarily from Mexico. A retrospective study of 20 patients over a period of 13 years reported that 95% had either travelled to Mexico or consumed unpasteurized milk products from Mexico. Fever was an initial complaint in 80% of the patients and 50% of the patients presented with arthritis (Shen, 2008).

Studies conducted in North Africa and in the Middle-East reported that brucellosis in humans was attributed to the presence of *B. melitensis* in livestock ( Al-Ani *et al,* 2004; Jennings *et al,* 2007) whilst in sub-Saharan African *B. abortus* was mainly implicated (Hendricks *et al,* 1995; Swai and Schoonman., 2009). Human brucellosis is often misdiagnosed in developing countries resulting in underreporting (Paul *et al,* 1995). A study in Tanzania showed that medical professionals, especially those in rural areas had poor knowledge of zoonotic diseases (John *et al,* 2008). In areas where *B. abortus* is a major problem in cattle, seroprevalence levels in humans are estimated to be in the range of 1–5% (Schelling *et al,* 2003; Swai and Schoonman, 2009). In contrast in areas where *B. melitensis* is endemic higher prevalence rates are expected in humans (Pappas *et al,* 2006).

Although *Brucella* can be transmitted directly and indirectly from its animal reservoir to humans, indirect transmission remains the highest overall risk and mainly occurs through the consumption of unpasteurized milk or dairy products (Godfroid *et al,* 2005; Pappas *et al,* 2006; Makita *et al,* 2008). *Brucella melitensis* infection in cattle has emerged as a serious public health problem since *B. melitensis* is capable of colonizing the bovine udder (Banai, 2002; Ashford *et al,* 2004; Lamontagne *et al,* 2010).

Moreover, in some South American countries, cattle are now believed to be more important than pigs as a source of *B. suis* biovar 1 infection for humans, because *B. suis* biovar 1 is also capable of colonizing the bovine udder (Corbel, 1997). The incubation period of the disease in humans varies greatly (Nicoletti, 1980), ranging from weeks to months (Ray and Steel, 1979) and often has an insidious onset. The acute stage of the disease is usually accompanied by bacteremia and spreading of the organism to various organ systems, mainly to reticuloendothelial tissues. Hence, brucellosis in humans is a systemic infectious disease resulting in varying clinical manifestations (Corbel, 2006). Acute brucellosis is characterized by nonspecific systemic signs and clinical symptoms consistent with a flu-like or septicemic illness. Clinical manifestations may comprise osteoarticular, dermal, gastrointestinal, respiratory, cardiovascular and neurologic disorders mimicking many other infectious and non-infectious diseases (Corbel, 2006).

Few cases of brucellosis in humans caused by *B. canis* have been described (Lucero *et al*, 2010; Nomura *et al*, 2010). However, canine brucellosis in humans might be underdiagnosed due to low awareness of the disease and a lack of valid serological tests. Human infections by marine mammal strains have a severe course but have rarely been reported (Brew *et al*, 1999; Sohn *et al*, 2003; McDonald *et al*, 2006). The clinical importance of *B. inopinata* and the atypical *Brucella* strain (BO2) closely related to *B. inopinata* is still unclear despite the fact that both agents have been isolated from diseased humans (De *et al*, 2008; Scholz *et al*, 2010). Little is also known about the human pathogenicity of *B. microti*, although in experimental cellular and murine models of infection *B. microti* exhibited a significantly higher virulence than other *Brucella* species (De Bagues *et al*, 2010).

In Oman, the disease was reported in animals in 1979 in the southern region (Nicoletti,

1986). Subsequently, the disease has affected both animals and humans. A higher number of human cases was reported in 1998 when more than 300 cases were recorded (MOH, 1998). During the period from 1998 until 2002 many human cases (307, 316, 307, 162 and 133 cases, respectively) were reported in both the southern and northern regions of the Sultanate (MOH, 2002). The highest incidence was reported in the southern region with 305, 309, 302, 159 and 127 cases reported each year, respectively. The study reported in this chapter was designed to highlight the incidence and pattern of human brucellosis in Oman through examination of records and data available from the Ministry of Health for the period from 1995 to 2012.

## 7.2 Materials and Methods

This study primarily examined data sourced from different hospitals in Oman through the Department of Surveillance and Disease Control. The forms used to collect the information included data on the region of origin, age, gender and nationality of the patient and the year of the case.

## 7.3 Results

The status of brucellosis in Oman before 1984 was unknown (Idris *et al*, 1993). The study conducted by Ismaily *et al*, (1988) reported the first laboratory-confirmed case of brucellosis in animals and the prevalence varied between (0 and 1.6%) in the northern region and (3.3-8%) in the Southern region. Since 1985 until 1992, several human cases were also identified by the Serum Agglutination Test (SAT) with most of these cases (96.5%) being located in the Southern region (Figure 7.1). The number of human cases increased over the study period, especially in the southern region city of Salalah (Table 7.1).

**Table 7.1 Cases of human brucellosis (1985-1992) as reported by the Department of Surveillance and Disease Control, Ministry of Health, Oman**

| Year | Total number of cases in Oman | Total number of cases from the Southern Region | Percentage of all cases from the Southern Region |
|---|---|---|---|
| **1985** | 260 | 219 | 84.2 |
| **1986** | 186 | 180 | 96.8 |
| **1987** | 229 | 224 | 97.8 |
| **1988** | 292 | 284 | 97.3 |
| **1989** | 224 | 220 | 98.2 |
| **1990** | 183 | 180 | 98.4 |
| **1991** | 350 | 347 | 99.1 |
| **1992** | 371 | 368 | 99.2 |
| **Total** | **2095** | **2022** | **96.5** |

The symptoms of the disease mainly presented as the typical undulant fever, musculoskeletal malaise, headache, fatigue and weakness. However two other prominent features were also reported: severe night sweats and the feeling of tiredness in the afternoon (Idris *et al,* 1993).

In 1995 the 2-Mercapto-ethanol test was introduced to diagnose disease and the reported incidence has fluctuated since then making the interpretation of results more difficult. Data on the reported incidence between 1995 and 2012 in Oman are presented in Table 7.2 and Figure 7.2.

