# An epidemiological study of the impact of *Toxoplasma gondii* and *Brucella melitensis* on reproduction in sheep and goats in Dohuk Province, Iraq

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# Author’s declaration

I, Ali Jadaan Ali Al-Hamada, declare that the work presented in this thesis has not previously been submitted to any university for the award of a degree of Doctor of Philosophy.

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Ali Jadaan Ali Al-Hamada

24/08/2021

# Abstract

Brucellosis and toxoplasmosis are very important zoonoses in many countries of the world, including Iraq. These diseases are considered economically important due to the negative effects on reproduction of small ruminants, which are a critical part of livestock enterprises in Iraq. Prior to the study outlined in this thesis, few studies on the epidemiology of brucellosis and toxoplasmosis and their effect on the reproduction in Dohuk Province had been undertaken. Consequently, the aims of the study were to determine the epidemiological characteristics, economic impact, and effect of brucellosis and toxoplasmosis on reproduction in small ruminants in Dohuk Province.

A cross-sectional study of 432 small ruminants (335 sheep and 97 goats) belonging to 72 farms in six districts in Dohuk Province, northern Iraq, was undertaken to investigate risk factors associated with brucellosis seropositivity. Sera were tested using the Rose Bengal test (RBT) and an indirect enzyme-linked immunosorbent assay (iELISA). Using parallel interpretation, RBT and iELISA results showed that 31.7% (95% confidence interval (CI): 26.1, 36.3) of sheep and 34.0% (95% CI: 24.7, 44.3) of goats in the study had antibodies against *Brucella*. A random-effects multivariable logistic regression model indicated that a higher chance of being seropositive (odds ratio (OR) = 1.7; 95% 1.4; 2.2) was associated with an increase in the age of animals. The odds of *Brucella* seropositivity in flocks where sheep and goats grazed together was 2.0 times higher (95% CI: 1.08; 3.9) compared to flocks where sheep and goats grazed separately. The odds of *Brucella* seropositivity in small ruminants was 2.2 higher (95% CI: 1.2; 4.3) for animals originating from farms with a history of goat abortion in the preceding 12 months. In contrast, for every 1000 Iraqi Dinars (US$ 0.85) spent by the farmers on control of *Brucella* in their flocks, the odds of *Brucella* seropositivity decreased

significantly (OR = 0.9, *p*-value = 0.021). The final model also indicated significant differences in *Brucella* seropositivity between the different districts of Dohuk province.

The small ruminants were also tested for the presence of antibodies against *Toxoplasma gondii,* using a latex agglutinin test (LAT) and an indirect enzyme-linked immunosorbent assay (iELISA). When the test results were interpreted in parallel, 42.1% (95% CI: 36.7, 47.7) of sheep and 36.1% (95% CI: 26.6, 46.5) of goats were found to have antibodies against *Toxoplasma*. A multivariable logistic regression model was developed to determine the risk factors for *Toxoplasma* seropositivity in small ruminant flocks. Factors which increased the risk of infection included the presence of cats near the feed of animals (OR= 6.3; 95% CI 1.6, 24.6) and a history of abortions in sheep in the preceding 12 months (OR=13.4; 95% CI 2.1; 86.7). For every ten goats aborting in the preceding 12 months the odds of seropositivity increased significantly (OR=6.7; 95% CI 1.3; 32.9). In contrast, for every 1000 Iraqi Dinars (US$ 0.85) spent by the farmers on the prophylactic treatment in their flocks, the odds of *Toxoplasma* seropositivity decreased significantly (OR = 0.94; 95% CI 0.90, 0.98).

Sera from 240 small ruminants (192 sheep and 48 goats) from 12 farms in Dohuk Province, northern Iraq, were collected to investigate relative risk of pregnancy loss associated with brucellosis and toxoplasmosis seroconversion during pregnancy. All the selected pregnant animals were examined by ultrasonography twice, at the time of blood collection (approximately 2 and 4 months of gestation). For detection of antibodies to *Brucella*, serum samples were tested using the Rose Bengal test (RBT) and an indirect enzyme-linked immunosorbent assay (iELISA), while the Latex agglutination test (LAT) and an indirect enzyme-linked immunosorbent assay (iELISA) were used to test for *Toxoplasma gondii* antibodies. There were significance differences in the seroprevalence in sheep and goats at the two sampling times for *Brucella* and *Toxoplasma* (P-value = 0.0003 and 0.03 in first and second sampling, respectively*).* The incidence risk of seroconversion to *Brucella* over the two months

was 10.6% (95% CI: 6.9 -15.3) and 7.3% (95% CI: 4.3 - 11.6) for *Toxoplasma*. The analysis indicated that animals that seroconverted to *Brucella* were more likely to lose their pregnancy (OR: 2.9, 95%CI 1.6-5.5).

An economic evaluation of mass vaccination programme for brucellosis indicated that the financial loss overall from brucellosis would decrease from 1.75 to 0.55 US$ per adult female. The net present value of the mass vaccination program was estimated at US$ 10,564,828 (95% CI: -16,203,454 to 37,049,245), the benefit-cost ratio was estimated to be 4.25 (95% CI: 0 to 11.22), and the internal rate return (IRR) was estimated at 91.38% (95% CI:11.71 to 190.62%). The seroprevalence in small ruminants was predicted to decrease from 9.22 to below 0.73 % after 20 years of the implementation of the proposed mass vaccination program.

It is concluded that, identifying the putative risk factors for both pathogens with implementing a mass vaccination program of small ruminants with Rev. 1 for brucellosis will inform the development of more effective control programs to reduce the impact of the infection and advocate for adequate resources to implement the programs.

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# List of publications

1. Al Hamada, Ali, et al. "Risk factors associated with *Brucella* seropositivity in sheep and goats in Dohuk Province, Iraq." Veterinary Sciences 4.4 (2017): 65 - **PUBLISHED**

2. Al Hamada, Ali, et al. "Risk factors associated with seropositivity to *Toxoplasma* among sheep and goats in Northern Iraq." *Veterinary Parasitology: Regional Studies and Reports* 15 (2019): 100264" - **PUBLISHED**

3. Al Hamada, Ali, et al. " Seroconversion to *Brucella spp*. and *Toxoplasma gondii* in Sheep and Goats in Dohuk Province, Iraq and Its Association with Pregnancy Loss. *Animals*, (2021) *11*(3), 836." - **PUBLISHED**

4. Al Hamada, Ali, et al. "Cost–Benefit Analysis of a Mass Vaccination Strategy to Control Brucellosis in Sheep and Goats in Northern Iraq." *Vaccines* (2021) 9(8): 878." - **PUBLISHED**

# Statement of ethics approvals

## HUMAN ETHICS

This project was conducted under Murdoch University Human Research Ethics Committee approval (2016/002).

## AINMAL ETHICS

This project was conducted under Murdoch University Animal Research Ethics Committee approval (2015/R2805).

# A note on thesis layout

This thesis consists of seven chapters, some of which have been prepared as stand-alone manuscripts and includes four published papers. To maintain the contents and formatting consistency throughout the thesis, the chapters presented differ slightly from the corresponding published manuscripts. Chapter 1 is the Introduction. Chapter 2 and Chapter 3 explore relevant literature on the impact, epidemiology, diagnosis, and control of *B. melitensis* and *T. gondii*, respectively. Chapter 4 presents the Material and Method utilized in this study. Chapter 5 and 6 contribute to the epidemiology of brucellosis and toxoplasmosis in small ruminants in Northern Iraq. Chapter 7 focuses on the influence of seroconversion to *Brucella* and *Toxoplasma* on the reproductive outcome of pregnant sheep and goats. Chapter 8 focuses on the benefit of implementing an animal mass vaccination program of small ruminants with Rev.1 to reduce the prevalence of brucellosis. Chapter 8 summaries the general finding of this project, discusses potential gaps in the field and future directions.

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# List of acronyms and abbreviations

| | |
|---|---|
| B. | *Brucella* |
| BCR | Benefit-cost ratio |
| cELISA | Competitive ELISA |
| CFT | Complement fixation test |
| cfu | Colony-forming unit |
| CI | Confidence interval |
| °C | Celsius |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| FAO | Food and Agriculture Organization of the United Nations |
| iELISA | Indirect ELISA |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IRR | Internal rate of return |
| LAT | Latex Agglutination Test |
| MRT | Milk ring test |
| NPV | Net present Value |
| OIE | World Organization for Animal Health |
| OR | Odds Ratio |
| PCR | Polymerase chain reaction |
| pH | Stands for 'potential of Hydrogen' |
| PV | Present value |
| RBT | Rose Bengal test |

| | |
|---|---|
| Rev-1 | *Brucella melitensis* Rev-1 |
| RR | Relative Risk |
| S. | *Salmonella* |
| S19 | *Brucella abortus* strain 19 |
| SAT | Serum Agglutination Test |
| SC | Subcutaneous |
| S-LPS | Smooth lipopolysaccharides |
| Spp. | *Species* |
| T. | *Toxoplasma* |
| VMC | Veterinary medical centres |
| WHO | World Health Organization |
| Y. | *Yersinia* |

# CHAPTER ONE: INTRODUCTION

## 1.1 Introduction

Sheep and goats are important livestock species, and their products (meat and milk) are used in various parts of the world (Corazzin et al., 2019; Kosgey et al., 2008; Vagnoni et al., 2017). In spite of the large numbers of small ruminants, the productivity is commonly low in resource-limited settings, due to several factors that include parasitic and infectious diseases (Martins et al., 2012). *Toxoplasma gondii* and *Brucella melitensis* are important pathogens that cause abortion in small ruminants (Gazzonis et al., 2019; Ntirandekura et al., 2018).

The intracellular parasite *T. gondii* is the causative agent of toxoplasmosis, which can infect virtually all warm-blooded animals, and is distributed worldwide (Zhang et al., 2016b). Felids are the only definitive hosts of *T. gondii*, and up to one third of the world population is infected with this pathogen (Khan & Grigg, 2017; Shapiro et al., 2019). Infection by this pathogen can be transmitted naturally through the ingestion of infective oocysts (Shapiro et al., 2019). In both small ruminants and humans, if the parasite is encountered during pregnancy it may cause seroconversion, and abortion and congenital malformation; foetal infection in small ruminants has a major economic impact upon small ruminant farming (Sager et al., 2003; Tonouhewa et al., 2019). Consumption of meat of infected lambs and goats is considered as the main source of human infection in developing countries (Asgari et al., 2011). Most *T. gondii* infections in small ruminants occur through the ingestion of oocysts, a stage of the parasite which is very stable and can survive in favourable condition in the environment for over 12 months, contaminating pasture, feeds and drinking water (Innes et al., 2009a). Sheep and goats are considered as biological indicators for the contamination of the environment with *T. gondii* oocysts (Al-Kappany et al., 2018).

Central Asia, the Middle East, and adjacent subtropical geographies are among those with the highest incidence of brucellosis in humans and livestock worldwide (Pappas, 2010). Brucellosis, a

neglected tropical food-borne disease, has a negative impact on both animal and human health as well as tremendous socio-economic impact in developing countries where rural income relies largely on small ruminants' breeding and dairy products (Aworh et al., 2017). Infection with *B. melitensis* is one of the most important causes of abortion in goats and sheep, and also causes severe systemic disease in exposed humans (Tekle et al., 2019). Animals and humans acquire infection mainly through direct contact with infected livestock, or the consumption of unpasteurized milk and other contaminated products (Ning et al., 2013). The symptoms of brucellosis include abortion and decreased milk production in small ruminants (Rossetti et al., 2017). In general, *B. melitensis* infection exhibits low fertility rate; however, it relapses frequently if left untreated (Rossetti et al., 2017). In developing countries, farmers have lower awareness of the prevention and treatment of brucellosis, compared to the farmers in developed countries (Sharma et al., 2015). Therefore, it can lead to serious economic loss and cause significant medical challenges in domestic animals and humans. However, the control of *B. melitensis* is proving challenging in most endemic area (Blasco, 2010).

Control of these diseases is the best way to reduce the damage caused by them. However, successful control is based on sufficient knowledge of their epidemiology (Mathew et al., 2015). In Iraq, brucellosis and toxoplasmosis are considered as notifiable infectious diseases across all provinces. These diseases have a seasonal notification pattern and abortion of pregnant animals continues to occur each year. A review conducted for ways of management of sheep and goats in Iraq identified a key knowledge gap in assessing risk of transmission of both brucellosis and toxoplasmosis, and in economic loss data due to brucellosis infecting sheep and goats in Iraq. managing with same risk factors over the years from the farmers, and their potential link with abortion outbreak of brucellosis and toxoplasmosis in small ruminants in Iraq. Awassi sheep and goats are the most common small ruminants reared in the Dohuk province (FAO, 2018); thus, the proposed study will focus on the epidemiology of brucellosis and toxoplasmosis in these ruminants.

Therefore, the over-arching aim of this PhD theses was to gain a more thorough understanding of the risk these two diseases pose to small ruminants in Dohuk Province, with a view to then being

able to recommend effective and economically beneficial management strategies to reduce the impact of the diseases.

Specifically, this project aimed to:

1- Determine the risk factors associated with *Brucella* seropositivity in sheep and goats in Dohuk province, Iraq

2- Determine the risk factors associated with seropositivity to *Toxoplasma* among sheep and goats in Northern Iraq.

3- Determine the seroconversion to *Brucella melitensis* and *Toxoplasma gondii* during pregnancy and its association with abortion in sheep and goats in Dohuk province, Iraq.

4- Conduct a cost-benefit analysis of a mass vaccination control strategy against brucellosis in sheep and goats in Dohuk province, Iraq.

### 1.2 Study site

Dohuk province is located in the northern part of Iraq, bordering three countries, Syria, Turkey, and Iran. It is divided into seven districts, and each district is further subdivided into sub-districts and villages. The district has approximately 1.2 million people (Ibrahim et al., 2015), and approximately 1 million sheep and goats (Zangana et al., 2013).

Dohuk Province is located in the northwest of Iraq within the region of Kurdistan approximately 470 km from Baghdad. The climate is semi-arid; it is hot and dry in the summer and cold and wet in the winter. The mean maximum temperature in the summer is 41 °C, and the mean temperature minimum in the winter is 4 °C (Jamal & Mohammed, 2015).

Livestock, agriculture and fisheries are among the oldest and most significant industries in the economy of Dohuk (Al-Barzinji et al., 2011). Sheep and to a lesser extent goats are traditionally important to producers and the community. The number of goats has declined since 1970 (Brown, 2012) because of the damage they inflict upon the environment as well as their reduced marketability;

most people in Dohuk prefer meat from sheep rather than from goats. In addition, the people of Dohuk generally believe that milk from goats is the source of *Brucella* as opposed to milk from sheep (Unegbu & Okanlawon, 2015).

Traditionally, the most important animals have been sheep and goats. Generally, goats are no longer a readily marketable commodity. They are now raised for their hair and wool, particularly the Angora goat, which is the source of mohair. Their numbers, once in millions, are steadily dropping because of the shrinkage of the market and the extensive damage they cause to the pastures and wood lands (Izady, 2015). In addition, the low prices commanded by mohair, coupled with the breed's slow reproductive capacity (i.e., its growth rates and milk production) further reduce the popularity of raising goats (Unegbu & Okanlawon, 2015). The reduction in available range land has further affected the population of goats in the area (Iñiguez, 2004; Unegbu & Okanlawon, 2015). In contrast, sheep not only are expanding in numbers but also are gaining in importance as an export commodity. The meat can now travel refrigerated or frozen to distant internal and external markets, generating vast amounts of income for the Kurdish producers (Izady, 2015). The Middle Eastern market's preference for fresh meat guarantees a ready market for Kurdish lamb, which is presently denied to geographically remote international producers, such as Argentina, New Zealand and Australia, whose live sheep export to the area is economical only after the local annual live supplies have run out (Izady, 2015).

### 1.3 Importance of toxoplasmosis and brucellosis in study area

Dohuk province, in the Northeast of Iraq, is considered the most important area in Iraq for raising animals, especially sheep and goats, which are the most abundant domestic animals in this region. The main breed of sheep in Iraq is the Awassi. The sheep industry is considered an important source of income for people in Dohuk Province. The sheep flocks in this study were classed semi-nomadic because they moved away from their grazing areas in the in the different area during early

March until the end September. For the remainder of the years, these flocks were housed and group-fed about 500g barley, 1 kg straw, and 200 g wheat bran per head per day.

Abortion outbreak in sheep and goats can lead to loss of milk production, decreased numbers of lambs and kids, loss of replacement or marketable stock, and cause significant risk of human disease (Lokamar et al., 2020; Martínez-Rodriguez et al., 2020). Many abortions in small ruminants occur in the last 6 weeks of gestation and during the dry period, resulting in lost lactation in addition to lost offspring (Menzies, 2011). Management of abortion involves implementing steps to reduce transmission of causes of abortion, with more than one agent or factors often involved in the same outbreak, as well as implementing a plan to reduce human exposure to zoonotic agents of abortion (Menzies, 2011).