**Table 7.2 Number of cases of human brucellosis reported in different governorates of Oman, 1995 -2012**

| Governorate | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Batinah** | 0 | 2 | 1 | 1 | 3 | 0 | 1 | 0 | 1 | 1 | 0 | 2 | 5 | 0 | 3 | 0 | 1 | 4 | **25** |
| **Al Buraimi** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | **4** |
| **Al Dakhiliyah** | 0 | 1 | 2 | 0 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 2 | 0 | 0 | **11** |
| **Al Dhahirah** | 1 | 1 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **8** |
| **Al Sharqiyah** | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 3 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | **14** |
| **Alwusta** | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | **4** |
| **Dhofar** | 112 | 69 | 196 | 305 | 309 | 303 | 159 | 128 | 193 | 97 | 114 | 66 | 81 | 90 | 75 | 89 | 105 | 139 | **2630** |

| Governorate | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Musandam** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** |
| **Muscat** | 0 | 0 | 3 | 1 | 2 | 2 | 1 | 3 | 3 | 4 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | **21** |
| **Total** | 113 | 74 | 203 | 307 | 316 | 308 | 162 | 134 | 198 | 107 | 116 | 71 | 89 | 94 | 79 | 93 | 107 | 147 | **2718** |
| **Incidence in Dhofar** | **99.1** | **93.2** | **96.6** | **99.3** | **97.8** | **98.4** | **98.1** | **95.5** | **97.5** | **90.7** | **98.3** | **93.0** | **91.0** | **95.7** | **94.9** | **95.7** | **98.1** | **94.6** | **96.8** |

In 1991, the disease monitoring programme was initiated in the sultanate by the Ministry of Health and all data were collected from hospitals by the Department of Surveillance and Disease Control. Although cases were reported in most of the governorates, the southern region (Dhofar) had the majority of cases (96.8%) (Table 7.3).

Over the period 1995 to 2012 the annual incidence of human brucellosis in Oman was 9.79/10000 (). As expected, a highest incidence was recorded in the Dhofar governorate (104.23/10000) compared with 0.35/10000 per year in the other governorates. More than 300 cases per annum were observed for three consecutive years (1998-2000) although subsequent to this period the number of cases has reduced (Figure 7.1).

In Figure 7.2 the change in the annual incidence from 1995 to 2012 is presented. At the start of the period of study (1995) the overall annual incidence was 0.51/10000. This then started increasing from 1996 (0.33/10000) and for the 4 consecutive years (1997-2000) remained elevated (0.90 to 1.40/10000). Subsequently it started decreasing for the next two years (2001 to 2002) but an increase of 45.7% was observed in 2003. From 2004 until 2012, the annual incidence of human brucellosis fluctuated between 0.51 to 0.29 per 10,000 people.

**Figure 7.1 The number of cases of human brucellosis reported in different governorates (1995-2012)**

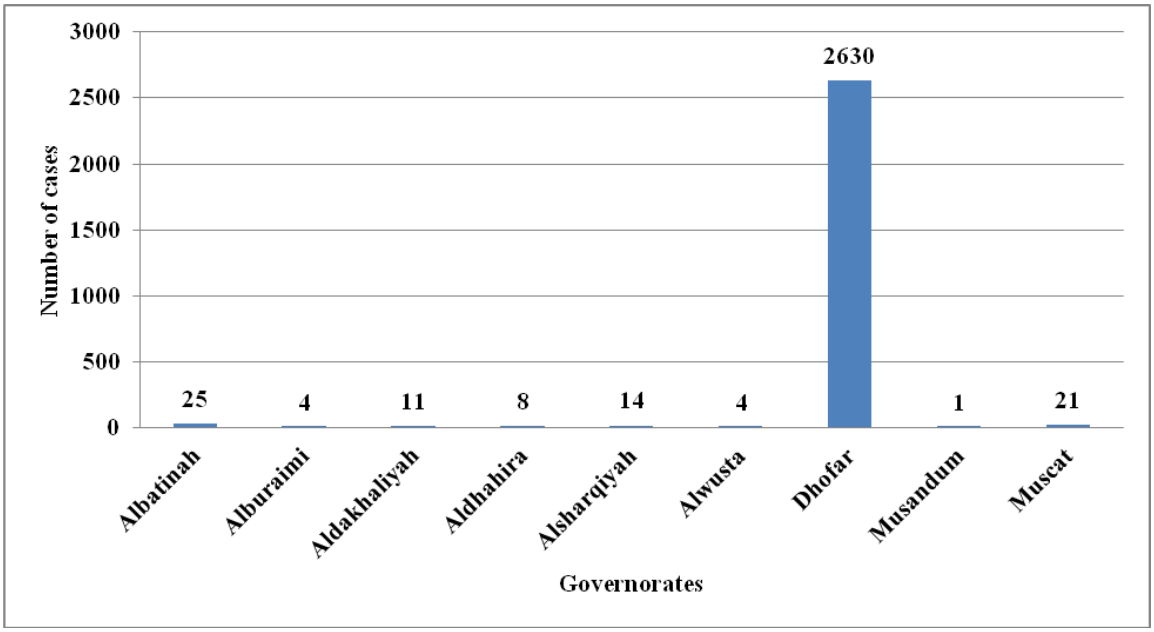



**Figure 7.2 Yearly Incidence/10000 of human brucellosis in Oman (1995-2012)**

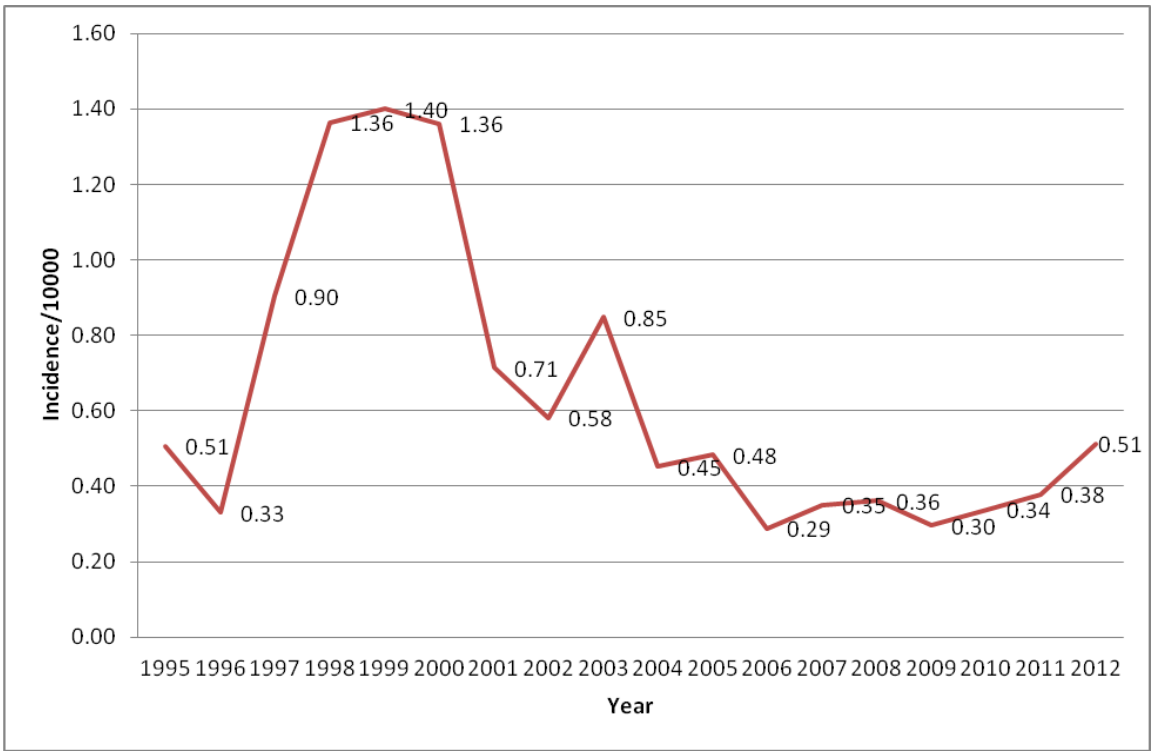


**Table 7.3 Incidence of human brucellosis in Oman (1995-2012)**

| Governorate | *Brucella* Positive | Population (Census, 2010) | Incidence per 10000 people |
|---|---|---|---|
| **Al Wusta** | 4 | 42111 | 0.95 |
| **Al Sharqiyah** | 14 | 350514 | 0.40 |
| **Batinah** | 25 | 772590 | 0.32 |
| **Al Buraimi** | 4 | 72917 | 0.55 |
| **Al Dhahirah** | 8 | 151664 | 0.53 |
| **Al Dakhiliyah** | 11 | 326651 | 0.34 |
| **Musandam** | 1 | 31425 | 0.32 |
| **Muscat** | 21 | 775878 | 0.27 |
| **Dhofar** | 2630 | 249729 | 104.23 |
| **Total** | **2718** | **2773479** | **9.79** |

The overall annual incidence of human brucellosis per 10000 people was recorded as 6.31 and 6.03 in female and males, respectively between 1995 and 2012. In total 733 (43%) females and 972 (57%) males were recorded with brucellosis in Oman. The risk of having brucellosis was similar for males and females (1.05; 95%CI 0.95, 1.15). The highest annual incidence was recorded in females (70.9/10000) and males (62.1/10000) residing in the Dhofar governorate (Table 7.4).

The overall incidence of brucellosis was recorded as 8.3/10000 and 1.08/10000 in the native Omani and expatriate population, respectively (Table 7.5) (OR 7.66; 95%CI 6.18, 9.50). The highest incidence for both the local (94.8/10000) and expatriate (9.81/10000) population was again recorded in the Dhofar governorate.

**Table 7.4 Influence of gender on the incidence of human brucellosis in Oman (1995-2012)**

| Region | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | Cases | Population | Annual incidence per 10,000 population | Cases | Population | Annual incidence per 10,000 population |
| **Al wusta** | 3 | 10117 | 2.97 | 1 | 31994 | 0.31 |
| **Al Sharqiyah** | 4 | 160715 | 0.25 | 9 | 189799 | 0.47 |
| **Batinah** | 11 | 338557 | 0.32 | 7 | 434033 | 0.16 |
| **Al Buraimi** | 2 | 29713 | 0.67 | 2 | 43204 | 0.46 |
| **Al Dhahirah** | 1 | 64220 | 0.16 | 4 | 87444 | 0.46 |
| **Dakhilyah** | 2 | 144696 | 0.14 | 4 | 181955 | 0.22 |
| **Dhofar** | 705 | 99358 | 70.96 | 934 | 150371 | 62.11 |
| **Musandam** | 1 | 12960 | 0.77 | 0 | 18465 | 0.00 |
| **Muscat** | 4 | 300732 | 0.13 | 11 | 475146 | 0.23 |
| **Total** | **733** | **1161068** | **6.31** | **972** | **1612411** | **6.03** |

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**Table 7.5 Incidence of human brucellosis reported in the Omani and expatriate population of Oman (2000-2012)**

| Governorate | Omani Nationals | | | Expatriates | | |
|---|---|---|---|---|---|---|
| | No. Pos. | Population | Incidence x $10^{-6}$ | No. Positive | Population | Incidence x $10^{-6}$ |
| **Batinah** | 17 | 620950 | 0.27 | 1 | 151640 | 0.07 |
| **Al Buraimi** | 4 | 43026 | 0.93 | 0 | 29891 | 0.00 |
| **Al Dakhiliyah** | 4 | 269069 | 0.15 | 2 | 57582 | 0.35 |
| **Al Dhahirah** | 5 | 118877 | 0.42 | 0 | 32787 | 0.00 |
| **Al Sharqiyah** | 12 | 293394 | 0.41 | 1 | 57120 | 0.18 |
| **Al Wusta** | 4 | 19043 | 2.10 | 0 | 23068 | 0.00 |
| **Dhofar** | 1555 | 164073 | 94.77 | 84 | 85656 | 9.81 |
| **Musandam** | 1 | 21898 | 0.46 | 0 | 9527 | 0.00 |
| **Muscat** | 15 | 407006 | 0.37 | 0 | 368872 | 0.00 |
| **Total** | **1617** | **1957336** | **8.26** | **88** | **816143** | **1.08** |

Of 1705 human cases of brucellosis recorded from 2000 to 2012, 94.8% (1617) were Omanis, followed by expatriates from Bangladesh (1.8%), India (1.1%), Egypt (0.7%), Pakistan (0.5%), Yemen (0.4%), Saudi Arabia (0.3%), Sudan (0.1%) and Jordan (0.1%). The nationality of 0.3% of patients was not recorded (Figure 7.3).

When analysed for age more cases (n=771, 45.2%) were observed in the youngest age group (0-10 years) followed by patients in the 11 to 20 year age group (23.9%). This

value further decreased with age and the lowest percentage was recorded in patients above 60 years of age (Figure 7.4).