Most contagious causes of abortion are spread efficiently through direct exposure to placenta, birth fluids and lochia from aborting does and ewes (Alemayehu et al., 2021). Brucellosis and toxoplasmosis are considered as the main causes of abortion in sheep and goats in Dohuk Province (Ilyas, 2019; Issa & Omer, 2011). Both diseases spread quickly throughout the flock during initial exposure. Long term strategies are needed to reduce the level of flock exposure and increase flock immunity (Lokamar et al., 2020; Martínez-Rodriguez et al., 2020). Toxoplasmosis and brucellosis are zoonotic diseases; therefore, immediate actions are needed to minimize the risk of human infection if an infectious abortion is suspected (Legesse et al., 2018; Shaapan, 2016).

Brucellosis, known as malta fever or Mediterranean fever, is one of the most common bacterial zoonotic disease caused by *Brucella spp*., which can result in serous health issues (Abedi et al., 2020). Brucellosis is endemic among humans and ruminants in developing countries such as Iraq (Hegazy et al., 2011), and as a bacterial zoonotic disease of global significance has detrimental impacts on public health and food production (Bagheri Nejad et al., 2020).

Small ruminant brucellosis has detrimental socioeconomic effects in vulnerable low –income communities, particularly in the Middle East such as Iraq (Franc et al., 2018; Hotez et al., 2012; Musallam et al., 2016; Pappas & Memish, 2007). The disease is principally manifested with late-

gestation abortion, foetal death, infertility, and reduced productivity in livestock (Poester et al., 2013a). However, the real economic losses imposed by the disease on livestock production in endemic resource –poor areas go beyond these apparent direct effects, leading to devastating impacts on socioeconomic development and the promotion of poverty (Franc et al., 2018).

Humans are considered incidental hosts that can be infected through contact with animals and animal products (Corbel, 1997; Godfroid et al., 2005). Acute *Brucella* infection manifests as disabling flu-like syndrome with nonspecific clinical signs including an undulating fever, sweating, chills, myalgia, arthralgia, and fatigue (Dean et al., 2012; Pappas & Memish, 2007). If the disease is not properly diagnosed in its acute phase and is left untreated, it can become chronic and persist for years (Baldi & Giambartolomei, 2013). The chronicity of infection results in localization of the bacteria in various tissues and organs causing debilitating complications such as osteoarticular, hepatobiliary, central nervous system, and cardiovascular involvement (Baldi & Giambartolomei, 2013). The *Brucella melitensis* is the most frequently identified cause of human brucellosis in endemic regions around the globe (Georgi et al., 2017), and it is considered an important foodborne pathogen in developing countries such as Iraq. Although brucellosis in people is not associated with high mortality, it triggers a broad spectrum of tangible and intangible costs that affect both individuals and the community (Franc et al., 2018).

Brucellosis continues to be an important animal and public health burden in the Middle East (ME) (Franc et al., 2018; Musallam et al., 2016; Pappas & Memish, 2007), a group of nations with common geohistorical, developmental, and cultural features (Pappas & Memish, 2007). Similar social, educational, and health conditions prevailing in the region favour endemicity of the disease (Godfroid, 2017; Pappas & Memish, 2007). Traditional customs, such as the consumption of unpasteurization dairy products (Barkallah et al., 2017; Gwida et al., 2010). Additionally, social and political instability and lack of required financial and manpower resources hinder the development and implementation of continuous programmes for the control of disease (Gwida et al., 2010), although considerable gains in knowledge have been achieved through decades of experience in

combatting the disease in different parts of the worlds. It is well knowing that for a control programme to be effective and successful, it needs a sustainable and a truly One Health approach (Godfroid et al., 2013; Godfroid et al., 2014) . The One Health concept is based on the interdependence of human health, animal health, and environmental health, and focuses on the need for cooperative decision-making, planning, and actions to address health problems (Evans & Leighton, 2014). Such an approach requires encompassing necessary discipline – specific and interdisciplinary collaborative arrangements and activities (Godfroid, 2017; Godfroid et al., 2013; Godfroid et al., 2014), whose prerequisite infrastructures are not likely to be provided in the near future in the ME because they demand time, coordinated organization, and authorization (Shiferaw et al., 2017), as well as economic resources that are currently unavailable. Therefore, national awareness and educational programmes addressing all population sectors from consumers to decision–makers seem to be a next logical, sustainable, and economically viable approach to improve disease status in this region.

Toxoplasmosis is a zoonotic disease of global distribution and importance. It is caused by intracellular, apicomplexan protozoan parasite *Toxoplamsa gondii*, the only species in the *Toxoplasma* genus. Felids (domestic and wild) are the definitive hosts and the only hosts able to shed oocysts to the environment. *Toxoplasma gondii* is considered as one of the most important foodborne and waterborne parasites of veterinary and medical importance (Havelaar et al., 2012). This parasite can infect most warm-blooded animals, including human and small ruminants (Almeria & Dubey, 2020). Sheep and goats are economically important in many countries due to the production of meat and milk for human consumption (Ragozo et al., 2010). Sheep rearing constitutes an important agricultural industry with huge economy in Kurdistan Region. Small ruminants are commonly infected with tissue protozoa parasite; in this regard, the *T. gondii* infection of sheep and goats poses a risk to public health in the world and causes significant economic losses to farm animals due to reproductive failure (Buxton et al., 2007a; Edwards & Dubey, 2013). Reproductive failure has a negative influence on animal production, welfare, health, and rural economy.

*Toxoplasma gondii* is a cause of concern for public health, particularly, in immunocompromised people and pregnant women. Although healthy adults generally are asymptomatic, severe clinical disease, even fatal, can be observed mainly in neonates and immunocompromised individual (such as HIV infection, long term-treatment with corticosteroids, hematologic, malignancies and transplant recipient) (Cong et al., 2015). Another important clinical manifestation of toxoplasmosis is ocular, with has shown a prevalence in infected people of around 2% in Europe and 17 % in south America (Greigert et al., 2019).

Toxoplasmosis is a major cause of reproductive failure associated with abortion in sheep and goats (Tenter et al., 2000). In sheep, the abortion or prenatal mortality of lambs occurs when ewes suffer a primary infection during pregnancy (Tenter et al., 2000). Abortion and neonatal mortality are the most important economic losses of sheep and goats industries worldwide (Dubey et al., 1996). The lambs or kids may die in the uterus and after delivery (i.e., stillbirth) or succumb within a few days of birth. Sheep are globally considered important in the epidemiology of *T. gondii* infection, especially in Europe (Buxton et al., 2007a). Sheep are essential to the economy of most countries because they are considered sources of food for human consumption.

The sheep and goat population in Dohuk Province is growing; 65% of these animals are raised for meat and 35% for milk production. Sheep and goats in Dohuk Province are under traditional husbandry systems; and the infection with *T. gondii* is common in this Province. However, there are limited studies conducted to determine the seroprevalence of *T. gondii* in small ruminants and no control programme exists (Al-abodi, 2021). Consequently, the design of strategies for control of infection are needed. This should include access to technical assistance programmes for farm owners, in order to improve husbandry practices in these farms, along with epidemiological surveillance and evaluation of the zoonotic impact of infection on the population. Therefore, the current project investigated the risk factors associated with seroprevalence of *T. gondii* in sheep and goats located in the Dohuk province.

Livestock, agriculture and fisheries are among the oldest and most significant industries in the economy of Dohuk (Al-Barzinji et al. 2013). Sheep and to a lesser extent goats are traditionally important to producers and the community. The number of goats has declined since 1970 (Brown 2012) because of the damage they inflict upon the environment as well as their reduced marketability; most people in Dohuk prefer meat from sheep rather than from goats. In addition, the people of Dohuk generally believe that milk from goats is the source of Brucella as opposed to milk from sheep (Unegbu and Okanlawon 2015).

Traditionally, the most important animals have been sheep and goats. Generally, goats are no longer a readily marketable commodity. They are now raised for their hair and wool, particularly the Angora goat, which is the source of mohair. Their numbers, once in millions, are steadily dropping because of the shrinkage of the market and the extensive damage they cause to the pastures and wood lands (Izady 2015). In addition, the low prices commanded by mohair, coupled with the breed's slow reproductive capacity (i.e., its growth rates and milk production) further reduce the popularity of raising goats (Unegbu and Okanlawon 2015). The reduction in available range land has further affected the population of goats in the area (Iñiguez 2004; Unegbu and Okanlawon 2015). In contrast, sheep not only are expanding in numbers but also are gaining in importance as an export commodity. The meat can now travel refrigerated or frozen to distant internal and external markets, generating vast amounts of income for the Kurdish producers (Nation-States and Kurdish Nationalism, 2015; Izady 2015). The Middle Eastern market's preference for fresh meat guarantees a ready market for Kurdish lamb, which is presently denied to geographically remote international producers, such as Argentina, New Zealand and Australia, whose live sheep export to the area is economical only after the local annual live supplies have run out (Izady 2015). In addition, because the cities close to Dohuk have been affected by war, many farmers have relocated their animals to this region (Nation-States and Kurdish Nationalism, 2015). The need for frozen and refrigerated meat from sheep has grown, and there has been an increased demand for wool by the expanding local cloth industries (Nation-States and Kurdish Nationalism, 2015).

# CHAPTER TWO: BRUCELLOSIS IN SMALL RUMINANTS

**LITERATURE REVIEW**

**(*Brucella melitensis* in sheep and goats)**

## 2.1 Introduction

Brucellosis is an important infectious disease resulting in significant reproductive losses to the livestock industry, as well as posing a major zoonotic hazard to humans (Franc et al., 2018; Pappas et al., 2006). Although brucellosis and its means of transmission were first recognised over 100 years ago, the disease still remains a problem throughout many parts of the world, especially in developing countries (Franc et al., 2018; Mantur & Amarnath, 2008).

*Brucella melitensis*, the main aetiological agent of brucellosis in small ruminants, was the first species of *Brucella* described. It was discovered by Bruce in 1887 (Alton, 1990b) in the spleens of soldiers dying of Mediterranean fever on the island of Malta. Bruce named the bacterium *Micrococcus melitensis*, however it wasn't until approximately 20 years after its discovery that goats were recognised the source of infection for humans (Minda & Gezahegne, 2016; Wyatt, 2005).

## 2.2 Aetiology

*Brucella* belong to the family Brucellaceae in the order Rhizobiales of Alpha Proteobacteria (Sankarasubramanian et al., 2016). They are Gram-negative, non-motile, facultative intracellular coccobacilli (Eisenberg et al., 2012; Garrity et al., 2001; Jezi et al., 2019). The genus *Brucella* contains thirteen known species: *B. abortus* (predominantly infecting bovines), *B. melitensis* (ovines

and caprines), *B. suis* (swine), *B. canis* (canines), *B. ovis* (ovines), *B. neotomae* (desert wood rats, *Neotomae lepida*), *B. ceti* (cetacean), *B. pinnipedialis* (pinnipeds), *B. inopinata*, *B. microti* (the common vole, Microtus arvalis), *B. papionis*, *B. vulpis* and an 'amphibian' type strain *Brucella* sp infecting a human (Whatmore, 2021) (Whatmore & Foster, 2021).

Seven biovars are recognized for *B. abortus*, five for *B. suis*, and three (1, 2 and 3) for *B. melitensis*. The three biovars of *B. melitensis* cause similar clinical disease in small ruminants; however, their geographic distribution varies (Behroozikhah et al., 2012). Infection with *B. abortus* or *B. suis* can also occasionally occur in small ruminants, although clinical disease appears rare (Spickler, 2018). The most frequently recorded biovars of *B. melitensis* are 1 and 3 (Yespembetov et al., 2019).

### 2.3 Survival of *B. melitensis* in the environment

*Brucella melitensis* can survive for several months in the environment, especially under cold, wet conditions in the absence of direct sunlight and UV light (Al-Majali et al., 2009; Saxena et al., 2018).The organisms have been reported to survive for 40 days in dry soil, 60 days in moist soils, 144 days at 20 °C and 40% relative humidity in soil, for several months in drinking water maintained at 4 to 8 °C, and two and a half years at 0 °C in water, 30 days in urine, 75 days in aborted foetuses, more than 200 days in uterine secretions and several years in frozen tissues or culture media (Coelho et al., 2015). Most commonly available disinfectants, including 70% alcohol, will destroy *Brucella* on contaminated surfaces (Worth Calfee & Wendling, 2012).

### 2.4 Clinical signs, pathology and pathogenesis

#### 2.4.1 Clinical signs in sheep and goats

*Brucella melitensis* is the principal cause of brucellosis in sheep and goats, although infections with *B. abortus* occasionally occur and *B. ovis* is commonly isolated from sheep but not from goats

(Davies, 2019). Brucellosis in goats has many similarities to bovine brucellosis; however, the disease in sheep is usually less protracted and spontaneous recovery is fairly common several weeks or months after infection (Maxie & Miller, 2015).

The main effect of *B. melitensis* in sheep and goats arises from its impact on productivity, in particular reproduction (Tekle et al., 2019). In females the disease is characterized by abortion, retained placenta, low fertility rates and the birth of weak offspring (Blasco & Molina-Flores, 2011; Khan & Zahoor, 2018; Rossetti et al., 2017). Abortion usually occurs in the last trimester of pregnancy (Blasco & Molina-Flores, 2011) and animals that abort may retain their placenta. Although small ruminants usually only abort once as a result of infection, reinvasion of the uterus and shedding of *Brucella* can occur in subsequent pregnancies (Abu-Seida et al., 2015; Rossetti et al., 2017).

Sheep and goats which abort also have a reduced milk yield (Rossetti et al., 2017); however, clinical signs of mastitis are uncommon (Saxena et al., 2018). The inflammatory changes in the infected mammary gland can result in a 10% reduction in milk production (Tilahun et al., 2010).

In the male, localisation to the testes and accessory sex organs is common (Ridler et al., 2014). This may result in acute orchitis and epididymitis and lead to testicular atrophy, infertility and abnormalities of the sperm (Megid et al., 2010). Affected testes may have unilateral or bilateral visceral to parietal tunica adhesions and show signs of necrotizing intratubular orchitis (Alizadeh et al., 2013; Olsen & Palmer, 2014; Wegner et al., 1994). The epididymes usually have unilateral or bilateral granulomatous epididymitis (Navarro-Martinez et al., 2001).

The disease may also affect the musculoskeletal system (Adams, 2002) resulting in arthritis (Khan & Zahoor, 2018; Ridler et al., 2014). Affected joints can display a fibrinous and granulomatous synovitis with proliferative villous projections typical of hygromas (Bracewell & Corbel, 1980; Lluch et al., 2015; Lowbeer, 1959).

## 2.5 *Brucella melitensis* in other species

*Brucella melitensis* can also occasionally infect cattle, camels and dogs, as well as humans, although infections in horses and pigs have been reported rarely (Spickler, 2018).

Dogs working with infected sheep/goat flocks can potentially become infected with *B. melitensis*, although the reported prevalence has been low (3.6%) (Rezaei-Sadaghiani et al., 1996), and they generally rid themselves of the infection relatively quickly (Mikolon et al., 1998). However, abortion, orchitis and epididymitis have been reported in infected dogs (Hinić et al., 2010). Infection of a dog with *B. melitensis* that was identical to the vaccine strain Rev.1 has also been reported (Hinić et al., 2010).

Outbreaks in cattle due to *B. melitensis* have reportedly become an emerging problem in many countries of the world, particularly when these animals are co-housed or co-grazed with infected small ruminants (Álvarez et al., 2011). Although the serological response arising from infection of cattle with *B. melitensis* is similar to that observed with *B. abortus* infection (Ewalt et al., 1997), S19 vaccine does not protect cattle from infection with *B. melitensis*, and *B. melitensis* Rev. 1 vaccine has not been validated for use in cattle (Álvarez et al., 2011).

Although clinical signs of infection are rare in camels, infected camels may shed the bacterium in their milk (Benkirane et al., 2014; Musa et al., 2008), and hence camel milk is considered a potential source of infection for humans (Garcell et al., 2016). Camels are reportedly very susceptible to *B. melitensis* (Gwida et al., 2012), with the prevalence influenced by the prevalence in small ruminants that share their habitat and the type of husbandry/management practices adopted (Musa et al., 2008). Zowghi and Ebadi (1988) isolated *B. melitensis* biovar 1 from camels in Iran and Radwan et al. (1995) isolated biovars 1, 2 and 3 from camels in Saudi Arabia.

The main clinical signs of *B. melitensis* in camels includes abortions, arthritis and hygroma (Musa et al., 2008). As in cattle and other species, in camels *Brucella* localize in the lymph nodes,

and specifically localization in the supra-mammary lymph node is of public health significance resulting in contaminated milk (Musa et al., 2008).

Infection of pigs with *B. melitensis* only occurs sporadically and again occurs where pigs are managed or grazed with small ruminants (Seleem et al., 2010).