**Figure 7.3 Number of cases of human brucellosis in different nationalities in Oman (2000-2012).**

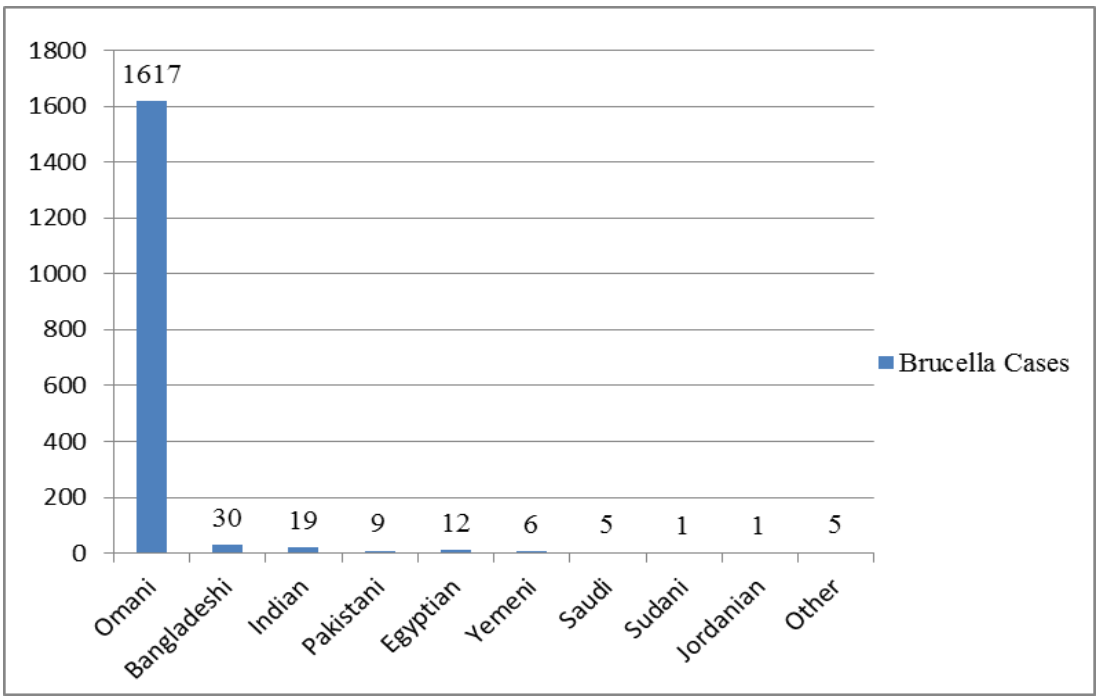
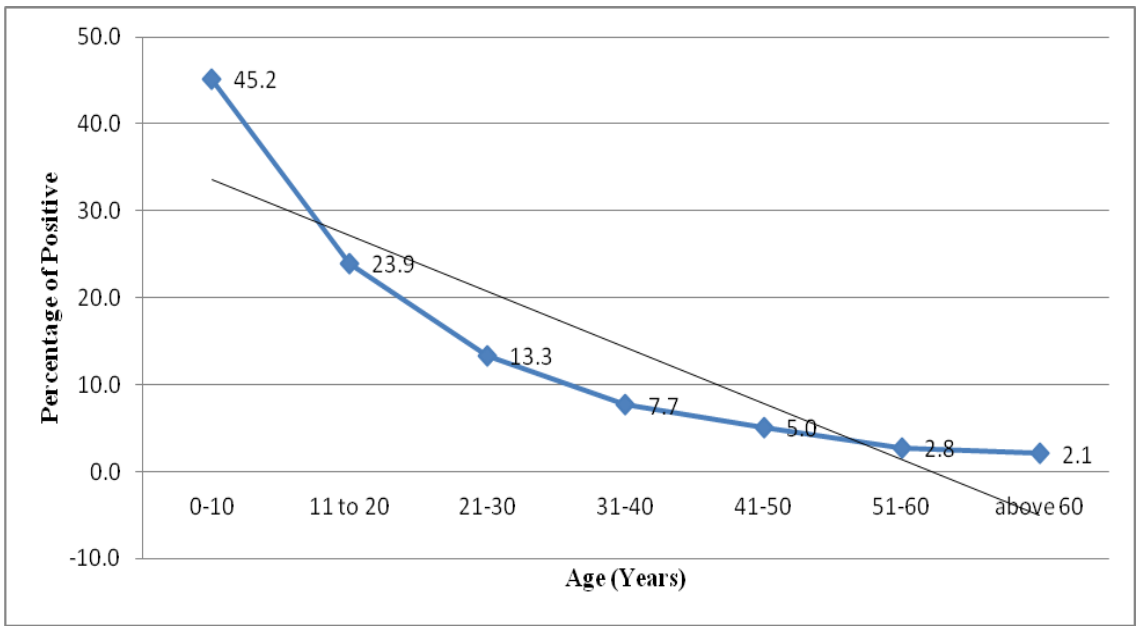


**Figure 7.4 Percentage of human brucellosis cases found in different age groups in Oman (2000-2012).**




## 7.4 Discussion

The number of cases of brucellosis in humans varies between Middle Eastern countries. Several countries have reported the occurrence of the disease since the 1980s, including Egypt, Ethiopia, Iraq, Iran and Jordan (Refai, 2002). The disease has also been reported in Gulf countries, with reports of the disease in Kuwait in 1983, Saudi Arabia in 1985 and UAE in 1996 (Refai, 2002). In Oman, brucellosis is considered the second most important zoonotic disease after rabies (Personal Communication, Director of Animal Health). Diagnosis of the disease in Oman depends primarily on serological tests (Idris *et al*, 1993). The only biotype isolated from both humans and animals has been *B. melitensis* biovar 1 (Ismaily *et al*, 1988; Idris *et al*, 1993; Adam and El-Rashied, 2013). Not surprisingly most human cases have been reported among farmers and their families and also in veterinarians as these groups are at greater risk of exposure to the

pathogen through their daily activities (Refai, 2002). Unfortunately in the current study there were no available data recorded on the occupation of the affected individuals. In this study the high number of cases reported in Dhofar was also expected as the disease is mainly transmitted from animals (Kozukeev *et al*, 2006). As reported in Chapter 4 the highest seroprevalence of livestock brucellosis in Oman was in Dhofar and consequently the risk of transmission would be expected to be higher in this governorate than in others.

The habit of consuming raw milk in Dhofar has played an important role in the dissemination of infection to humans (Idris *et al*, 1993). The fluctuation and big variation in reported cases between 1995 and 2012 does not necessarily indicate that the disease is being successfully controlled in the animal population in Dhofar, but may be artificial through changes in the diagnostic tools used (the 2-Mercapto-ethanol test was introduced in 1995). As the control program in animals was started in 2003 and has continued since then, it is not clear why the incidence has again increased in 2011 and 2012. This may be due to the shedding of the vaccine strain through milk (Longo *et al*, 2009) which, if so, will complicate the issue in Dhofar. However the data could also be biased and be influenced by a large number of uncontrolled factors which may have elevated disease reporting in recent years. However the cases discussed in this chapter were those presented to hospitals and are consequently likely to under-represent the real situation in Oman as many cases may go undiagnosed.

Brucellosis was previously believed to be very rare in children, however it is now well accepted that people of all ages can be affected (Mehmet and Bilgehan, 2003). The higher number of cases in younger ages (0-10 years) could be related to the consumption of more raw milk at this age, although congenital infection has also been

reported **(**Corbel *et al*, 2006; Mesner *et al*, 2007). The chances of contact between children and animals or their secretions may also be possible due to the inquisitive nature of children. Similar findings were also reported by Idris *et al*, (1993) where 4 out of 525 school children were positive for the disease. Furthermore, an observational study undertaken in the Dhofar hospital for 3 years revealed that 63% of cases in children were due to consumption of raw milk and 83% of cases had contact with live animals (El-Amin *et al*, 2001).

The higher incidence in males than females may be due to an increased likelihood of males having contact with animals and their excretions than females. In Oman men usually take care of animal feeding, watering, disposal of foetal membranes and grazing. In contrast women are mainly involved in manual milking after washing the udder with water and disinfectant, which may decrease the chances of contact with the bacterium. Furthermore females may be more careful to prevent unnecessary contact with animal discharges, unlike men who often have less strict personal hygienic standards as reported by Blackmore and Schollum (1982).