Infection of horses with *B. melitensis* is very rare. The main clinical sign observed, if any, in infected horses is local abscess formation in the supraspinous or supra-atlantal bursa (Corbel, 2006). This results in the disease condition known as fistulous withers or poll evil. *Brucella*-associated abortions have been reported in horses, but seem to be uncommon (Spickler, 2018).

Any wild ruminants that come into direct contact with infected sheep or goats or have indirect contact with aborted foetuses or discharges from infected small ruminants can potentially become infected with *B. melitensis* (Saxena et al., 2018). Infection has been reported in Alpine ibex (*Capra ibex*) in Italy (Ferroglio et al., 2007) and in chamois (*Rupicapra rupicapra*) in the Southern French Alps (Mick et al., 2014). In infected Alpine ibex significant clinical disease with signs of orchi-epididymitis, polyarthritis, blindness and neurological signs can develop (Garin-Bastuji et al., 2014). Although there is the potential for spill-over infection into wild ruminants; there is no evidence to suggest that these animals serve as reservoir hosts for the bacterium (Ferroglio et al., 2007).

### 2.6 Pathology of *B. melitensis* in sheep and goats

The presence of erythritol in the placenta of small ruminants is responsible for the localization of *Brucella* in the uterus and the subsequent accumulation of large numbers of bacteria resulting in abortion (Petersen et al., 2013). How *Brucella* respond to the presence of erythritol is not fully understood, but it has been hypothesized that it may involve nutritional, immune and/or hormonal factors (Barbier et al., 2017; Letesson et al., 2017). In natural infections the presence of erythritol may “encourage” *Brucella* to leave the intracellular niche of the trophoblasts in the placental region

and replicate extracellular, potentially resulting in large numbers of bacteria. These bacteria induce inflammation resulting in abortion and ejection of the placenta (Petersen et al., 2013).

In males lesions are mainly found in the tunica vaginalis and testes. The tails of the epididymides are more commonly affected than the head, with either unilateral or bilateral epididymal enlargement. Extensive adhesions, and thickened and fibrous tunica vaginalis can result in reduced mobility of the testes within the scrotum in the chronic form of the disease (Coetzer et al., 1994; Xavier et al., 2009).

## 2.7 Pathogenesis

*Brucella melitensis* enters susceptible animals by inhalation, through skin abrasions, across mucous membranes, particularly those of the oropharynx and conjunctiva, by ingestion, or via semen during natural mating or artificial insemination (Aparicio, 2013; Głowacka et al., 2018). After entry into an animal neutrophils and macrophages transport the bacteria to the regional lymph nodes where they may remain for two weeks to several months (Tadeg et al., 2015). The organism is then transported via the lymphatic and circulatory system to a range of organs, particularly the pregnant uterus, mammary gland, spleen and supramammary lymph nodes of females, and the testes and accessory gland of males (Poester et al., 2013b; Sutherland & Searson, 1990; Tadeg et al., 2015).

*Brucella* have a number of virulence factors to overcome the immune system which facilitates their survival in host cells (Oliveira et al., 2008). The bacteria enter host cells via lipid rafts and once internalized, the bacteria localise in a *Brucella*-containing vacuole (BCV). An important *Brucella* virulence factor is its unconventional lipopolysaccharide (LPS) that is required for entry into host cells and in the early development of BCV (Arellano-Reynoso et al., 2005; O'Callaghan et al., 1999).

In pregnant small ruminants, over 85% of bacteria can be found in the placental membranes, cotyledons, and amniotic liquid, reaching up to $1 \times 10^{10}$ colony-forming units (CFU)/ml in allantoic fluid and $1 \times 10^{13}$ CFU/g of tissue in the cotyledons (Martínez-Núñez et al., 2010). Although not all

infected goats abort, in one study all goats which did abort shed *B. melitensis* in uterine discharges (Higgins et al., 2017).

The mammary gland is also a very important predilection site for *B. melitensis*. Infection in lactating non-pregnant goats is likely to lead to colonisation of the gland with excretion of *B. melitensis* in the milk (Khan & Zahoor, 2018). This excretion is short, only for a few weeks, in sheep, compared to several months or even years in goats (Maxie & Miller, 2015). In goats, about two thirds of acute infections acquired through pregnancy result in infection of the udder and subsequent intermittent or persistent excretion of the bacteria in the milk (Alton & Elberg, 1967; Alton et al., 1985a; Alton et al., 1985b), resulting in a significant decrease in milk yield after abortion (Rossetti et al., 2017). In contrast, sheep that abort, rarely excrete the bacteria for more than two months in the milk (Alton, 1990a).

Some animals develop self-limiting infections, while others become asymptomatic latent carriers with the potential to shed the organism resulting in infection and transmission to previously free populations (Hensel et al., 2018; James et al., 2017).

## 2.8 Epidemiology

### 2.8.1 Geographical distribution

Brucellosis arising from infection with *B. melitensis* is an important zoonotic disease in Africa, southern Europe, the Middle East, Central and Southeast Asia, India, China and Central/South America (Menzies, 2012; Rossetti et al., 2017) (Figure 2.1). Ovine brucellosis caused by *B. melitensis* is present in five of the seven continents (South and North America, Europe, Asia, and Africa). Biovar 1 is predominant in Latin America (Benkirane, 2006; Lucero et al., 2008), biovar 2 in the Middle East and biovar 3 in the Middle East, African and Mediterranean countries, Eurasia, and China (Benkirane, 2006; Musallam et al., 2016; Refai, 2002; Sun et al., 2016). Biovars 1 and 3 are equally common in India (Barua et al., 2016; Singh et al., 2013).


**Figure 2.1 Worldwide distribution of Brucellosis. Accessed 25th December 2021. (https://wahis.oie.int).**

Ovine and caprine brucellosis caused by *B. melitensis* is considered endemic in countries of the Mediterranean basin and the Middle East, in particular Iran, Turkey, Syria, Jordan and Iraq (Al-Mariri et al., 2011; Bechtol et al., 2011; Ebrahimi et al., 2014; Samadi et al., 2010).

## 2.9 Distribution of *Brucella melitensis* in Iraq and neighbouring countries

Brucellosis has been endemic in Iraq since 1966 when first Nielsen reported the disease in sheep and goats from Baghdad (Karim et al., 1979). Karim et al. (1979) reported the first study of the prevalence of brucellosis among sheep and goats of the northern region of Iraq. In Table 2.1 the results of studies to determine the distribution and seroprevalence of brucellosis in sheep and goats from different parts of Iraq and from neighbouring countries are listed.

**Table 2.1. Prevalence of *Brucella melitensis* in Iraq and neighbouring countries to Iraq**

| Country | Location | Animals tested | Year of study | Prevalence (%) | Diagnostic test(s) used | References |
|---|---|---|---|---|---|---|
| Jordan | Northern part of Jordan | Goats | 2010 | 27.7 | RBT and the complement fixation test (CFT) | Al-Majali (2005) |
| Saudi Arabia | Alkamil Province | Sheep<br>Goats | 2013 | 8.09<br>5.88 | RBT, indirect enzyme-linked immunosorbent assay (I-ELISA) | Kandeel et al. (2014) |
| Turkey | Kirikkale Province | Sheep | 2005 | 6.47 | RBT | Apan et al. (2007) |
| Iran | Sarab city | Sheep<br>Goats | 2007 - 2008 | 4.18<br>5.0 | RBT, serum agglutination test (SAT) and 2-mercaptoethanol test (2ME) | Akbarmehr and Ghiyamirad (2011). |
| | Sulaimani Province | Sheep<br>Goats | 2006 | 1.34<br>3.36 | Tube agglutination test (TAT) | Shareef (2006) |
| | Northern part of Iraq (Sulaimani, Dohuk, Mosul, and Kirkuk) Provinces | Sheep<br>Goats | | 0.93<br>4.47 | TAT and SAT | Karim et al. (1979) |
| Syria | Damascus | Sheep | 2011 | 66 | RBT | Al-Mariri et al. (2011) |

RBT: Rose Bengal Test

## 2.10 Modes of transmission

*Brucella* species can be transmitted directly or indirectly. The pathogen is typically transmitted when susceptible animals come into direct contact with tissues or discharges from infected animals. Infection can also occur through the grazing of pastures contaminated with the bacteria or from contaminated water sources (Hegazy et al., 2015). Most contamination of food and water arises from aborted foetuses/material or excretions from the female reproductive tract and these also form the major source of infection for humans (Ebrahimi et al., 2014). Animals infected via the oral route may subsequently shed *Brucella* in the faeces after infection of the gut associated lymphoid tissue draining the gastro –intestinal tract, particularly the Peyer's patches and mesenteric lymph nodes (Grilló et al., 1997; Paixão et al., 2009; Saxena et al., 2018).

*Brucella* shed in the milk or colostrum can also result in infection of offspring (Grilló et al., 1997). The vaginal excretion of *B. melitensis* in goats is greater and more prolonged than sheep, lasting for 2-3 months. In sheep, the period of excretion is generally shorter than for goatsand normally ceases within 3 weeks of parturition or abortion (Alemneh, 2018; Blasco, 2001).

The number of *Brucella* excreted in milk is important for the transmission of the bacterium to humans (Likov et al., 2010). Not all small ruminants that are seropositive shed *B. melitensis* in their milk. However, persistent infection of the mammary glands and supramammary lymph nodes can lead to constant or intermittent shedding of the organisms in the milk in succeeding lactations. Consequently milk is considered an important source of infection for humans as well as lambs and kids (Xavier et al., 2009).

Although a small proportion of lambs or kids can be born infected *in utero,* the majority are believed to be infected with *B. melitensis* through ingestion of contaminated colostrum, milk or from the infected mothers (Robi, 2020). This latter method of transmission is considered to be a

major source of latent infections (Zhang et al., 2018). Latent carriers are difficult to detect with routine serological tests and constitute a major problem in the eradication of brucellosis because they are a potential source of relapses in previously infected flocks (Saxena et al., 2018).

The bacteria can also be shed in semen (Amin et al., 2001; Ebrahimi et al., 2014), although the risk from natural mating would appear lower than other routes of transmission (Pal et al., 2017). It is possible that males may transmit the bacterium mechanically at the time of mating (Ogugua et al., 2014). However, it is generally believed that the male does not play a major role in the transmission of the disease (Ogugua et al., 2014).

Subsequent to abortions soil can become heavily contaminated with *Brucella* (Ebrahimi et al., 2014). The use of communal pastures allows frequent contact between animals, and provides increased opportunity for environmental exposure to infectious materials, for instance arising from parturition. Previous studies have reported that contact between goats and sheep at the flock level was one of the most important risk factors for infection with *Brucella* (Al-Majali et al., 2007). Transmission of brucellosis occurs in ruminants through the excretion of contaminated materials from the female genital tract, which constitutes the main form of transmission to other animals and humans. In most circumstances, the main source of pathogen occurs via the placenta, foetal fluids and vaginal discharges expelled after delivery or abortion. At that time, large numbers of *Brucella* are released (Hensel et al., 2018; James et al., 2017).

## 2.11 Risk factors for infection

**Table 2.2 Risk factors for brucellosis infection**

| **Variable** | **Reference** |
|---|---|
| Small ruminants aged between one and four years of age have been reported to be more susceptible to infection than animals less than 1 year of age | (Boukary et al., 2013) |
| The seroprevalence in female sheep and goats has been reported to be higher in males | (Ogugua et al., 2014) |

| | |
|---|---|
| The goat is considered the principal host of *B. melitensis*, with sheep less likely to be infected, even when kept in close contact with infected goats | (Teshale et al., 2006). |
| The goat is more susceptible to infection than sheep | (Quinn et al., 2011) |
| The system of husbandry and management adopted, as well as environmental conditions, greatly affect the potential for the spread of infection with *B. melitensis* on and between farms | (Megersa et al., 2011a; Teshale et al., 2006). |
| Purchasing animals from saleyards is also considered to be a major risk factor for the entry of *B. melitensis* into herds | (Kabagambe et al., 2001). |
| The illegal movement of small ruminants between Iraq and neighbouring countries, especially during the summer months for grazing and watering, has resulted in epizootic outbreaks of disease and the disease subsequently becoming endemic | (Al-Griw et al., 2017) |
| Implementing poor hygienic practices and an absence of veterinary services were shown to contribute to the higher risk of dissemination and transmission of brucellosis in small ruminants | (Boukary et al., 2013) |
| Owners are often poorly informed about how the disease is spread and transmitted and about methods to adopt to control its impact | (Al-Majali, 2005; Samadi et al., 2010) |

## 2.12 Diagnosis of brucellosis in animals

The diagnosis of brucellosis, based on presenting clinical signs alone, is difficult because of the non-specific nature of these signs and the fact that similar signs are shared with other febrile illnesses including infection with *Coxiella burnetii* and *Salmonella abortusovis* (Roshan et al., 2018). Furthermore, the slow growth rate of the causative agent in culture media and the complexity of some of the classical serological diagnostic tests requiring specific complex and expensive equipment, adds to the difficulty of diagnosis of brucellosis in many countries and regions (Smirnova et al., 2013). Although there are numerous serological assays for the disease, the gold standard still remains culture (Manual, 2008; Saxena et al., 2018), even though it has low sensitivity, is expensive and time

consuming (Al Dahouk & Nöckler, 2011; Espinosa et al., 2009; Franco et al., 2007). Because of these disadvantages most diagnoses of the disease in clinical cases or in control and eradication programs, are made via the careful interpretation of the results of serological tests (Kaltungo et al., 2014; Sammartino et al., 2006).

## 2.13 Methods for brucellosis diagnosis

### 2.13.1 Microscopic examination and culture methods

A smear of tissue or biological fluid stained with the Stamp's modification of the Ziehl-Neelsen method can be useful as a presumptive diagnosis of brucellosis; however, this diagnosis should still be confirmed through serology (Habtamu et al., 2013). On microscopy after staining with the Stamp's modification of the Ziehl-Neelsen method, *B. melitensis* can often be confused with other organisms that cause abortion, such as *Chlamydophila abortus* or *Coxiella burnetiid* (Saxena et al., 2018). A fluorochrome or peroxidase-labelled antibody conjugate-based technique is an alternative staining technique for the bacterium (Roop et al., 1987; Vitry et al., 2014). The fluorescence polarization assay (FPA) is a homogeneous assay which does not require removal of unreacted reagents and can, therefore, be performed very quickly and conducted both in the laboratory and the field (Nielsen & Gall, 2001). However, a significant disadvantage of fluorochrome-labelled antibodies is the high background scattering (noise) and non-specific fluorescence arising from other biological molecules that may be present in the sample. These can interfere with the measurements and limit the use of such probes.

Samples of the placenta, lymph nodes, milk, stomach contents, spleen and lungs of aborted foetuses, and vaginal swabs, semen or fluid from arthritic lesions or hygromas are often cultured to confirm a diagnosis of brucellosis (Ahmed et al., 2010; Padilla Poester et al., 2010). *Brucella melitensis* does not require serum or $CO_2$ for growth and can be isolated on ordinary solid media under aerobic conditions at 37$^{o}$C, with colonies becoming visible after 3 to 5 days of incubation (Scott McVey et al., 2013). Isolation and culture of *Brucella* is generally undertaken on basal media such as

Trypticase soy (BBL®), Bacto Tryptone (Difco®), Tryptic soy (Gibco®) and Tryptone soya (Oxoid®) (Corbel, 2006). Nevertheless, due to the frequent overgrowth by other bacteria which are frequently present in field samples, selective media are recommended for isolation purposes (Scott McVey et al., 2013). Farrell's media inhibits the growth of contaminants better than other *Brucella* agar, and is has been recommended for potentially contaminated samples (Vicente et al., 2014).

For culturing milk, colostrum and other body fluids, it is preferable to use broth or a biphasic medium. Many methods have been developed for culturing *Brucella* spp. from blood specimens, such as the conventional Ruiz-Castaneda (Castaneda) method, automated systems, lysis concentration (LC) method and clot culture method, because of the low concentration of *Brucella* in these samples (Espinosa et al., 2009; Mantur & Mangalgi, 2004; Padilla Poester et al., 2010).

### 2.13.2 Identification of *Brucella*

There are several methods used to identify the bacteria to the genus level. After incubation at 37ºC in either the absence or presence of 10% $CO_2$ for up to 2 weeks, *Brucella* suspected colonies are characterized by their typical round, pinpoint, glistening, and honey drop-like appearance (Alton et al., 1988; Araj, 2010). Initially, the colonies of smooth strains are small, round and convex. When viewed from above, colonies initially appear convex and pearly white and subsequently they become larger and slightly darker in colour (Sulayman et al., 2020). The bacteria are small Gram-negative or Stamp's positive coccobacilli, or short rods with rounded ends and slightly convex sides (Scott McVey et al., 2013). Biochemical tests, including the requirement for $CO_2$, production of $H_2S$, dye sensitivity, and urease, oxidase and catalase tests are also used to identify *Brucella* to the species level (Affi et al., 2011). An absence of growth on MacConkey agar and a non-haemolytic appearance on sheep blood agar is also characteristic of the genus (Geresu et al., 2016; Schurig et al., 1991).