The higher incidence among Omani's is not surprising for a number of reasons. Firstly they own most of the animal farms and consequently having more chances to have contact with potentially infected animals. Secondly they follow traditional habits which include drinking raw milk which has been associated with many cases of disease. The relative high number of Bangladeshi's affected may be due to their preference to work as farmers or farm labourers compared with other nationalities. However, the biggest population working on farms in Oman, after Omani's, are the Bangladeshi's and again this would explain the large number of cases in this group.

There was a significant correlation (Pearson's correlation=0.925, $p<0.001$) between human and animal brucellosis in the different governorates of Oman. This study has demonstrated that brucellosis is a common disease in humans in Oman with the disease being more common in regions with high levels of brucellosis in animals. It is likely that implementation of a control program in animals would result in a commensurate decrease in brucellosis in humans.

# CHAPTER EIGHT

## GENERAL DISCUSSION

*Brucella melitensis*, the first species in the genus *Brucella* to be described, causes abortions in pregnant animals and Malta fever in humans (Alton, 1990a). The organism is prevalent in Mediterranean and Middle Eastern countries, through Central Asia to China and the southern areas of the former Soviet Union. Some areas of Africa and India, as well as Central and South America, are also affected (Corbel *et al*, 2006). Although the natural hosts for the organism are goats and sheep, it is considered to be the least species-specific organism of the *Brucellae* (Alton, 1990a). In Europe, small ruminants and their products have been reported to be the main source of infection of humans with *B. melitensis* (Díaz-Aparicio, 2013). However, other animal species have emerged as important carriers of the pathogen in several countries, such as cattle in Israel, Kuwait, and Saudi Arabia and camels in the gulf countries of Oman and UAE (Hashim *et al*, 1987; Abbas and Agab 2002; Al-Majali *et al*, 2009).

During 1979, the first case of the disease in animals was reported by the Ministry of Agriculture and Fisheries in goats from the southern region (Dhofar) of Oman and subsequently human cases were reported in the same region. The consumption of contaminated raw milk was believed to be the source of human infection (Ismaily *et al*, 1988). In contrast to the southern region, only sporadic cases have been reported from the northern region which was previously considered to be a free area. However, a study undertaken by Alrawahi *et al.* in 2006 (MAF, 2006) reported several cases from the northern region of Oman. Moreover, the number of human cases has also increased in the northern region, strengthening the demand for implementation of a control program

in this region.

In 2003, a national program to control brucellosis in the southern region of Oman was approved by the government. This 12 year program was divided into four phases each of 3 years. The first phase concentrated on vaccination of cattle and small ruminants using the Rev-1 vaccine. However, the programme was not designed based on accurate and essential information regarding the prevalence and risk factors for the disease and did not take into consideration the situation of the disease in other regions.

The specific aims of the study outlined in this thesis were to:

1- Map the distribution of disease in Oman using geographical information systems (GIS).

2- Determine the seroprevalence of brucellosis in Oman.

3- Establish the species and types of *Brucella* present in Oman and their genetic relationship.

4- Determine the susceptibility of *Brucella* to the currently available antibiotics.

5- Evaluate different diagnostic tests for the diagnosis of brucellosis in Oman.

6- Highlight the disease situation in humans through an examination of historical hospital data

This study documented the overall herd/flock seroprevalence of brucellosis in animal holdings as 2.4% (95% CI 1.6, 3.4) with 30 infected holdings of 1267 holdings tested. As expected, the southern governorate (Dhofar) had significantly more seropositive flocks (n= 20, 8.6%) than the northern governorates (n= 10, 0.97%), $\chi^2$ (2df)

=47.74, p<0.001. Possible reasons for this might be the endemic nature of the disease, the previous importation of live animals from other countries in the Horn of Africa without prior monitoring of their brucellosis status, more inter-species contact, sharing of common pastures, a larger herd size and the presence of low biosecurity and unhygienic conditions in farms from the southern governorate. This finding of a higher seroprevalence in Dhofar is supported by the reports of other researchers (Alton *et al,* 1988; Grilló *et al,* 1997; European Commission, 2001; WHO, 2006b).

The containment of disease and lower seroprevalence observed in the northern governorates may be explained by the restriction of the exportation and movement of female animals from Dhofar to other regions in Oman and the approved importation of castrated males only. Furthermore, the practice of tethering animals in the northern region may help reduce spread within and between herds by reducing animal to animal contact.

Although there were no significant differences in the seroprevalence between cattle herds of different governorates, the herd level seroprevalence in cattle herds from Dhofar (4.9%) was higher and comparable to that reported by the BDU in 2003 prior to the implementation of a vaccination campaign (6.5%). However the seroprevalence found was lower than that reported in 2010 (24%) (MAF, 2003b; 2010). There are several reasons for the higher seroprevalence reported in 2010. Firstly, the vaccination protocol was not followed as recommended. Secondly, the veterinarians were not trained in handling and delivering the vaccine properly. There was the potential for contamination of the vaccine vials which may have led to infections or a failure to develop protective immunity. Finally, ear tagging was not implemented as planned and differentiation between the vaccinated and non-vaccinated herds was not obvious.

Therefore, the higher percentage of 2010 study may reflect a lower vaccination coverage, inadequate protection and false positive animals in case of unidentified vaccinated herds. In the current study the vaccination history of the herd was taken into consideration, and samples were predominantly from non-vaccinated animals/herds to minimise the probability of false positive results.

Among small ruminants, seropositive sheep were detected in only three flocks (0.6%; 95% CI 0.1, 1.7) and there was no significant difference in the herd level seroprevalence between districts. In contrast 2.3% (95% CI 1.2, 3.9) of goat herds were seropositive and the herd prevalence was significantly higher in Dhofar (16.2%) than in the other governorates. The higher number of seropositive goat holdings were expected in Dhofar as more goats are kept per herd as opposed to few farmers keeping sheep. The higher seroprevalence in goats could also be associated with the reported higher susceptibility for *B. melitensis* in this species. In contrast, sheep are reported to be more resistant to infection and may eliminate infection (Alton, 1990a; Durán-Ferrer, 1998; European Commission, 2001). Secondly, the sheep are mainly used for meat purposes in Oman and these breeds also may have enhanced resistance to infection as reported by Corbel and Brinley-Morgan (1984). The fact that vaginal excretions of goats are more copious and last for at least 2 to 3 months post-partum and approximately two thirds of the naturally acquired infections result in an infection of the udder with excretion of *Brucella* in the milk during subsequent lactations in goats, increases the probability of infection in this species (Alton, 1990a). In contrast, shedding of organisms from infected sheep may last for a maximum of three weeks post-abortion or post-partum ( European Commission, 2001). In Oman, small ruminants (sheep and goats) are usually treated as one group and the results of most previous studies did not separate out the

prevalence for the two species. Given the different epidemiological pattern and susceptibility, data for these species should be analysed separately in future studies.

The herd prevalence of disease among small ruminants in Dhofar was also determined by BDU at 16.9 and 7.9% in 2003 and 2010, respectively (MAF, 2003b, 2010). However, in the current study 16.2% of sampled goat herds contained seropositive animals, while no sheep flock contained seropositive animals in Dhofar. The difference in the prevalence in 2003 and the results of the current study could be due to failure of the vaccination campaign or may reflect the failure in differentiation between infected and vaccinated herds as identifying ear tagging was not widely adopted in earlier studies. The high percentage in small ruminants may also be related to the higher number of goats when compared to sheep, especially in Dhofar, which as discussed previously, are more likely to be seropositive.

In camels, the overall herd seroprevalence was (1.4%, 95% CI 0.6, 2.8) and infection was detected in only eight herds. There was also no significant difference in the herd prevalence between the northern (0.5%) and southern (3.7%) regions of Oman. Dhofar, where brucellosis would appear endemic, contains approximately 50% of the total camel and cattle population in Oman. Consequently most cases of brucellosis in humans have also been recorded in this region. In 2009, the BDU conducted a pilot study to determine the herd prevalence among camel holdings (4.3%), however the study was limited to only one area of Dhofar (Salalah).

This study found serological evidence of infection in camels, especially in those having contact with small ruminants. Similar observations have been reported in other studies ( European Commission, 2001; Musa and Shigidi, 2001; Abbas and Aqab, 2002).

However, clinical signs, especially abortions, were rarely reported by veterinarians or by the owners of camels in positive camel holdings. The low exhibition of clinical disease in camels infected with *B. melitensis* was also in agreement with the findings of others (Abbas and Aqab, 2002; Teshome *et al,* 2003). Although clinical disease is seldom observed in camels, the role of milk from infected camels plays a major role in the transmission of disease to humans (Ismaily *et al,* 1988).

The overall individual animal seroprevalence of brucellosis in Oman was generally at a low level (< 1%). The animal level seroprevalence of cattle in Dhofar in 2003 was reported to be 3.7% and this increased to 8% in 2010 (MAF, 2003b, 2010). However, in the current study, the individual animal seroprevalence of cattle in Dhofar was only 1.5% (95% CI 0.3, 4.4). The overall low animal level prevalence in the current study may be explained by the low prevalence in the northern region which contains the highest number of susceptible animals (goats). The high number of sheep throughout the northern regions may also have resulted in a reduction in the individual level prevalence as sheep have been reported to be more resistant to infection. Cases in sheep and goats were also reported in Dhofar with the individual seroprevalence reported by the BDU dropping from 4.5% in 2003 to 1.3% in 2010. However in the current study the individual seroprevalence of brucellosis in goats in Dhofar was only 1% (95% CI 0.6, 1.8) and was significantly higher than that found in other governorates. In contrast the individual animal seroprevalence in camels was only 0.8% (95% CI 0.3, 1.6). However, there was no significant difference between the seroprevalence in camels from the southern (0.14%) and northern regions (0.77%). In Dhofar, the individual seroprevalence in camels was 0.8% which was comparable to the 1% reported in 2009 (MAF, 2010).

The individual animal seroprevalence was compared with other studies from countries in the region. However, other countries in the Middle East are not isolated, as trade in animals and animal products are significant (Al-Majali *et al*, 2005). The southern governorate of Dhofar shares a border with the Republic of Yemen while the northern region of Oman shares borders with UAE and Saudi Arabia. The individual level seroprevalence in cattle (0.4%) was higher than that reported in Yemen (0.06%), whilst lower than that reported in UAE (1.3%), Saudi Arabia (18.7%), Iraq (3%) and Iran (0.8%) (Gul and Khan, 2007). This finding is probably associated with the lower number of goats and cattle imported from the Horn of Africa into Oman for consumption and breeding purposes when compared with Saudi Arabia and the UAE. However the numbers imported into Oman would be expected to be higher than that into Yemen and this is likely to explain the higher prevalence in Oman compared to Yemen.

The individual animal seroprevalence in sheep sampled in this study (0.1%) was also slightly lower than that reported in Yemen (0.6%) (AlShamahy *et al*, 2000) and significantly lower than that reported from the UAE (3.4%), Saudi Arabia (9.7%) and Iran (10.8%) (Gul and Khan, 2007). This may partly be explained by the overall lower number of sheep in Oman, compared to these other countries. Unlike goats, the limited numbers of sheep imported mainly go directly to slaughter houses and very few are imported for breeding purposes. Generally Omanis prefer the meat of goat and cattle to that of sheep, and this accounts for the low number of sheep imported into the country which would be associated with a reduced probability of disease introduction.

Although goats comprise approximately 70% of the total livestock population of Oman (Agriculture Census, 2004), the animal level seroprevalence for brucellosis in goats was

lower than that reported in neighboring Middle Eastern countries. For instance, the prevalence of disease among goats in Jordon was reported at 27% (Al-Majali, 2005) and 9.7% in Saudi Arabia. These values are markedly higher than that of Oman (0.4%) and Yemen (1.3%) (Gul and Khan, 2007). Good quarantine measures, along with limited importation of goats into Oman, when compared with Saudi Arabia, may play a role in accounting for these differences.

In camels, the individual prevalence found in this study (0.4%) was much less than that reported in Iraq, Saudi Arabia and the UAE (17.2, 8 and 2%, respectively) (Gul and Khan, 2007). In Oman, the importation of camels has been banned for a long time (> 20 years) with the exception of camels entering for competitive races (MAF, 2013). The main purpose of rearing camels in the southern region of the country is for meat production and there is limited transfer of live camels to the northern region, where camels are kept mainly for racing.

Several studies have investigated factors associated with the presence of brucellosis ( European Commission, 2001; Al-Majali, 2005). This study is believed to be the first study to investigate the risk factors associated with the disease in Oman. The logistic-regression analysis indicated an association of breed, age, herd size and production system with the presence of disease. The higher seroprevalence found in imported animals in this study may be due to their susceptibility to infection as they were imported from disease-free-areas. Alternatively this may be due to false positive reactions as they may have previously been vaccinated against brucellosis in their country of origin (Thrusfield, 2005). The higher prevalence reported in crossbred cattle may be attributed to the susceptibility of such breeds to infection or to differences in regions where crossbred cattle are commonly found (Dhofar) where they are primarily

reared under a semi-intensive system which facilitates transmission of infection (Haileselassie *et al,* 2010).