### 2.13.3 Serological tests

Antibodies induced to the bacterial cell wall O-polysaccharide (OPS) component of the smooth lipopolysaccharide (sLPS) are generated in infected animals and are used in the serological diagnosis

of brucellosis (Corbel, 2006). However serial testing has been recommended for screening due to the occurrence of false negative results early in infection (Godfroid et al., 2011; Herrera et al., 2011; Khan & Zahoor, 2018). A range of serological tests have been developed for the diagnosis of brucellosis, each with potential advantages and disadvantages.

#### 2.13.3.1 *Rose Bengal Test*

The Rose Bengal test (RBT) is a simple, inexpensive test that can be conducted in the field (Muma et al., 2009). It is highly sensitive in acute and long standing brucellosis cases which is related to its ability to detect IgM, IgG and IgA (Diaz et al., 2011). The test has a reported sensitivity and specificity in small ruminants of 95 and 100%, respectively (Ferreira et al., 2003) and is recommended for herd/flock tests but is not recommended for testing individual animals (Alton et al, 1988). Although the test reportedly gives few false negative results, it can give many false positive reactions after vaccination with Rev. 1 vaccine, primarily due to the presence of IgM induced by vaccination (Kaltungo et al., 2014).

#### 2.13.3.2 *Complement fixation test*

The complement fixation test (CFT) is a technically challenging diagnostic test to perform due to the process and number of reagents required. It requires experienced operators and regular quality control systems to be implemented and can only be performed under strict laboratory conditions. The results of the test depend upon the antigen used, along with the incubation conditions (Khan et al., 2011; Khan et al., 2014). Despite these potential challenges it is widely used as a confirmatory test for brucellosis in cattle and small ruminants due to its high sensitivity and specificity (Nielsen et al., 2008; Ruckerbauer et al., 1984). The CFT is recommended for testing animals for international trade by the World Organisation for Animal Health (OIE) (Elschner et al., 2019).

A sensitivity of 100% has been reported and a specificity of 77.45% for animals originating from areas where the disease is endemic compared with 93.75% sensitivity and 94.79% specificity for animals from non-endemic areas (Khan et al., 2012). The high sensitivity of the CFT is a key

reason why this test has been considered one of the most superior serological tests (Al-Sherida et al., 2020; Garin-Bastuji et al., 2006). For large-scale surveillance/eradication purpose, the CFT is considered the most reliable serological test (Al-Sherida et al., 2020; Garin-Bastuji et al., 2006). However, as with other serological tests, it is not capable of distinguishing antibodies induced by vaccination with Rev.1 and antibodies resulting from natural infection in small ruminants (Al-Sherida et al., 2020; Gall & Nielsen, 2004) and along with the technical difficulties in performing the tests results in a reluctance or inability to use this test in many countries (Nielsen, 2018).

#### 2.13.3.3 *Enzyme-linked immunosorbent assay*

There is considerable literature on the diagnostic value of the enzyme-linked immunosorbent assay (ELISA) for confirming infection with *B. abortus* in cattle and infection with *B. ovis* in sheep (De Bagüés et al., 1992), and it has been reported to be more effective for detecting infections of small ruminants with *B. melitensis* than the RBT and SAT (Cloeckaert et al., 2001; El-Razik et al., 2007; Jacques et al., 1998).

There are several forms of ELISAs available, including direct, indirect and competitive forms (ELISA, iELISA and cELISA, respectively). ELISAs have also been used to detect antibodies in milk (Altun et al., 2017), and have been evaluated in naturally infected and vaccinated small ruminants (Altun et al., 2017{Wainaina, 2020 #699)}, and also in humans (Yagupsky et al., 2019). A milk ELISA used in lactating ewes, has been reported to be more sensitive than the Milk Ring Test (MRT) in ewes (Biancifiori et al., 1996; Khan & Zahoor, 2018). However the iELISA can cross-react with antibodies to *Yersinia enterocolitica* O:9 that bear a smooth lipopolysaccharide (S-LPS) similar to *Brucella* spp, resulting in false positive reactions (reported 93% specificity) (Cloeckaert et al., 2001; Munoz et al., 2005; Nielsen et al., 2006).

De Bagüés et al. (1992) used an iELISA to differentiate natural infection with *B. melitensis* in sheep from the immune responses resulting from vaccination with Rev. 1 by either the conjunctival or sub-cutaneous route. Others have also reported that the ELISA was more effective than the RBT or CFT for differentiating the immune response from natural infection in small ruminants with that

induced following vaccination with Rev. 1 (Cloeckaert et al., 2001; Nielsen et al., 2004). Under field conditions it has been proposed that the combined use of conjunctival vaccination of young animals with Rev. 1 vaccine and testing of sera with an iELISA could improve the speed of eradication of *B. melitensis* from sheep flocks (Alshwany, 2019; De Bagüés et al., 1992).

#### 2.13.3.4 *Milk ring test*

The milk ring test (MRT), which can detect IgM and IgA antibodies bound to fat globules, is inexpensive, easy to perform and large numbers of samples can be tested in a short time (Cadmus et al., 2008) and is commonly used for detecting brucellosis in dairy cattle (Kumar et al., 2016). However, like serological tests, its use in sheep and goats has limitations (Garin-Bastuji & Blasco, 2008; Khan & Zahoor, 2018). The MRT may not be sensitive enough to detect antibodies in milk with a low concentration of IgM and IgA or that lack the fat clustering factors (Patterson & Deyoe, 1977). In milk of sheep and goats the fat globules are smaller than with cattle, and the agglutinated antigen usually falls to the bottom of the test tube or remains suspended in the milk column (Kolar, 1984) and consequently it is not routinely recommended for the detection of *Brucella* antibodies in sheep and goat milk when compared with cattle (Khan & Zahoor, 2018; Tittarelli et al., 2006). An additional problem with the MRT is the low content of antibodies in goat and sheep milk (Kolar, 1984). Another obvious disadvantage with this test is that its use is limited to lactating animals. Additionally, the lactation period is relatively short, particularly in sheep, and some breeds are not milked at all, restricting its use in these animals (Kolar, 1984).

#### 2.13.3.5 *MALDI-TOF mass spectrometry (MS)*

MALDI-TOS mass spectrometry (MS) has been suggested as a fast and reliable method for the identification of different types of bacteria (Ferreira et al., 2010b; Seng et al., 2009). *Brucella* species still an important pathogen in a wide area around the world, and some databases used for this purpose lack reference profiles for this bacteria. MALDI-TOF is useful for identifying *Brucella* from culture plates and blood cultures. The use of this method in routine bacterial identification from plate culture. Rapid automated bacterial identification systems must be interpreted with caution because brucella

has been misidentified with some of these systems (Elsaghir & James, 2003). PCR has shown high sensitivity and specificity, but its use remains infrequent, mainly due to standardized problems (Queipo-Ortuño et al., 2005). Nevertheless, *Brucella* has not been still incorporated to some of the primary databases available because of problems derived from their potential bioterrorist use. The importance of this technician tool can be able to direct diagnosis of the blood culture in countries where brucellosis is still frequent (Ferreira et al., 2010a).

#### 2.13.3.6 *Polymerase chain reaction (PCR)- based methods*

These methods for the identification of *Brucella* bacteria in blood samples are becoming very important tools for the identification of *Brucella* at both species and biovar level (Yu & Nielsen, 2010). The techniques require minimum biological contamination and can provide results in a very short time. Moreover, genetic fingerprinting of isolates aid in epidemiological methods used to identify *Brucella* spp., and new methods for *Brucella* spp. (Wang et al., 2014). Identification and typing are still being developed. However, the sensitivity, specificity, and issues of quality control and quality assurance using these methods must be fully validated on clinical samples before PCR can be used in routine laboratory testing for brucellosis (Dal et al., 2019; Yu & Nielsen, 2010).

## 2.14 Treatment, prevention, and control of brucellosis

The control and prevention of brucellosis in livestock provides significant production and financial benefits to farmers, as well as producing human health benefits through reduction of cases of this zoonotic disease.

### 2.14.1 Treatment

The treatment of brucellosis requires the use of a combination of antibiotics (Ariza et al., 2007) and the prompt diagnosis and treatment of brucellosis during pregnancy can be lifesaving for the foetus in sheep and goats (Olsen & Palmer, 2014). As *Brucella* replicates within the cell, any treatment must be capable of entering the cell and targeting the bacterium in this location (Głowacka

et al., 2018). In one study, *B. melitensis* was successfully eradicated from 480 naturally infected sheep and goats using a combination of oxytetracycline (OTC) and streptomycin (ST), (OTC 25 mg/kg i.m. every 2 days for 4 weeks, combined with ST 20 mg/kg i.m. every 2 days for 2 weeks), as evidenced by cessation of shedding of bacteria in milk and their absence in any tissues at necropsy post treatment (Radwan et al., 1992). However, in livestock it is preferable to prevent its entry or to control the disease rather than to treat infected animals. In contrast in humans treatment is obviously undertaken. The recommended treatment is a long course (at least 6 weeks) of combinations of oral rifampin (600–900 mg/day) and doxycycline (200 mg/day) (Mile et al., 2012; Solera, 2010). However, treatment of brucellosis in animals is not recommended (Acharya et al., 2017).

### 2.14.2 Prevention and control

Brucellosis is a serious threat to public health, as well as resulting in significant losses to the livestock industries (Khan & Zahoor, 2018; Singh et al., 2015), however control in developing countries can be challenging due to: a lack of facilities and trained personnel; insufficient preventive vaccines; and a low priority for control of this disease by many governments (Deka et al., 2018; Donadeu et al., 2019; McDermott et al., 2013). One of the most important means to decrease the burden of any endemic disease is to reduce its prevalence and incidence (Banai, 2002; Lubroth et al., 2007). As there is no vaccine available to protect humans from infection, it is essential that to prevent cases in humans control focuses on the relevant animal reservoir(s) (Godfroid et al., 2011).

## 2.15 Vaccination

Several vaccines have been developed and trialled for use in small ruminants to protect them from brucellosis. There is no universal *Brucella* vaccine that would provide protection against all pathogenic species of *Brucella* (Jain-Gupta et al., 2019).

### 2.15.1 Live attenuated vaccine Rev. 1

There are a few available live attenuated vaccines for animal immunization against brucellosis (Gheibi et al., 2018). The live vaccine, Rev. 1, is widely used for the control of brucellosis in small ruminants (Kornspan et al., 2019). Although this vaccine is extensively used to protect small ruminants, it can induce serological responses that potentially interfere with the diagnosis of natural infection (Shome et al., 2014). Furthermore: it is resistant to streptomycin, one of the antibiotics of choice used to treat brucellosis (Moriyón et al., 2004); it is pathogenic for humans (Perkins et al., 2010; Salmon-Divon et al., 2018); and its use is prohibited in countries free of the pathogen (Jiménez et al., 1994). In the last several decades, much research has been performed to develop safer *Brucella* vaccine the disease mainly in animals. Till now, no effective human vaccine is available (Avila-Calderón et al., 2013) .Vaccination via the sub-conjunctival route is considered safer than administration via the subcutaneous route; however, it is still not considered safe to use in pregnant animals (Hensel et al., 2020; Zundel et al., 1992). In sheep, whole-flock vaccination via sub-conjunctival administration of the standard dose containing 1 x $10^6$ organisms during lactation has been recommended (Blasco, 1997a).

Although vaccination can induce protective antibodies it is important to accompany this control measure with adoption of good management and husbandry practices, including improved flock and regional biosecurity (Kwaghe et al., 2016).

### 2.15.2 Other live vaccines used in the past

*Brucella melitensis* strain M5-90, a live attenuated vaccine strain cultured from the virulent strain M28, has been shown to be effective in the control of brucellosis in goats, sheep and cattle in China (Cosivi & Corbel, 1998; Deqiu et al., 2002; Wang et al., 2011). However, it retains pathogenicity for humans and represents an occupational health risk to vaccinators (Strausbaugh & Berkelman, 2003). However, as with Rev. 1, M5-90 is not safe for use in pregnant animals which potentially may abort after vaccination, and the immunity induced can also interfere with the interpretation of serological assays.

### 2.15.3 Genetic marker vaccines

One approach to overcome the lack of differentiating the response induced by vaccination from a natural infection immune response is to develop a marker vaccine by deleting virulence or antigenic genes from the parental vaccine strains whilst still maintaining good immunogenicity and hence vaccine efficacy (Zhang et al., 2013). As the most protective vaccines compromise serodiagnosis, this creates policy dilemmas, and these often result in the failure of eradication and control programs. Detection of antibodies to the *Brucella* bacterial cell wall O-polysaccharide (OPS) component of smooth lipopolysaccharide is used in diagnosis of this disease, and the same molecule contributes important protective efficacy to currently deployed veterinary whole-cell vaccines. This has set up a long-standing paradox that while *Brucella* OPS confers protective efficacy to vaccines, its presence results in similar antibody profiles in infected and vaccinated animals. Consequently, differentiation of infected from vaccinated animals (DIVA) is not possible, and this limits efforts to combat the disease. Recent clarification of the chemical structure of *Brucella* OPS as a block copolymer of two oligosaccharide sequences has provided an opportunity to utilize unique oligosaccharides only available via chemical synthesis in serodiagnostic tests for the disease. These oligosaccharides show excellent sensitivity and specificity compared with the native polymer used in current commercial tests and have the added advantage of assisting discrimination between brucellosis and infections caused by several other bacteria such as *Y. enterocolitica* O:9, *V. cholera* O1, *E. coli* 0157, *E. hermanii*, *P. maltophilia* 555, *S. urbana*, *S. godesberg*, *F. tularensis* with OPS that share some structural features with those of *Brucella* (Bundle & McGiven, 2017).

### 2.15.4 DNA vaccines

A number of studies have been conducted evaluating DNA vaccines of *Brucella*; however, the majority of these have been conducted in mice (Oliveira & Splitter, 1996). These DNA vaccines have been shown to induce some protection against *B. melitensis* in mice (Al-Mariri et al., 2001; Bowden et al., 2000), and induce both humoral and cellular (Th1 and CTL) immune responses against a wide range of diseases, such as malaria, leishmaniasis and tuberculosis (Robinson, 1999). However, it is

not clear if DNA vaccines can induce long-term protection against brucellosis (Ko & Splitter, 2003) and if protection demonstrated in mouse models can be translated to protection in livestock (Yang et al., 2005). However Zhang et al. (2019) reported that the DNA vaccine against brucellosis induced a good protective immune response against *B. melitensis* in a mouse model. Because of all the above conflicting results with DNA vaccines, more studies with these vaccines are required in small ruminants.

## 2.16 Control

Control of brucellosis, especially at the end stage of an eradication programme, requires an efficient and accurate surveillance system along with accurate diagnostic tests and highly discriminatory methods to characterize strains present to determine the source of infection and transmission routes (Ferreira et al., 2012). A surveillance system needs to be specifically developed to suit the particular country and region(s) (Robinson, 2003). In order to reduce the incidence of infection in healthy animals, vaccination is one of the most efficacious and cost-effective means of control. Adoption of good hygienic practices, including: pen disinfection and disposal of aborted and placental material; isolation of newly introduced animals; and quarantine infected flocks is recommended (Pérez-Sancho et al., 2015). Quarantining animals which have aborted can reduce the spread of disease within and between farms in endemic regions (Li et al., 2017; Musallam et al., 2015; Refai, 2002). Restrictive quarantine measures have been recommended to be lifted if the animals pass three consecutive negative tests at 21 days intervals (Li et al., 2017; Musallam et al., 2015; Refai, 2002). Isolation of pregnant females before parturition (particularly primiparous animals), regardless of their test status, is recommended until offspring are weaned. Two months after weaning it is recommended that all lambs/kids selected as potential replacements breeders are tested, and all animals which test negative are segregated and continued to be tested every 2 to 3 months until the entire flock has achieved two consecutive whole flock negative tests (Minnesota Board of Animal Health, 2019). Developing community outreach programs involving education, implementing

vaccination campaigns of at-risk or infected flocks/regions, applying disease surveillance for imported livestock, and quarantining infected properties should be developed and involve input from farmers, health educators, and veterinary professionals to help control *Brucella* infection (Li et al., 2017).

The importance of a strong veterinary service and good animal care has been shown to reduce the likelihood of a flock or region being infected with brucellosis (Dadar et al., 2021). Adoption of good husbandry and management systems, including the isolation of animals that abort, and establishing specific areas or pens for lambing/kidding which are subsequently disinfected, have also been recommended to reduce infection within a flock (Dadar et al., 2021; Salman & Meyer, 1987b). Isolation of pregnant ewes and does during and after parturition is critical because these animals potentially can shed *Brucella* resulting in spread of infection to other animals within the flock (Havas, 2012). Any aborted foetus should be disposed of under strict biosafety precautions. Deep burial is recommended to prevent the foetus being dug up by dogs with potential for dissemination of the bacteria. Entrances to sheds/barns where animals are housed should have a footbath containing suitable disinfectant to prevent transmission of the pathogen between groups in infected flocks (Islam et al., 2013).