Generally in this study, the seroprevalence increased with age, possibly because of the higher risk of contracting the disease after puberty due to increased contact with potentially infected animals ( European Commission, 2001; Kebede *et al,* 2008; Tolosa *et al,* 2008; Dinka and Chala, 2009) and due to the massive excretion of pathogens during active infection (Durán-Ferrer *et al,* 2004). However, the similar prevalence in males and females is not unexpected due to the similar management practices these animals are subjected to, especially in Dhofar. Nevertheless, males are usually kept for a shorter period of time than females which may reduce the chance of interaction with infected females or other herds and hence the probability of infection could be expected to be lower in males (Kebede *et al,* 2008; Dinka and Chala, 2009; Teklue *et al,* 2013).

Diagnosis of brucellosis is usually made by antigen detection or through serological assays, however no test is 100% sensitive or specific (Martin, 1977). Only *B. melitensis* biotype 1 was cultured from the different species sampled in the Dhofar governorate. The molecular characterization of the isolates in this study suggested a closely related strain has existed in all governorates over the past three decades. Others (Verger *et al,* 1985; Hill and Cook, 1994) have also reported that *Brucella* are genetically similar elsewhere.

In the current study although there were no significant differences observed in the prevalence as determined by the cELISA (sensitivity >97%, specificity >99%) (Perrett *et al,* 2010), the iELISA and the RBPT, more samples were positive on the ELISAs than on the RBPT. This may reflect the higher sensitivity of the ELISAs as has been reported

by others (Burriel *et al*, 2004; Durán-Ferrer *et al*, 2004). However, the rapid test (Immunochromatography Assay - ICA) appears to have a lower sensitivity. Both the cELISA and iELISA seem to be the preferred tests for investigating the seroprevalence in herds and for evaluating control and eradication programs, especially in a country where vaccination is undertaken. In an experimental infection study in pregnant ewes by Durán-Ferrer *et al*, (2004), large differences in the immune response of previously vaccinated and non-vaccinated groups were observed. Most of serological techniques were able to detect antibodies in the non-vaccinated group two weeks post infection and this immunity was detected until the end of the experiment (30 weeks). In contrast, although most tests were able to detect weak antibodies before experimental challenge, the response post-inoculation was variable and few tests were capable of consistently detecting the antibody responses in previously protected animals.

The control of brucellosis has been a subject of debate among scientists for several decades and no one specific program is recommended for every country/territory or region. To plan a program and action plan, several considerations for each situation have to be undertaken. Firstly it is very important to specify whether the goal of the program is the control or eradication of the disease and this mainly depends upon the financial situation of the country and the impact of the disease. Secondly, it is critical to have accurate information about the factors associated with the disease. These factors include the location and environment where the disease is distributed, the animal population and the management and husbandry practices adopted, the expected prevalence, the culture of the owners in each region and their willingness to cooperate in a control program. Once the situation with respect to the disease is known, the country may go for a specific strategy or a combination of strategies. Ideally,

identification of animals by ear tagging or another method and screening of animals with a suitable test, such as the RBPT or ELISA, is recommended before selecting a specific control program. In situations where the prevalence is low, a test and slaughter program could be adopted using the simple, rapid and inexpensive RBPT. However, the same test may not be ideal in populations with a high prevalence or in a country with a low prevalence but with limited financial resources. Therefore, a confirmatory process using more accurate tests and different controlling methods has been adopted in many countries (Mylrea, 1991).

The failure of a vaccination program (2003 until 2012) with strain Rev-1 vaccine in the Dhofar governorate could be explained by several reasons. Firstly, several factors were not taken into consideration as have been mentioned previously. One of the major factors was the absence of accurate data about the real situation among the different species in the regions of Dhofar. Limited funds for the project were reported by a number of teams and staff, resulting in low vaccination coverage and a poor level of animal identification. As a consequence it was difficult to differentiate the serological response from vaccinated and infected animals by the laboratory. Adopting a vaccination strategy without adequate staff training and without receiving owner cooperation is likely to have hindered the progress of the program. Furthermore the project had poor management and the vaccination strategy adopted was altered by different managers. Initially all species and all ages of animals were vaccinated, however this was changed to one where only younger animals were vaccinated and subsequently it was decided to vaccinate all animals except for camels.

With the information gathered in the current study, it is possible to develop a strategy to control brucellosis in Oman. However, the problems with previous programs need to be

considered when developing such a program. The country could be treated as two regions (the higher prevalence area in the south - Dhofar Governorate and the lower prevalence area in the north). In the southern region, initially implementing a vaccination campaign with animal identification and screening is the preferred control procedure; however, it is essential to include all animal species in this program after evaluation of the use of Rev-1 vaccine in camels. Subsequently a test and slaughter method with vaccination of young, disease-free animals could be implemented. According to Alton (1977b), it is likely that vaccination would need to be continued for at least 10 years to reduce the level of infection to a low level. On the other hand, as the prevalence is already low in the northern region, a test and slaughter program could be implemented immediately. Lastly the animals that are actually vaccinated in a herd is controversial. Several alternatives exist including vaccinating all animals in a herd (both adults and young animals), or vaccinating only adults or only young animals. Although vaccinating both young and adult animals would appear easier than repeated vaccination of calves, several authors have highlighted that vaccination of sexually mature cattle induced higher levels of immunity in cattle than vaccination of calves (Alton *et al*, 1980; Corner and Alton, 1981).

In conclusion, the work described in this thesis has demonstrated that *B. melitensis*, biotype (biovar) 1 is the sole cause of animal brucellosis in the Sultanate of Oman. Serological testing using different assays revealed evidence of the infection in different animal species. This study also highlighted that brucellosis is a serious zoonosis in the endemic region (Dhofar). It is recommended that both a vaccination program and a test and slaughter policy be adopted to control the disease. These control measures would result in significant benefits to the economy as well as to public health.

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## APPENDICES

Appendix 1

**Questionnaire for recording epidemiological data at farm level**

Epidemiological study of Brucellosis in the Sultanate of Oman
Farmer Questionnaire – Management and economic evaluation - English translation
Region: Area Village:
Farmer Name: Herd Number: Date:
1-1 How many animals are in the herd ( )
1-2 please fill in the following

| Cattle | | | | | Camels | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| > 48 mo | 25-48 mo | 13-24 mo | 6-12 mo | < 6 mo | > 48 mo | 25-48 mo | 13-24 mo | 6-12 mo | < 6 mo | Age |
| | | | | | | | | | | Number of females |
| | | | | | | | | | | Number of males |
| | | | | | | | | | | Total |

| | Goats | | | | | sheep | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age | < 6 mo | 6-12 mo | 13-24 mo | 25-48 mo | > 48 mo | < 6 mo | 6-12 mo | 13-24 mo | 25-48 mo | > 48 mo |
| Number of females | | | | | | | | | | |
| Number of males | | | | | | | | | | |
| Total | | | | | | | | | | |

1-3 Please complete the following table (enter all available information):

| |
|---|
| Are animals confined in the farm - or do they go outside for grazing? |
| If confined-Are they fed together in the same place? |
| What are the animals used to go for grazing? |
| When do these animals go for grazing and when do they return? |
| Are animals separated from other species on the farm or are all species together? |
| Are any animals tied up? If so, which animals (species/type) and how many? |
| Are sheep and goats kept together or are they separated? Are they grazing freely or are they tied up? |
| Is AI used on cattle on the farm? |
| How many offspring have been produced over the last 3 years (approximate)? |
| How many abortions have occurred during the last three years and in which animals (species and groups)? |
| Did all the abortions result in the birth of dead fetuses or did some survive for a period of time? |
| How many of the aborted fetuses died immediately after birth according to species for the last three years? |
| At what stage (month) of the pregnancy did the abortion occur? If unsure of the age what size were the aborted fetuses? |
| Did any animals have a retained placenta after delivery? If so in which species? |
| How did you dispose of aborted fetuses and/or placenta (Burn- bury- leave it where it was-gave to dogs, other please specify)? |

| |
|---|
| Have you sold any animals from your farm/herd during the last three years? If yes please specify the species-numbers sold and their gender and price received? |
| What type of food do you give to your animals? Is the same ration (type of food) given to the animals throughout the year or is it changed? |
| In the past year, did you buy any extra food, medicine or vitamins for your animals? If yes approximately how much did you spend each month? Please provide details of extra food that you gave? |
| What is the source of water for your animals? Have you ever had to buy in water from outside for the animals for drinking? If so how much did the water cost, how often did you do it and how long did it last? |
| Do you take the animals for drinking outside the farm? How many times daily and how far is the source? What is the time needed? |
| Is there any electricity used in farm? Is it private or governmental? How much does it cost per month? |
| Are there any expatriate workers in the farm? How many and what are their salaries? |
| How many diseased animals were there last year? Who treated them? How many veterinarians visited the farm last year? |
| If a private veterinarian did the treatment, how much does it usually cost? What was the total cost last year? |
| Was the herd vaccinated-who did it- how many times annually? |
| If you sold animals last year, who bought them (Market-farmers-meat consumers-abattoir)? |
| Did you introduce any animals in last three years? What was the source? What was their age? How much did they cost? |
| Do you isolate newly purchased animals before mixing with your herd? If yes for how long? |
| Have you ever purchased animals from neiboring countries especially during the Eid celebration? What were their species, sex and age? |
| What have been the major health problems in the herd for the last two years? |

| |
|---|
| Was the herd vaccinated against Brucella? Which species? When was the last vaccination performed? |
| What kind of camels are there (Racing-breeding or meat)? Where are they from? How are they fed? |
| Do you take your camels to neiboring countries? Why? When? For how long? |
| What other expenses does the farmer have? |

Appendix 2

**Excel based proforma for recording of epidemiological information**

| Proforma for Disease Field Sampling of Map Project, Veterinary Research Center, Ministry of Agriculture & Livestock Regarding Brucellosis, Jhone's Disease and Echinococcosis | | | | | | | | | | Page-I |
|---|---|---|---|---|---|---|---|---|---|---|
| Serial No. | | | Date | / / | | Location | Region/_____ Sub Region _____ | | | |
| VRC No. | | | | | | Lattitude | | | | |
| | | | | | | Longitude | | | | |
| Animal | Cattle | Sheep | Goat | Camel | | | | | | |
| Sex | Male | Female | | | | Age | < 1yea | 1-3 y | 3-5 y | > 5 year |
| | | | Tag No. | | | Breed | Local | Imported | Crossbred | |
| Ownership and Managemental Details | | | | | | | | | | |
| Name of Owner | | | | | Contact No. | | | | | |
| Address | | | | | | | | | | |
| Total Number of Animals | | | | | | Type of He | Dairy | Meat Production | Mixed | |
| 1 | Cattle | < 10 | 10 to 20 | 20 to 30 | 30 to 40 | 40 to 50 | > 50 | Managed | Separatly | Mixed | |
| 2 | Sheep | < 10 | 10 to 20 | 20 to 30 | 30 to 40 | 40 to 50 | > 50 | Replaceme | Home Reared | Purchasing | |
| 3 | Goat | < 10 | 10 to 20 | 20 to 30 | 30 to 40 | 40 to 50 | > 50 | | | From | |
| 4 | Camel | < 10 | 10 to 20 | 20 to 30 | 30 to 40 | 40 to 50 | > 50 | | Local Market | Yes | No |
| 5 | Dog | < 10 | 10 to 20 | 20 to 30 | 30 to 40 | 40 to 50 | > 50 | | If No: | Specify Region | |
| 6 | Others | | | | | | | | | | |
| Breeds Kept | | | | | | Housing | Shed | loose | Open | | |
| | Cattle | Local | Cross Bred | Imported | | Feeding | Pastures | Pen | Mix | | |
| | Sheep | Local | Cross Bred | Imported | | Water Sc | Communal | Separate | Others | | |
| | Goat | Local | Cross Bred | Imported | | | | | | | |

| Animal Health Informations | | | | | | | | | | | Page-II |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Animal** | Cattle | Sheep | Goat | Camel | | | **Age** | < 1 year | 1 - 3 y | 3 - 5 y | > 5 year |
| **Sex** | Male | Female | | | | | **Breed** | Local | Imported | Crossbred | |
| | | | | | **Tag No.** | | | | | | |
| **Yaccinated for Bruc** | Yes | No | | | | | **Appearence** | Bright | Dull | Lethargic | Others |
| | | | | | | | **Appetite** | Normal | Reduced | off feed | |
| **Temperatu** | F | | | | **Pulse** | /min | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | **Female** | Cattle | Sheep | Goat | Camel |
| **Status** | Cattle | Sheep | Goat | Camel | | Abort Hist | Abort Hist | Abort Hist | Abort Hist |
| | Lactating | Lactating | Lactating | Lactating | | Never Abort | Never Abort | Never Abort | Never Abort |
| | Pregnant | Pregnant | Pregnant | Pregnant | | Lameness | Lameness | Lameness | Lameness |
| | Dry | Dry | Dry | Dry | | Other | Other | Other | Other |
| | Bull | Ram | Buck | Buck | | | | | |
| | | | | | **Male** | Cattle | Sheep | Goat | Camel |
| **Diarrhea** | Yes | No | | | | Orhchitis | Orhchitis | Orhchitis | Orhchitis |
| **if yes** | Loose | **Frequency** | | Intermittent | | Infertility | Infertility | Infertility | Infertility |
| | watery | | | Continuous | | Lameness | Lameness | Lameness | Lameness |
| | bubbles | | **Treatmen** | Yes | No | Other | Other | Other | Other |
| | Bloody | | **Respons** | Yes | No | | | | |
| | | | | | **Any other information** | | | | |
| **Bottle Jaw** | Yes | No | | | | | | | |