Both passive and active surveillance systems (Robinson, 2003) are required to control brucellosis, however information obtained from active surveillance systems is preferential to that from passive surveillance systems, which are often limited to data sourced from diagnostic laboratories, farm personnel and veterinary records (Adone & Pasquali, 2013). Establishment of unique property identification codes, particularly when used in conjunction with radio frequency identification (RFID) ear tags, can help in the trace-back and trace-forward of disease outbreaks and allow more effective surveillance systems to be adopted for livestock at saleyards and abattoirs (Radunz, 2006). However, such identification systems are expensive and are often outside the financial capabilities of many developing nations and their livestock owners. The routine evaluation of surveillance data is important to evaluate the effectiveness of brucellosis control efforts and to

identify areas where a targeted response may be required (Peck et al., 2018). As brucellosis affects both humans and other animals, in particular livestock, surveillance systems should be designed to detect the disease's presence as early as possible (Hadorn & Stark, 2008). Any delay in the early diagnosis of the disease can result in spread to other susceptible animal populations, as well as to other regions (Saegerman, 2006).

Control of brucellosis in developing countries is not only a regional concern but also a global issue as international visitors can become infected when visiting endemic countries (Dahouk et al., 2005; Godfroid et al., 2005). Another challenge for the control of brucellosis can arise from transmission of *Brucella* through the wildlife-livestock interface. Wildlife reservoirs close to flocks/herds of domestic animals are important as infections may "spill-over" from wildlife to farmed animals and subsequently spill-back from domesticated animals to wildlife (Assenga et al., 2015; Rhyan & Spraker, 2010). To prevent loss of wildlife species, it is easier to control the disease in livestock; however, in some instances, culling of wildlife to control brucellosis emergence or spill-back of infections to livestock is essential (Godfroid, 2017). However, there have been no published studies investigating the maintenance of *Brucella* in wildlife in Iraq and very limited studies have been conducted in neighbouring countries (Esmaeili, 2014).

In Iraq the uncontrolled movement of sheep and goats between Iraq, Syria and Iran and Iraq across the porous borders results in disease spread regionally. These animals are often sold in the open market without any form of animal identification or registration. Introduction of infected animals into a herd or flock is the major cause of disease introduction to a previously free herd/flock resulting in significant economic losses for the farmers (Al-Griw et al., 2017; Zamri-Saad & Kamarudin, 2016). Animals introduced to a flock, region or country should be tested negative for brucellosis prior to introduction and then isolated for a minimum of 30 days prior to mixing with the destination flock (Giasuddin et al., 2018; Larson, 2007).

The control of brucellosis in Dohuk Province is challenging because of: a lack of permanent identification of animals; ineffective control and recording of animal movements; and poor veterinary services and infrastructure (Alshwany, 2019).

## 2.17 Economic impact of brucellosis in small ruminants.

Infection of flocks with *B. melitensis* can result in a large economic loss for livestock farmers as well as a community. Direct impacts of the disease are associated with reduced milk production, loss of kids and lambs and reduced fertility. Indirect costs of the disease include the costs of vaccination, testing and compensating farmers for slaughtered animals (Montiel et al., 2015; Mustofa & Nicoletti, 1993). Losses arising from brucellosis in small ruminants estimated from a range of studies are summarised in Table 2.3. Control of brucellosis, as opposed to eradication, is recommended in most developing countries to avoid the sizeable expenditure required with implementing eradication programs (McDermott et al., 2013).

**Table 2.3. Economic losses arising from infection of sheep and goats with *Brucella melitensis* from different countries.**

| Country and Region | Type of animal (Sheep/Goats) | Estimated direct economic losses (US$) per year | References |
|---|---|---|---|
| India | Goats | $71 million | Singh et al. (2015)<br>Sulima and Venkataraman (2010) |
| In the north of Portugal | Sheep and goats | $ 110,000 | Coelho et al. (2011) |
| In Peninsula Malaysia | Goats | $2.6 million | (Bamaiyi et al., 2015) |
| Turkey | Sheep and goats | $ 61.71 million | Can and Yalcin (2011) |
| In Borno and Yobe States of arid northeastern Nigeria | Sheep and goats | $ 3.3 million | Brisibe et al. (1996) |

## 2.18 Conclusions

Brucellosis in small ruminants from infection with *B. melitensis* is a neglected disease in many regions of the world, particularly for low-income farmers who do not own land, are nomadic, or graze poor quality land (Boukary et al., 2013; Franc et al., 2018; Rizzo Naudi, 2006). However, knowledge about the risk factors associated with the disease, its impact on the local community, the distribution of the disease, and means for controlling the disease are critical for the local control of this disease. Consequently the study outlined in this thesis was developed to improve the understanding of the epidemiology of brucellosis in sheep and goats in Dohuk Province in the north of Iraq. The specific aims of this research were to: estimate the seroprevalence of brucellosis in small ruminants; identify

risk factors associated with flock level and individual animal level seropositivity; and to compare the financial benefit of controlling the disease through a mass vaccination program compared with the current program of vaccinating lambs and kids between 3 and 6 months of age. A prospective study was also conducted to estimate the incidence of seroconversion and to evaluate the impact of infection on reproductive output in pregnant sheep and goats. This information is critical prior to developing a control program against brucellosis in small ruminants. Implementing disease control program should lead to a reduced incidence and prevalence in both the human and livestock population, resulting in improved profitability for the small ruminant industry and reduced disease impact on humans.

# CHAPTER THREE: TOXOPLASMOSIS IN SMALL RUMINANTS

## LITERATURE REVIEW

**(*Toxoplasma gondii* in sheep and goats)**

### 3.1 Introduction

*Toxoplasma gondii* was first described in the tissues of the gundi (*Ctenodactylus gundi*) of a rabbit (Weiss & Dubey, 2009). Infection with this protozoan is reportedly one of the most common parasitic infections of humans and warm-blooded vertebrate hosts (Ma et al., 2019). It has a heteroxenous life cycle with *Felidae* acting as definitive hosts and other warm-blooded animals, including humans, as the intermediate hosts (Dubey & Beattie, 1988; Opsteegh et al., 2012). However a recent study found that reptiles could also infected with *Toxoplasma* (Nasiri et al., 2016).

Infection of humans with *T. gondii* was first reported in 1938 with the diagnosis of congenital toxoplasmosis in a newborn girl (Wolf et al., 1939). Subsequently the protozoan was recognised as having veterinary importance when it was linked with abortion storms in sheep in 1957 (Dubey, 2008). The effect of *T. gondii* on the placenta of aborted sheep and on the aborted fetuses was first reported in New Zealand (Hartley & Marshall, 1957); however the method of infection was not understood at that time. In the late 1960s, *T. gondii* was found to be shed in the faeces of cats (Hutchison, 1965) and a decade later cats were recognized as the definitive hosts of *Toxoplasma* (Frenkel et al., 1970). The resistant oocysts of *T. gondii*, shed by cats, were considered to be the main source of infection for humans and other animals (Dubey et al., 2004b). The discovery of *T. gondii* oocysts helped to explain the method of transmission to herbivores (Dubey & Beattie, 1988).

Subsequently the role of paratenic hosts and vertical transmission of the parasite were also recognised to play a role in the transmission and life cycle of *T. gondii* (Tenter et al., 2000).

## 3.2 Aetiology

*Toxoplasma gondii* is an important pathogen that causes abortion and significant economic losses in small ruminants (sheep and goats) (Asgari et al., 2013; Buxton et al., 2007a; Danehchin et al., 2017; de Wit et al., 2020). This protozoa belongs to the Phylum Apicomplexan, Class Coccidian, Subclass Coccidiasina, Order Eimeriida, Suborder Eimeriorina, Family Sarcocystidae, (Sarcocystis), Subfamily Toxoplasmatinae and Genus *Toxoplasma* (Mcauley et al., 2015).

Infections of domestic animals with *T. gondii* in the United States of America and Europe are typically characterized as Types II and III strains (Dubey et al., 2004a). In Iraq, Type II strains have been recovered most commonly from sheep that aborted (60% of isolates), with types III and I representing 30% and 10% of the remaining isolates, respectively (A'aiz, 2016). In contrast, another recent study in south Iran reported that Type I was the predominant type in sheep from that region (Armand et al., 2017).

Investigations in other parts of the world show similar diverse results; for example of 46 *T. gondii* isolated from sheep in France, 45 (98%) were Type II with only one isolate belonging to Type III (Halos et al., 2010). In addition, in two studies in the UK and Switzerland, all isolated *T. gondii* were classified as Type II based on PCR-RFLP (Berger-Schoch et al., 2011).

## 3.3 *Toxoplasma* genotypes in sheep and goats

Three types/lineages of *T. gondii* have been described (Types I, II and III). Type I is reportedly highly virulent in mice (Sibley et al., 2009), Type II is the most common type occurring in persistently infected animals (Alanazi, 2013; Dubey et al., 2008), and Type III is the second most prevalent type

in animals (Dubey et al., 2008). Clinical human infections are more often associated with Type II strains than with other types (Halos et al., 2010).

Subsequently genotypes were recognised with the major ones being BrI, BrII, BrIII and BrIV, genotype 12, Africa 1 and Chinese 1 (Pena et al., 2008). Among these, genotype BrI was identified as highly virulent, genotype BrIII as nonvirulent, and genotypes BrII and BrIV of intermediate virulence (Chen et al., 2011; Mercier et al., 2011; Pena et al., 2008). A total of 231 genotypes of *T. gondii* have been identified around the world, comprising 1,457 strains (Fu et al., 2015).

## 3.4 Structure and Morphology of *Toxoplasma gondii*

### 3.4.1 Oocysts

Sporulated oocysts of *T. gondii* are sub-spherical to ellipsoidal in shape and measure between 10–13 μm in diameter (Dubey, 2016). The oocyst stage is highly resistant (Dumètre et al., 2013) and is capable of surviving ultraviolet (UV) light treatment (Dumetre et al., 2008; Wainwright et al., 2007), storage at 4°C for up to 54 months and in soil for 18 months at various temperatures (Frenkel et al., 1975; Innes, 2010). Although room temperature promotes sporulation, temperatures below 4°C may slow the sporulation process, but are not always sufficient to prevent sporulation (Dumètre et al., 2013).

### 3.4.2 Tachyzoites

More recently, the tachyzoite of *T. gondii* has been described *in vitro* to be surrounded by a parasitophorous membrane (PVM), which consists of both host- and parasite-derived proteins (Krishnamurthy & Saeij, 2018). Tachyzoites are crescent-shaped and approximately 6 μm in length (Dubey et al., 1998). They enter the host cell by actively penetrating the cell membrane. Tachyzoites multiple intracellularly by using a unique form of cell division called endodyogeny, and assemble two daughter cells within a mother cell until their growth can no longer be sustained by the host cell (Berry et al., 2018). The infected cell then ruptures releasing the tachyzoites. The released parasitic

stages may then continue to infect neighbouring uninfected cells or can spontaneously convert into the slowly replicating bradyzoite stage within infected cells and also can be passed from maternal blood to the fetal tissue resulting in congenital *T. gondii* infections (Appleford & Smith, 1997; Robert-Gangneux & Dardé, 2012). Unlike the oocyst stage, tachyzoites are killed by pasteurisation and heating, and they rapidly die outside of the host (Saridewi et al., 2013; Tenter et al., 2000).

### 3.4.3 Bradyzoites

Structurally, bradyzoites differ slightly from tachyzoites. The nucleus is located toward the posterior end, whereas in tachyzoites it is more central (Dubey et al., 1998). Most bradyzoites have one to three rhoptries, which are looped back on themselves. Bradyzoites contain several amylopectin granules, which are seen during latent infection. In contrast, these granules are usually seen in acute infection in tachyzoites (Sugi et al., 2017). Tissue cysts grow and remain intracellularly and the bradyzoites also divide by endodyogeny, as well as by endopolygeny (Berry et al., 2018; Sullivan Jr & Jeffers, 2012).

The cyst wall is thought to be important for the cyst to remain in the host cell for long periods, and it is 200 to 850 nm thick (Zhang et al., 2001). Cysts are described to be of spherical shape (Jack et al., 2011) but of variable size (Silva et al., 2013).

The conversion from fast-dividing tachyzoites to slow-dividing bradyzoites seems to be a spontaneous process occurring when the replication rate of the tachyzoites slows down (Mahamed et al., 2012). Bradyzoites, which form tissue cysts, can be found in mice 10 to 15 days after challenge (Mahamed et al., 2012). Bradyzoites can also form when a host ingests oocysts, and to a small extent when tachyzoites are ingested or meat contaminated with tissue cysts is eaten (Tenter et al., 2000). It is believed that tissue cysts can periodically rupture, thus releasing parasites which re-invade host cells and establish new tissue cysts (Reiter-Owona et al., 2000). The biological mechanisms of the variable persistence of bradyzoites that within tissue cysts are not fully understood (Watts et al.,

2015). The glycosylated cyst wall is thought to play a crucial role in survival of bradyzoites during chronic infection, as well as in the oral transmission of infection (Tomita et al., 2013).

### 3.5 Sexual stages

Sexual stages can only develop in cats (wild or domestic *Felidae*), the definitive host of *T. gondii*. Felids acquire *T. gondii* by ingesting tissues of infected intermediate hosts, such as rodents (Ajzenberg et al., 2004; Su et al., 2003). In both cases, enzymes and the acidic environment of the stomach and enzymes of the intestine result in the release of bradyzoites or sporozoites. In turn, the cell walls surrounding bradyzoites, oocysts or sporocysts will be digested. Sexual stages undergo several rounds of asexual propagation in epithelial enterocytes followed by the formation of sexual stages through the process of gametocytosis. Large numbers of macrogametocytes (that produced macrogametes) (females) are formed, along with a few microgametocytes (males) containing 20–30 microgametes. Mature female macrogametes contain all the nutrients required for oocyst formation (Ferguson et al., 2005). The microgametes usually penetrate a single macrogamete thus fertilising it. This process leads to the formation of a diploid zygote that later develops into the oocyst stage. According to Ferguson (2002), only macrogametogony is associated with the synthesis of the wall-forming bodies that are required to form the oocyst wall. Following gametocytosis, oocysts are passed with feline faeces and undergo meiosis in the environment, generating four haploid sporozoites (Robert-Gangneux & Dardé, 2012).

The prepatent period for oocyst shedding in cats is 3–10 days post-infection with bradyzoites, and 13 days after ingesting tachyzoites (Dubey, 1998a, 2006). Millions of oocysts can be shed over a period of 14 days by a single cat (Dabritz & Conrad, 2010; Dubey, 1995). After sporulation in the environment (in water, on vegetables, feed stuffs, cat litter and soil) oocysts may be accidentally ingested by a wide range of intermediate hosts, such as wild and domesticated animals, birds or humans (Bahia-Oliveira et al., 2003; Bowie et al., 1997; Dubey & Jones, 2008). In addition invertebrates may also ingest oocysts and be a potential source of infection for mammals and birds

(Bettiol et al., 2000). In the intermediate host, tachyzoites are formed first, followed by the formation of tissue cysts. Tissue cysts are mainly found in the central nervous system (CNS), striated and nonstriated muscular tissue, and the eye (Dubey & Lindsay, 1998; Dubey & Beattie, 1988; Skariah et al., 2010). However, tissue cysts are considered the terminal life-stage in the intermediate host. They may result in a life-long latent infection in tissues, such as in skeletal and cardiac muscles, and the CNS (Mendez & Koshy, 2017). Importantly, tissue cysts are also infectious for other host species if the carrier is predated (Tenter, 2009).

What distinguishes *T. gondii* from other cyst-forming coccidia is the fact that horizontal transmission is not only restricted to oocysts (i.e. from definitive to intermediate hosts), but also via horizontal transmission by tissue cysts (i.e. between intermediate hosts) (Jones & Dubey, 2010). In addition, vertical transmission of tachyzoites from a mother to offspring can also occur. This has been demonstrated to be true for both definitive host species (felids) as well as several intermediate host species including rats, mice (Dubey et al., 2012b), sheep (Innes et al., 2009a) and humans (Dubey et al., 2012b).

### 3.6 Life cycle of *Toxoplasma gondii*

There are three life-stages of *T. gondii*: oocysts; tachyzoites; and bradyzoites. In the context of parasite transmission, *T. gondii* may be transmitted from definitive to intermediate hosts, from intermediate to definitive hosts, as well as between definitive hosts and between intermediate hosts (Figure 3.1). In places where domestic felids are absent, wild felids such as jaguars (*Panthera onca*) and bobcats (*Lynx rufus*) can serve as definitive hosts (Demar et al., 2008; García-Bocanegra et al., 2010; Millan et al., 2009; Mucker et al., 2006). Furthermore, even in the absence of a definitive host, *T. gondii* may persist in the environment by cycling only between intermediate host species. Due to this efficient life cycle, that includes not only domesticated but also wild animals, it has been suggested that the *T. gondii* life cycle consists of two elements: the domestic and the sylvatic (wild) cycle (Robert-Gangneux & Dardé, 2012; Sullivan Jr & Jeffers, 2012; Ullmann et al., 2010). Infection

in humans can arise from the ingestion of oocysts, consumption of undercooked meat containing tachyzoites or bradyzoites, via vertical transmission or through a contaminated blood transfusion or transplant with an infected organ (Foroutan-Rad et al., 2016).

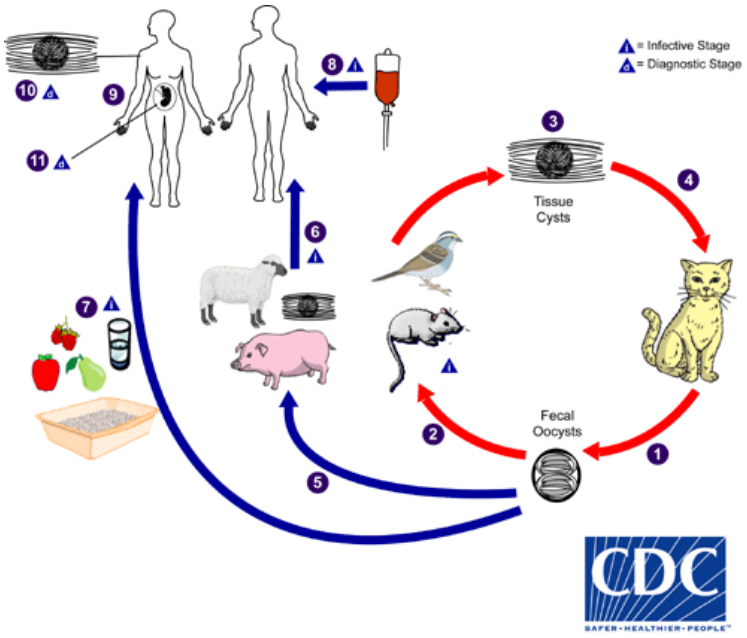


**Figure 3.1 *Toxoplasma* gondii life cycle (https://www.cdc.gov/parasites/toxoplasmosis/biology.html - accessed 12 November 2019).**

**1.** Unsporulated oocysts are shed in the cat's faeces. **2.** Oocysts take 1-5 days to sporulate in the environment and become infective. Intermediate hosts in nature (including birds and rodents) become infected after ingesting soil, water or plant material contaminated with oocysts. **3.** tachyzoites localize in neural and muscle tissue and develop into tissue cyst bradyzoites. **4.** Cats become infected after consuming intermediate hosts harbouring tissue cysts. **5.** Animals bred for human consumption and wild game may also become infected with tissue cysts after ingestion of sporulated oocysts in the environment. Humans can become infected by: **6.** Eating undercooked meat of animals harbouring tissue cysts; **7.** Consuming food or water contaminated with cat faeces or by contaminated environmental samples (such as faecal-contaminated soil or changing the litter box of a pet cat); **8.** Blood transfusion or organ transplantation; **9.** Transplacentally from mother to fetus. **10.** Diagnosis is usually achieved by serology, although tissue cysts may be observed in stained biopsy specimens. **11.** Diagnosis of congenital infections can be achieved by detecting *T. gondii* DNA in amniotic fluid using molecular methods such as PCR.

Source: Centers for Disease Control and Prevention. https://www.cdc.gov/parasites/toxoplasmosis/biology.html

## 3.7 Prevalence of *Toxoplasma*

### 3.7.1 Prevalence of *Toxoplasma* in small ruminants

Small ruminants are known to be reliable indicators of the prevailing rates of *T. gondii* infection as they are fully susceptible to infection, and, once infected, antibodies remain for a long time (Blewett & Watson, 1983). As a result, the seroprevalence in sheep increases with age (Dubey & Welcome, 1988). The prevalence of *T. gondii* in sheep and goats, not surprisingly, varies between countries. The age of the tested animals, season of sampling, and the diagnostic tests used are considered the principle reasons for this variation of prevalence between locations. The results of some studies that have reported the prevalence and/or outbreaks of *T. gondii* in Asia, Africa, and Mediterranean countries in sheep and goats are summarised in Table 3.1. Other published manuscripts have reviewed the prevalence of toxoplasmosis in small ruminants from North and South America and Europe (Belluco et al., 2016; Dubey, 2009b; Stelzer et al., 2019).

In 2011, a study conducted by Al-Barwary and Mikail (2014) in ewes that aborted revealed a seroprevalence of 97.4% to *Toxoplasma* in Dohuk Province in the north-west of Iraq.

### 3.7.2 Prevalence of toxoplasmosis in other animals

The prevalence of *Toxoplasma* in herbivores reportedly is relatively stable in individual locations because of the long survival of oocysts on pastures and the constant exposure of the grazing animals to the oocysts (Robert-Gangneux & Dardé, 2012).

**Table 3.1**. **The prevalence of *Toxoplasma gondii* in sheep and goats in Iraq and neighbouring countries to Iraq**

| Country/Region | Location | Host | Year of study | Prevalence (%) | Diagnostic test used | Reference source |
|---|---|---|---|---|---|---|
| **Mediterranean** | | | | | | |
| Iraq | Dohuk Province | Sheep | 2014 | 23.93 | ELISA and PCR | Mikaeel et al. (2015) |
| | Sulaimania Province | Sheep<br>Goats | 2006 | 57<br>54.6 | ELISA and LAT | Abdulla and Al-Taie (2011) |
| | Baghdad Province | Sheep | 2012- 2013 | 23.9 | ELISA | Al-Ethawi and Yakoob (2013) |
| Saudi Arabia | Riyadh City | Sheep<br>Goats | - | 36.4<br>35.3 | IFAT | Alanazi (2013) |
| Iran | South-eastern Iran | Sheep<br>Goat | 2005 | 24.7<br>15.8 | Modified agglutination test (MAT) | Bahrieni et al. (2008) |

| Country/Region | Location | Host | Year of study | Prevalence (%) | Diagnostic test used | Reference source |
|---|---|---|---|---|---|---|
| | Isfahan | sheep | 2013 | 8.47 | PCR | Khamesipour et al. (2014b) |
| | Mazandaran Province, North of Iran | Sheep<br>Goats | 2005 | 35<br>30 | IFAT | Sharif et al. (2007) |
| | Gilan Province, North of Iran | Sheep<br>Goats | 2012 | 36.8<br>12.9 | PCR | Azizi et al. (2014) |
| | South-eastern Iran | Sheep<br>Goat | 2005 | 24.7<br>15.8 | Modified agglutination test (MAT) | Bahrieni et al. (2008) |

## 3.8 Clinical Signs, Pathology and Pathogenesis

### 3.8.1 Clinical signs and pathology

Clinical ovine toxoplasmosis occurs following the ingestion of sporulated oocysts in previously unexposed pregnant sheep (Castaño et al., 2016). However recent research has suggested that vertical transmission from persistently infected ewes to the fetus may occur more frequently than previously thought (Buxton et al., 2006).

The clinical signs in sheep include early embryonic death and resorption, abortions, mummification, stillbirths, neonatal death or the birth of live but weak lambs (Buxton & Rodger, 2008; Dubey, 2009b). More than 50 percent of sheep may suffer fever, dyspnoea, tremor and abortion if infected in the mid stage of pregnancy (Innes et al., 2009a). *Toxoplasma gondii* is considered to be one of the most important agents causing ovine abortion and neonatal loss worldwide (Buxton et al., 2007b; Dubey, 2009b).

Pereira-Bueno et al. (2004) reported that toxoplasmosis was the cause of 23.1% of abortions in sheep in Spain. In contrast, another study conducted in the UK reported that only 2% of abortions in small ruminants were due to toxoplasmosis (Buxton et al., 2007b). Death of a newborn lamb may occur four to five days after birth with the presence of concurrent neurological signs (Movassaghi et al., 2016). Malformations in the fetus and physical deformities have also been reported, including the occurrence of lambs with two heads (Ismael et al., 2016; Morley et al., 2008).

Classically, abortion in cases of ovine toxoplasmosis usually occurs one month after infection (Castaño et al., 2014). In the acute phase of abortions, infarcts and thrombosis occur in the caruncular villi of the placentomes along with ischaemic lesions (periventricular leukomalacia) in the brain of fetuses resulting in hypoxic damage to the fetus and vascular lesions in the placenta (Castaño et al.,

2014). Fetal deaths and resorption can be present if the sheep are infected during the early pregnancy period (< 50 days) (Dubey, 2009b; Hartley, 1964).

Pathological lesions present in infected animals can include small, white foci and necrosis of the cotyledons and focal necrotic lesions in the fetal brain, liver, and lungs (Senegas et al., 2009). Placental cotyledons typically have characteristic “white spots” due to the dystrophic mineralization of necrotic foci caused by the multiplication of tachyzoites (Senegas et al., 2009). In aborted foeti, microscopic lesions are most commonly seen in the brain, and less commonly in the skeletal muscle and myocardium. Histological lesions observed in the brain typically consists of necrotic foci surrounded by a narrow line of glial cells (Adesse et al., 2018; Gual et al., 2018; Weissmann, 2003).

No significant difference in milk production and its composition were observed between 18 seropositive and 22 seronegative dairy ewes in Brazil (Klauck et al., 2016).

In dogs clinical signs of toxoplasmosis result from the localization of the tachyzoites in the neuromuscular tissue resulting in neurological signs including ataxia, circling, behavioural changes, twitching, tremors, and seizures (da Silva et al., 2005). *Toxoplasma gondii* can also result in abortions in dogs, even early during the infection (Bresciani et al., 2009).

### 3.8.2 Pathogenesis

When an intermediate host, such as a sheep or goat, becomes infected by grazing pasture contaminated with sporulated oocysts, the parasite will be released and actively infect and multiply within the intestinal cells. Subsequently the parasites are released when the cells rupture and the tachyzoites then invade more host cells, including neutrophils, macrophages, lymphocytes, and intestinal epithelial cells, within the lamina propria (Dubey et al., 2012a; Dubey et al., 1997; Lingelbach & Joiner, 1998; Speer et al., 1998).Tachyzoites may also spread to the local mesenteric lymph nodes (Dubey et al., 2004b; Gregg et al., 2013; Jokelainen et al., 2012) and then can spread to other host tissues and cross the biological barriers of the placenta, the blood-brain barrier and the blood-retina barrier (Barragan et al., 2005; Dubey et al., 1997; Speer et al., 1998).

In pregnant animals, the tachyzoites infect and multiply within the caruncular septa stage, with the stage of gestation at the time of infection influencing the outcome of the pregnancy and infection (dos Santos et al., 2016). When an infection in the placentome is initiated, multiplication of the parasite causes multiple foci of necrosis (dos Santos et al., 2016). These foci of tissue damage enlarge during gestation until abortion or birth occurs when they may be macroscopically visible as white spots on the cotyledons of the placenta, a feature used to aid in the diagnosis of the disease (Dubey & Jones, 2008; Dubey, 2008; Senegas et al., 2009). Most researchers believe that after a sheep aborts from infection with *T. gondii*, the animal develops protection against further *Toxoplasma* induced abortion, however others believe this protection is not absolute (Edwards & Dubey, 2013).

Tissue cysts may survive in the visceral organs of the intermediate host, including the liver, lungs, and kidneys. They have been reported to be more prevalent in neural tissues, including the brain/cerebellum, spinal cord and retina, as well as skeletal and cardiac muscles (dos Santos et al., 2016). When cats ingest meat infected with tachyzoites, bradyzoites or sporozoites, the wall of the cyst is destroyed by the proteolytic enzymes in the stomach of the cat, and in the small intestine bradyzoites are released (Dubey et al., 2004b). Some of these bradyzoites cross the lamina propria of the intestine and multiply. Within a few hours, *T. gondii* may disseminate to extra-intestinal tissues. Other bradyzoites that penetrate small intestinal epithelial cells begin to multiply asexually (Dubey, 1998a; Ferguson et al., 2002; Jones et al., 2017). Oocysts of *T. gondii* are only formed in domestic and wild felids. Cats infected for the first time can shed millions of oocysts per gram of faeces (Schares et al., 2008; Zulpo et al., 2017). As less than 200 sporulated oocysts are required to induce congenital disease in previously unexposed sheep (Abu-Dalbou et al., 2011), the large numbers of oocysts shed in cat faeces can result in many sheep, and other intermediate hosts, becoming infected. Cats usually shed oocysts only once in their lifetime and for a limited period of time (a few weeks) (Elmore et al., 2010). However, Zulpo et al. (2018) reported more cats were found to re-shed oocysts after experimental challenge with heterologous strains than with homologous strains. Oocysts can survive for 12–18 months in the environment, depending on the presenting climatic conditions, and

are a primary source of infection for grazing animals (Innes, 2010; Innes et al., 2009b; Tenter et al., 2000). More oocysts have been reported to be shed by younger than older cats (Buxton & Rodger, 2008; Jackson & Hutchison, 1989).

### 3.9 Vertical Transmission

It has long been recognised that the principle method of transmission in sheep was through ingestion of oocysts arising from the faeces of the felid definitive host after sexual recombination (Dubey, 2009b). In carnivores and omnivores, transfer of asexual bradyzoite stages can also occur through ingestion of prey animals and it has been suggested that this transmission pathway has led to the expansion and clonal population structure of the parasite (Su et al., 2003). Tachyzoites of *T. gondii* may also be transferred vertically to the fetus via the placenta or the neonate through milk (Klauck et al., 2016; Tenter, 2009). Vertical transmission in small ruminants has mainly been investigated in sheep (Chessa et al., 2014; Klauck et al., 2016; Moreno et al., 2012). In one study examining vertical transmission in sheep, *T. gondii* were detected in one ram orally dosed with oocysts and another ram injected subcutaneously with tachyzoites. When these were mated with seronegative ewes, 5 of 10 mated ewes seroconverted and *T. gondii* were isolated from the ewes and their offspring. This study highlighted the potential for both sexual transmission of the protozoa along with vertical transmission to lambs *in utero* (Lopes et al., 2013).

Beverley (1959) was the first to propose that serial vertical transmission of *T. gondii* in mice might sustain the parasite and this was subsequently confirmed by others (Johnson, 1997). Congenital transmission of *T. gondii* over repeated generations has also been demonstrated in experimentally infected mice (Grigg & Sundar, 2009).

Two different scenarios have been suggested for congenital transmission: primary exposure of the female during pregnancy followed by transfer to the fetus during pregnancy; or reactivation of chronic infection in the female during pregnancy, most likely as a result of the hormonal changes

associated with pregnancy, and subsequent transfer to the fetus (Elbez-Rubinstein et al., 2009). It is now believed that vertical transmission of *Toxoplasma* plays a more important role than was previously believed. This was highlighted by the finding that congenital (vertical) transmission may occur in up to 66% of pregnancies in ewes (Hide et al., 2009).

Once infection occurs, there is generally a delay of 4 weeks until the occurrence of the abortion (Dubey, 2016), and others have observed that abortion can occur up to 40 days after infection if it occurs during mid-pregnancy (Buxton et al., 1993). In an experimental study, where sheep were inoculated orally with sporulated oocysts of *T. gondii* at days 40 (G1), 90 (G2) and 120 (G3) of gestation, abortion occurred in all groups. However, in group G2 abortions were more frequent during the acute phase of the disease, whilst in G3 they occurred mainly after 20 days post infection (pi). Parasites and lesions in the placenta and fetus were detected from 19 days pi in G3, whereas in G2 or G1 they were detected only from 26 days pi (Castaño et al., 2016). These results highlight that the period of gestation at infection influences the parasites multiplication and subsequent development of lesions in the placenta and fetus, and hence the clinical course of the disease. The authors hypothesised that the parasite reached the placenta and fetus earlier in the group challenged at 120 days compared with the other two groups (G1 and G2) because of changes in maternal immune responses late in pregnancy (Tan et al., 2011).

### 3.10 Economic impact of toxoplasmosis

Toxoplasmosis in sheep is distributed worldwide resulting in increased production costs, reduced marketability of meat, fewer available animals for selection of replacements resulting in slower genetic improvement, and is also a primary source of human infection (Cenci-Goga et al., 2013).Toxoplasmosis in sheep can have a significant economic impact (Cenci-Goga et al., 2013) through reproductive failure as a result of abortions, early embryonic death and resorption, fetal death and mummification (Dubey, 2009a). However, published information on the costs associated with

infections in livestock production is scarce and difficult to determine, particularly because of fetal loss at early stages of pregnancy, which often may go undetected (Castaño et al., 2016).

The most recent peer reviewed reports from Great Britain and Uruguay suggest annual losses of between 5 and 15 million US $ per country (Stelzer et al., 2019). Bennett (2003) found the main effect of the toxoplasmosis is abortion or embryonic death in ewes (which represents a relatively high cost of output loss per affected ewe). With 1.2 to 2.2 percent of ewes assumed affected each year out of a British population of over 16 million ewes.

In Uruguay, Freyre et al. (1999) estimated that between 1.4% and 3.9% of ewes aborted due to toxoplasmosis in 1993. They assumed a loss of US$10 to US$12 per fetus, resulting in a country-wide loss of US$1.4–4.7 million per year. The annual economic losses due to lamb mortality and lost lactations in 4 million milking sheep in Sardinia, Italy was estimated at 10 million € (~US$11 million) during the period 1999-2002 in Sardinia (Masala et al., 2003).

Blewett and Trees (1987), concluded that in the UK the annual incidence of toxoplasmosis was 1% and 2% and it was responsible for losses averaging between UK£0.70 and UK£1.40 (~ US$1.72) per breeding ewe per annum. If similar losses were present in all regions of the EU other than in 2003 over 1.25 million lambs would have been lost.

In South Australia it has been estimated that the disease can result in an annual loss of up to AU$70 million (US$47.3 million) in the state's flock of 10.7 million sheep, or AU$6.54 per breeding ewe (Courtney, 2017).

### 3.11 Risk Factors for infection with *Toxoplasma*

Ingestion of raw or insufficiently cooked meat from small ruminants is regarded as an important source of *T. gondii* for humans, especially in countries and regions where mutton and goat meat is regularly eaten (Cook et al., 2000; Kijlstra & Jongert, 2008; Robert-Gangneux & Dardé, 2012). Elevated seroprevalence to *T. gondii* (up to 65%) have frequently been reported in small ruminants

from the Mediterranean region (Ntafis et al., 2007; Oncel et al., 2005; Panadero et al., 2010; Rinaldi & Scala, 2008; Shaapan et al., 2008). This not only poses a risk to humans but results in lowered productivity in the affected livestock (Lundén et al., 1994).

The consumption of unpasteurized goat cheese and goat' milk has also been identified as a risk factor for infection of humans (Dubey et al., 2014) as tachyzoites of *T. gondii* have been detected in the milk of both sheep and goats (Dubey, 2009a, 2009b; Tenter, 2009). Also, risk for human infection with *T. gondii* due to consumption of chicken and turkey meat (Geuthner et al., 2019).

Due to the regular consumption of products originating from small ruminants, particularly in the Mediterranean region, there is a high risk of human infection. As a consequence, there is a need to identify the risk factors for this zoonosis in small ruminants, as well as in humans (Saad et al., 2018; Tenter et al., 2000).

The presence of cats and their faeces can result in contamination of grazing pasture, feedstuffs and surface water with the resistant *Toxoplasma* oocysts (Dubey et al., 2020; Tenter et al., 2000). Furthermore, their access to buildings housing small ruminants can also increase exposure of the livestock to the parasite. Consequently it has been recommended to restrict the number of cats on a farm and to prevent them having access to feed, water and bedding used by the livestock to reduce the likelihood of environmental contamination with oocysts (Deng et al., 2016; Tilahun et al., 2018).

Rearing sheep under an extensive management system increases the risk of these animals becoming infected by *T. gondii*. This practice allows animals to come in contact with oocysts that are released in the vegetation by domestic and wild cats(Dubey et al., 2020). Compared with extensive and semi-intensive systems, sheep raised intensively may have a lower likelihood of ingesting oocysts in contaminated food or water (Ahmad et al., 2015; Anderlini et al., 2011). Under conditions involving grazing of poor quality vegetation, which is common in many developing countries, in conjunction with supplementary feeding of feedstuffs that have often been stored in areas where

rodents and hence cats often have access to, small ruminants can be readily exposed to large numbers of infective oocysts (Anastasia et al., 2013).

Skjerve et al. (1998), reported that in Norway small ruminants that grazed close to farms (presumably resulting in a greater risk of exposure to feed contaminated with cat faeces) were more likely to be seropositive for *Toxoplasma*.

Because infective stages of *T. gondii* can be present in placenta and aborted fetuses (Unzaga et al., 2014), it is important to ensure correct disposal of these materials by burning or deep burial to minimise the likelihood of ingestion by domestic and wild cats (Rêgo et al., 2016).

In some developing nations sheep owned by different farmers are co-grazed and managed under community based breeding programmes (CBBP) to improve access to animal facilities and veterinary services (Haile et al., 2013). However, in such situations a higher seroprevalence to *T. gondii* has been recorded, potentially due to the sharing of infected rams, which can shed tachyzoites in their semen (Anderlini et al., 2011; Moraes et al., 2010a; Moraes et al., 2010b; Mueller et al., 2015).

## 3.12 Diagnosis

### 3.12.1 Histopathology

Although multifocal necrosis can be present in the placenta of cases that abort (Moreno et al., 2012), there are usually no macroscopic lesions present in the cotyledons or membranes. There can be variable degrees of autolysis in the placenta of dead fetuses (Castaño et al., 2016) and parasites can be detected in the placenta and the fetal heart, brain, lung or liver (Dubey, 2008; Moreno et al., 2012).

Evidence of necrosis can be observed in the white matter of the brain and the cerebellum and cerebrum of the fetus. Focal lymphoid-cell proliferations and micro-necrotic foci may also be present in the fetal kidneys, liver, adrenal glands, lymph nodes or brain (Dubey & Jones, 2008; Dubey, 2008).

Chalky nodules have been reported in the fetal cotyledons, as well as necrosis and calcification of mesenchymal cells in the villi (Unzaga et al., 2014). In Saudi Arabia diffuse oedema associated with mononuclear and segmented leukocytes has been reported throughout the amniotic membrane, along with necrosis of chorionic membrane in naturally infected sheep (Ismael et al., 2016).

Immunohistochemistry is considered an important technique in the detection of *T. gondii*, as it allows the protozoa to be identified in aborted materials. It is reportedly a very sensitive method that is capable of detecting antigen to *Toxoplasma*, even in decomposed tissues (Buxton, 1998; Dubey & Jones, 2008). Although clinical toxoplasmosis can be diagnosed through presenting clinical signs along with examination of smears and detection of gross and microscopic pathology in sheep and goats (Abu-Dalbou et al., 2010), infection is usually confirmed with serological tests (CDC, 2019).

### 3.12.2 Serological tests

The most common serological tests used for the diagnosis of toxoplasmosis in small ruminants are the Toxo latex slide agglutination test (Latex), ELISA and PCR (Glor et al., 2013; Tegegne et al., 2016). The Sabin-Feldman dye test, which was previously the gold standard diagnostic assay for toxoplasmosis, is a simple serological method for confirming toxoplasmosis, it requires the presence of live parasites and accessory factors from blood donors, cannot differentiate between historical and recent exposure to the parasite and now is performed by very few laboratories (Ashburn et al., 2001; Robert-Gangneux & Dardé, 2012). The complement fixation test (CFT) is now rarely used for the diagnosis of toxoplasmosis due to its lower sensitivity and difficulty in performing when compared with other serological assays (Ondriska et al., 2003).

The indirect hemagglutination test (IHA) is a simple test to perform, but is not capable of detecting antibodies during the early phase of an infection (Webster, 2010). The direct agglutination test is another simple and cost-effective test but it reportedly has a lower sensitivity, although higher specificity, than the latex agglutination test (Johnson et al., 1989). Another test, the immunofluorescence test (IFT), has also been used in humans but was shown to result in low

specificity due to cross reactions with rheumatoid factors and the potential also for false negative results through competition between antibody types (Filice et al., 1983).

#### 3.12.2.1 *Latex agglutination test*

The latex agglutination test (LAT) is often used for screening purposes as it is a simple technique with a high sensitivity (Holliman et al., 1990; Kuraa & Malek, 2016). This test is a modified agglutination test as antigen is coated onto polystyrene latex particles and consequently is both a quantitative and qualitative test (Jiang et al., 2008). However a disadvantage of the LAT is that most commercial kits use antigen sourced from tachyzoites grown in tissue culture. These antigens can contain a variable amount of host material, which potentially affects the test's specificity, as well as the reproducibility of the test results (Jiang et al., 2008). The LAT has a relatively low sensitivity (78.6%) and specificity (61.9%) in small ruminants (Oncel et al., 2005). However its simplicity means it is frequently used as a screening tool in epidemiological surveys, however positive samples need further testing with other serological tests (Abdul-hussein & Al-Marsomy, 2020; Holliman et al., 1990).

#### 3.12.2.2 *Enzyme linked immunosorbent assays*

Various ELISAs using crude, fractionated, or recombinant antigens have been used to detect *T. gondii* antibodies in ovine sera (Caballero-Ortega et al., 2008; Ferra et al., 2020) and in particular antibody ELISAs have been adapted for use in most domestic animals, including sheep and goats (Dubey, 2009b; Dubey, 2008; Van der Puije et al., 2000). There are specific ELISA assays to detect both IgM and IgG immunoglobulin subtypes. These assays are ideally suited to screen large numbers of samples and to evaluate the IgM/IgG ratio (Ali et al., 2021). One commercial ELISA kit (PrioCHECKW *Toxoplasma* Ab SR, Prionics Schlieren-Zurich, Switzerland) assessed was reported to have a relative sensitivity between 93.3 and 100%, and a relative specificity between 96.9 and 100% (Glor et al., 2013).

ELISAs are almost predominantly used to detect anti- *T. gondii* IgG, IgM, and IgA antibodies rather than antigens (Filice et al., 1983; Tré-Hardy et al., 2021). Conventional indirect ELISAs, using tachyzoite lysate antigen (TLA) as the coating antigen, have been demonstrated to be sensitive for detecting IgG and IgM antibodies in both humans and other animals (Abdelbaset et al., 2017; Filice et al., 1983; Obwaller et al., 1995; Tomasi et al., 1986).

ELISAs developed for *Toxoplasma* primarily detect chronic (past) infection in animals, and do not identify the presence or prevalence of viable parasites. Hence, PCR-based techniques have been developed to detect *T. gondii* DNA in meat samples (Robert-Gangneux & Dardé, 2012).

#### 3.12.2.3 *Polymerase chain reaction (PCR)*

The gold standard for detecting *T. gondii* in meat samples is a bioassay involving the use of either mice or cats. These bioassays are laborious and time-consuming techniques, which are not desirable for screening large numbers of samples from an animal ethics point of view. Therefore, PCR-based methods for detecting *T. gondii* in meat samples have been developed. PCR is usually sensitive in detecting *T. gondii* DNA, but do lack sensitivity when compared to bioassays (da Silva & Langoni, 2001; Garcia et al., 2006; Hill et al., 2006). It was found that PCR was as sensitive as the bioassays, but lack sensitivity when used on meat samples, mainly due to the inhomogeneous distribution of *T. gondii* tissue cysts, in combination with the small size of the samples (normally 50 mg with commercially available DNA extraction kits used in PCR) (Opsteegh et al., 2010).

The PCR on the other hand offers an advantage over serology in its ability to diagnose infection at earlier stages of gestation when the fetus is not yet immunocompetent, and in lambs that have ingested colostrum *(Hurtado et al., 2001)*. It was concluded by Hurtado et al. *(Hurtado et al., 2001) that the* detection by a PCR of the internal transcribed spacer, ITS1, region in fetal tissues was a valuable and quick technique for the diagnosis of ovine abortion caused by *T. gondii.* The PCR has reportedly a detectable sensitivity of 230 tachyzoites per 100 g of meat sample assayed (Opsteegh et al., 2010).

Infection in sheep and goats has also been confirmed through testing blood samples with PCRs (Buxton, 1998; Tavassoli et al., 2013) and the PCR amplification of different target genes to detect *T. gondii* DNA in ovine fetal tissue and placental samples is a valuable tool for the diagnosis of congenital toxoplasmosis (Hurtado et al., 2001; Owen et al., 1998; Partoandazanpoor et al., 2019; Steuber et al., 1995).

Several PCR-based methods have been developed to detect *T. gondii* in ovine aborted fetuses by targeting the parasite's B1 sequence, the P30 and DNA ribosomal genes (Burg et al., 1989; Hurtado et al., 2001; James et al., 1996; Owen et al., 1998; Ramzan et al., 1997). Primers for the P30 gene can polymerize DNA of *Nocardia* and hence PCRs based developed with these primers are reportedly less specific than those targeting the B1 gene *(Kasper et al., 1983)*. Jones et al. (2000), demonstrated that applications using the B1 gene had both high specificity and sensitivity for detecting infection with *T. gondii* in aqueous Humor. This is due to the highly conserved nature of the B1 gene in all strains of *T. gondii* strains and the presence of multiple copies of it within the genome.

### 3.13 Prevention and control of toxoplasmosis in small ruminants

Although many studies on infections with *T. gondii* and clinical toxoplasmosis in small ruminants have been published, there are still many gaps in our knowledge about the disease and its impact and control (Stelzer et al., 2019). Irrespective of these gaps, a number of methods to control toxoplasmosis in small ruminants have been suggested (Dubey, 2009b). In particular management and husbandry practices should be adopted to prevent exposure of livestock to oocysts present in the environment. This primarily focuses on preventing sheep and goats from ingesting food, water and bedding material that are potentially contaminated with the faeces of cats, particularly young cats (Gazzonis et al., 2015). Furthermore, implementation of methods to control the numbers of unowned and feral cats, in conjunction with adoption of rodent-control measures to restrict this food source for cats, will reduce the potential for environmental contamination with oocysts (Othman & Alzuheir,

2014). Supplementing small ruminants during pregnancy with anticoccidial medications, which incorporate monensin or decoquinate, will also help reduce the impact of the protozoa (Pavlovic & Ivanović, 2005). However, such prophylactic (e.g, Oxytetracycline, Penicillin, Ivermectin, multivitamin, etc.), measures may be beyond the financial abilities of subsistence or nomadic farmers.

Measures to control the size of the cat population are a key component to reducing environmental contamination with oocysts. Such measures may include immunocontraception (Fischer et al., 2018), capture neuter and release programs and culling programs (Courchamp & Sugihara, 1999; Lazenby et al., 2015; Swarbrick & Rand, 2018). Maintenance of a small healthy population of mature neutered cats will both reduce oocyst excretion as well as help control rodents (Abu-Madi & Behnke, 2014).

Education is also a critical component of controlling most diseases, including toxoplasmosis, and involvement of sheep farmers and farmer's associations in specifically designed educational programs is required to enable a better understanding of the management and husbandry practices adopted, the knowledge of the farmers, as well as the general public, about the disease and how it is transmitted (Cenci-Goga et al., 2013). Such an education campaign for farmers should include information on ways to prevent access of cats to animal feed and water sources to reduce contamination with oocysts (Garcia et al., 2012; Gebremedhin et al., 2013; Stelzer et al., 2019). Overstocking should also be avoided as this has also been identified as a risk factor for seropositivity (Gebremedhin et al., 2013; Guimaraes et al., 2013). As fresh or frozen ram semen can be responsible for the transmission of *T. gondii* all semen and rams/billy goats used should be ideally sourced from non-infected flocks, however this is challenging in regions where the protozoa is endemic (Consalter et al., 2017).

In the case of an outbreak (abortion storm), all dead lambs and placenta should be disposed of by deep burial or burning to minimise access to by cats (Abu-Dalbouh et al., 2012).

### 3.13.1 Vaccines

A number of vaccines have been developed and trialled in sheep and cats to reduce the impact of toxoplasmosis on human and animal health. Vaccines offer multiple benefits, including reducing the impact of the disease in small ruminants and decreasing infection of humans (Innes et al., 2011). Although many vaccine candidates have been tested, none have successfully controlled the disease, primarily due to the presence of tissue cysts and the potential for vertical transmission of the parasite (Wang et al., 2019). In an attempt to overcome these problems, researchers have developed subunit, recombinant and DNA vaccines, however none have yet to provide complete protection against infection (Bout et al., 2002).

Some of the initial studies on vaccines against *Toxoplasma* were conducted in cats in an attempt to reduce or prevent oocyst shedding (Freyre et al. (1993) and Frenkel et al. (1991). These authors used a T-263 strain, which fails to produce oocysts, and orally administered sporozoites or bradyzoites. Although both stages induced a detectable humoral antibody response, only the orally administered bradyzoites induced immunity to oocyst shedding. A disadvantage of the use of this strain as a vaccine candidate is its short life and the need for it to be produced in mice.

Mishima et al. (2002) developed a recombinant viral vaccine composed of a feline herpesvirus Type 1 (FHVI) vector containing an immunogenic antigen of *T. gondii*. Vaccination of cats with this vaccine induced production of IgG against the *T. gondii* antigen and this was found to inhibit the *in vitro* invasion of tachyzoites.

In a study involving vaccination of kittens with 60 Co-irradiated tachyzoites of the Beverley strain, no oocysts were shed after challenge of these kittens with bradyzoites of the irradiated strain. In contrast, when kittens were vaccinated with a 60 Co-irradiated or fixed tachyzoites of the RH strain, oocysts were shed in animals challenged with bradyzoites of the Beverley strain. These findings indicate that the development of protective immunity in cats is strain dependent (Mishima et al., 2002; Omata et al., 1996).

The S48 strain of *T. gondii*, which has been used to reduce or eliminate tissue cysts in sheep, has also been administered to cats and has been shown to inhibit the sexual development of *T. gondii* (Innes et al., 2009b).

The S48 vaccine is highly effective because it is a live vaccine and because it will invade host cells and undergo limited multiplication and the *T. gondii* antigens are presented to the host immune system in the correct manner to enable induction of protective immune responses (Innes et al., 2011).

Toxoplasmosis is a most important disease, now considered as one of the most important food borne diseases in the world and also considered a major cause of production loss in livestock. A main health approach to improve a vaccination programme to treat toxoplasmosis is an attractive and realistic consideration. Knowledge of toxoplasmosis epidemiology, transmission routes and main risk groups has helped to target key host species and outcomes for a vaccine programme and these would be to prevent/reduce congenital disease in sheep and to prevent/reduce *T. gondii* oocyst shedding in cats (Innes et al., 2019).

The first vaccine (Toxovax$^{TM}$) developed for use in an intermediate host (sheep) was developed from a live, attenuated tachyzoite S48. In sheep vaccinated with S48 and then challenged with an oocysts orally, the parasite was prevented from spreading within the lymph system (Buxton et al., 1994). The vaccination primes the immune system limiting the spread of the parasite from the lymph to the circulation of the host. This results in less dissemination of the parasite to other tissues and importantly prevents it from reaching the placenta and causing abortion (Buxton, 1993; Innes et al., 2009a). Although this vaccine was found to induce immunity and decrease the incidence of abortion in sheep from congenital toxoplasmosis, it was expensive and the immunity induced (18 months) was reportedly not as long as that resulting from natural infection (Buxton, 1993; McColgan et al., 1988).

Strain S48 was originally isolated from an aborted lamb in New Zealand and was maintained through repeated passages in mice (Innes et al., 2011; O'Connell et al., 1988). Tachyzoites of S48, when administered by the intramuscular route, result in a short-lived infection in sheep of around 14

days. However, S48 vaccine, like other *Toxoplasma* vaccines such as DNA and T-263 strain, is not be able to eliminate tissue cysts and/or block vertical transmission in sheep (Hiszczynska-Sawicka et al., 2014; Innes & Wastling, 1995; Li & Zhou, 2018; Zhang et al., 2015). As this strain has lost the ability to differentiate into bradyzoites, it is not able to establish a persistent infection in the animal (Buxton, 1993) and has been used in vaccines to reduce tissue cyst development in sheep (Katzer et al., 2014). As it has lost the ability to form tissue cysts, it can be safely administered by intramuscular inoculation to sheep without posing a potential threat to food safety (Burrells et al., 2015). It has been recommended that, initially, the whole flock should be vaccinated at least 3 weeks prior to mating and in subsequent years all animals introduced into the breeding flock should also be vaccinated (Buxton & Innes, 1995). Another advantage of this vaccine is that only one injection is required during the life of the sheep (Buxton & Innes, 1995).

Tachyzoites of S48 are only able to undergo limited multiplication within the sheep, however they induce a cell mediated immune response involving CD4+ and CD8+ T-cells and IFNγ in vaccinated animals (Innes et al., 2019; Wastling et al., 1994; Wastling et al., 1995; Wastling et al., 1993). IFNγ plays an important role in limiting the intracellular multiplication of the parasite (Innes et al., 2019; Oura et al., 1993). The SAG1 antigen of tachyzoites and bradyzoites plays an important role in the parasite's motility, attachment, invasion and increase in immune activity of the host's response (Hiszczynska-Sawicka et al., 2014; Li & Zhou, 2018; Zhang et al., 2015). Some researchers reported that SAG1 damages the intestines after the tachyzoites were directly injected into the intestines of mice (Rachinel et al., 2004). The challenge with vaccines of S48 tachyzoites is to identify the important antigens that, when presented to the immune system, would induce life-long protective immunity (Buxton & Innes, 1995).

### 3.14 Conclusions

*Toxoplasma gondii* is found in most continents and countries of the world, however prior to the research outlined in this thesis no information was available on the seroprevalence in small ruminants

in Dohuk and no studies had been undertaken to determine risk factors for seropositivity. Consequently, a sero-epidemiological survey was conducted in the province to assess the seroprevalence in small ruminants and to identify potential risk factors for infection. Such studies help to inform the design of local and regional control strategies for this parasite. Reduction in the incidence of toxoplasmosis in livestock should result in improved profitability for farmers as well as reduce the number of cases in humans.

### 3.15 Hypotheses

The study will test the following hypotheses:

1. Brucellosis and toxoplasmosis are endemic in Dohuk Province.
2. The seroprevalence of the two pathogens varies between farms, depending on management, husbandry and demographic factors.
3. The level of knowledge of farmers about these diseases is low in Dohuk Province.
4. The diseases result in significant economic losses to farmers in Dohuk Province.

### 3.16 Research objectives

The main objective of this project is to further the knowledge of the epidemiology of *Brucella* and *Toxoplasma* in sheep and goats in Iraq's Dohuk Province.

The specific aims are the following:

1- Determine the risk factors associated with *Brucella* seropositivity in sheep and goats in Dohuk Province, Iraq

2- Determine the risk factors associated with seropositivity to *Toxoplasma* among sheep and goats in northern Iraq.

3- Determine the seroconversion to *Brucella melitensis* and *Toxoplasma gondii* during pregnancy and its association with abortion in sheep and goats in Dohuk Province, Iraq.

4- Analyse the benefit-cost comparing two different vaccination control strategies against brucellosis in sheep and goats in Dohuk Province, Iraq.

# CHAPTER FOUR: MATERIAL AND METHODS

## 4.1 General Materials and Methods

A cross-sectional study was conducted in selected herds in villages in Dohuk Province which included a serological study of brucellosis and toxoplasmosis in sheep and goats, and a questionnaire survey administered to farmers to obtain information about the putative risk factors of both diseases and their effects on reproductive performance. The sample size was determined using the software programme EpiTools (http://epitools.ausvet.com.au/content.php?page=2Means1).

### 4.1.1 Study area

Dohuk Province is located in the northwest of Iraq within the region of Kurdistan approximately 470 km from Baghdad. The climate is semi-arid; it is hot and dry in the summer and cold and wet in the winter. The mean maximum temperature in the summer is 41 °C, and the mean temperature minimum in the winter is 4 °C (Mohammed and Jamal 2015).

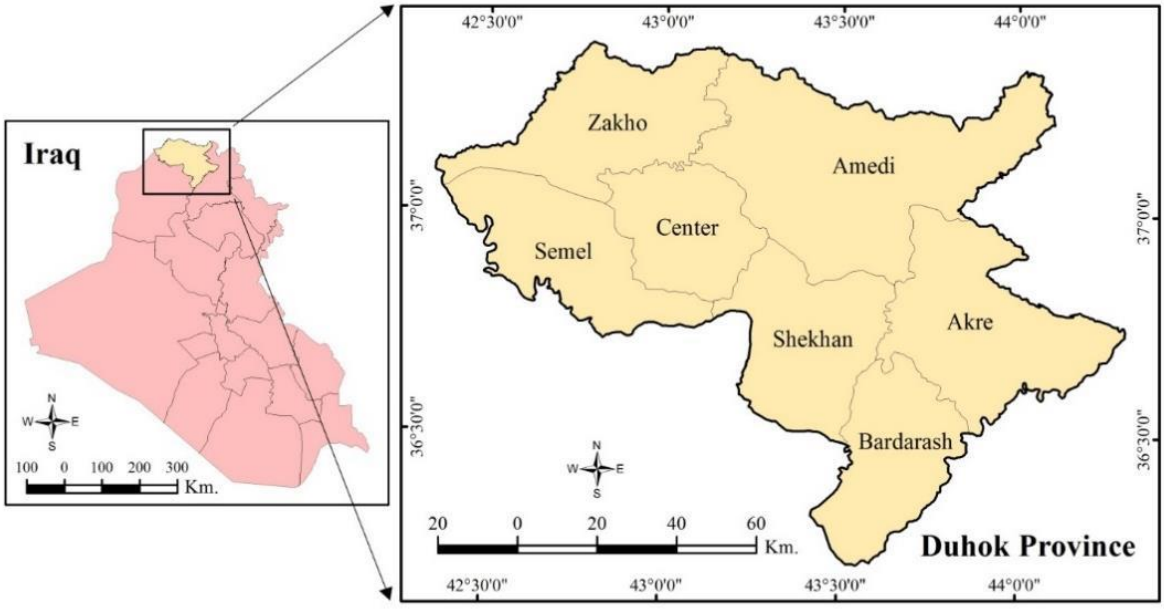


**Figure 4.1. Map of Dohuk Province (Ibrahim et al., 2021)**

A multistage sampling strategy was used. From each of the six districts in Dohuk Province, two sub-districts were randomly selected for sampling (i.e., 12 sub-districts). Then from each selected sub-district, two villages were randomly selected, resulting in 24 study villages. Three participants were randomly selected from each village for interviewing and sampling of their sheep (24 villages × 3 participants = 72 participants). Eight sheep and goats were sampled randomly from each participant (6 × 72 = 432 animals). This number was selected according to the EpiTools programme, using an expected prevalence of brucellosis based on research in Jordan, Syria and Iran (30%). (Khamesipour et al., 2014a)

Because sheep and goats are generally raised together and are similarly at risk for *Brucella* spp. and *T. gondii*, the sampling involved both species in proportion to their reported frequency (700,000 sheep and 300,000 goats). Therefore, the number of sheep was 432×70% = 302 sheep, and the numbers of goats was 432 × 30% = 130 goats which were sampled.

1. A serological study was conducted to determine the seroprevalence of *Brucella spp.* and *T. gondii*. Blood samples were collected and a questionnaire was administered to farmers whose animals were sampled to determine management and husbandry practices adopted.

Five ml to 10 ml of blood was collected aseptically from the jugular vein of each animal (without any anticoagulant) using sterile disposable syringes and needles. After collection, the blood was transferred into sterile plain tubes, which were left undisturbed for at least 30 minutes at room temperature in a slightly inclined position to facilitate clotting. The sera were then separated using a centrifuge (1,500 g for 15 min), removed by pipette and then stored at -20 °C until the testing was performed.

A commercial Rose Bengal plate test (RBPT) (Biolabo France) and an ELISA (Novatec Frankfurt Germany) was used to determine the seroprevalence to *Brucella* spp. An ELISA (Novatec Frankfurt Germany) was used to determine the seroprevalence to *Toxoplasma*.

Risk factor analyses by odds ratio and their confident intervals (95%) was calculated to measure the degree of association between the risk factor and presence of *Brucella* and *Toxoplasma.* An economic analysis was also conducted to determine the effects of the diseases.

2. A questionnaire was designed and administered to farmers whose animals were sampled. This was then developed in English and then translated into Kurdish for administration to the farmers. The interviews were completed before the blood samples were collected from the animals. In addition, a second questionnaire, also developed in English, was designed and given to veterinarians who provided veterinary care to the flocks. This questionnaire was designed to obtain information pertaining to any vaccination regimes for *Brucella*, such as when the last vaccination was conducted and in which villages the flocks were vaccinated. These surveys were used in conjunction with the results of the serological assays for brucellosis to reduce the likelihood of accepting false positives. Both surveys were conducted to collect qualitative and quantitative data about the surrounding area or villages in Dohuk city.

3. Cohort study: Based on the results of the cross-sectional study, the flocks were classified as seropositive or seronegative for the two pathogens. Animals were pregnancy tested by ultrasonographic examination (as will be explained in the following section), and 20 animals that were approximately 2 months pregnant were then randomly selected from each participating flock for inclusion in the study (until a total of 240 animals were obtained; 192 sheep, 48 goats). Each animal was identified with an ear tag. Information regarding the husbandry, management and animal characteristics was recorded.

Ten mL of blood was collected from the jugular vein into a sterile plain tube for the serological tests, and ultrasonography (Q3Vet Digital portable ultrasonic) was used to determine the pregnancy status of the sampled animals (the only pregnant ewe and does were enrolled in this study). The

selected sheep and goats were sampled at two-month intervals (2 and 4 months); data was collected on reproductive performance and serological response during the study.

## 4.2 Statement of the problem

Prior to the study outlined here, no previous research had investigated the epidemiology and economic effects of *T. gondii* and *B. melitensis* in small ruminants in Dohuk Province.

The results obtained from this study provided beneficial information on brucellosis and toxoplasmosis, which allowed for the development of preventive measures against both diseases and the implementation of suitable targeted surveillance programmes by the Iraqi government.

## 4.3 Aim and objective of our study

Both brucellosis and toxoplasmosis are considered important zoonotic diseases in small ruminants. Both diseases are widely distributed. Through their zoonotic capability, they can have significant economic effects on the profitability of rearing sheep and goats as well as on the welfare of the community. Brucellosis and toxoplasmosis reduce production by causing abortions, stillbirths and placentitis, which typically occur in the late stages of pregnancy. They also lead to mastitis and reduced milk production. The distribution of these diseases is worldwide, including Iraq and other countries in the Middle East. These diseases can have significant direct and indirect effects on livestock and human health, resulting in considerable losses to individual farmers and the community.

# CHAPTER FIVE: RISK FACTORS ASSOCIATED WITH *BRUCELLA* SEROPOSITIVITY IN SHEEP AND GOATS IN DOHUK PROVINCE, IRAQ

**ATTRIBUTION STATEMENT**

The following chapter has been drafted in accordance with the journal Veterinary Sciences

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Please note the different spelling of Duhok Province which is an accepted alternative; for consistency throughout the thesis the spelling is Dohuk Province.

The following authors contributed to this manuscript as outlined below.

| **Authorship order** | **Contribution (%)** | **Concept Development** | **Data Collection** | **Data Analyses** | **Drafting of manuscript** |
|---|---|---|---|---|---|
| Ali G. Alhamada | 60 | X | X | X | X |
| Ihab Habib | 20 | X | | X | X |
| Anne Barnes | 10 | X | | | X |
| Ian Robertson | 10 | X | | X | X |

Contribution indicates the total involvement the author has had in this project. Placing an 'X' in the remaining boxes indicates what aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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**Candidate** **Principal Supervisor**

## 5.1 In Brief

By serological screening of brucellosis in sheep and goats in Dohuk Province north of Iraq Alhamada et al. show that the seropositivity of *Brucella* in small ruminants is 31.7%; for every 1000 Iraqi Dinar (~0.85 US Dollar) spent by the farmers on control of *Brucella* in their flocks, the odds of *Brucella* seropositivity decreased significantly.

## 5.2 Highlights

- Risk factors for *Brucella* seropositivity were evaluated in small ruminants in Dohuk Province in northern Iraq.
- The likelihood for *Brucella* seropositivity was significantly higher in older animals, with mixed grazing between sheep and goats, and among goats originated from farms with a history of abortion in the past 12 months.
- The likelihood for *Brucella* seropositivity decreased with increase farmer' expenditure on control of *Brucella* in sheep flocks.

## 5.3 Abstract

Sera from 432 small ruminants (335 sheep and 97 goats) from 72 farms in Dohuk Province, northern Iraq, were collected to investigate risk factors associated with brucellosis seropositivity. Serum samples were tested using the Rose Bengal test (RBT) and an indirect enzyme linked immunosorbent assay (iELISA). Using parallel interpretation, RBT and iELISA results showed that 31.7% (95% confidence interval (CI): 26.1, 36.3) of sheep and 34.0% (95% CI: 24.7, 44.3) of goats had antibodies against *Brucella* in the study area. A random-effects multivariable logistic regression model indicated that a higher chance of being seropositive (odd ratio (OR) = 1.7; 95% 1.4; 2.2) was associated with an increase in the age of animals. The odds of *Brucella* seropositivity in flocks where sheep and goats grazed together was 2.0 times higher (95% CI: 1.08; 3.9) compared to flocks where sheep and goats graze separately. The odds of *Brucella* seropositivity in small ruminants was 2.2 higher (95% CI: 1.2; 4.3) for goats originating from farms with a history of abortion in the past 12 months. In contrast, for every 1000 Iraqi Dinar (~0.85 US Dollar) spent by the farmers on control of *Brucella* in their flocks, the odds of *Brucella* seropositivity decreased significantly (OR= 0.9, *P-value*= 0.021). The final model also indicated significant differences in *Brucella* seropositivity between the different districts of Dohuk Province.

## 5.4 Introduction

Brucellosis is one of the most important zoonoses affecting both human and animal health. The disease is endemic in many nations throughout the Middle East, Mediterranean regions, Central Asia and Latin America. *Brucella melitensis* (mainly infecting sheep and goats) is the most common cause of human brucellosis worldwide (Addis, 2015). In humans, the disease is manifested by acute febrile illness which, if not treated adequately, might develop complications include chronic hepatomegaly, splenomegaly and arthritis. The disease is classified as a risk group III disease due to its ease of airborne transmission. In livestock, brucellosis mainly affects the reproductive organs and causes abortion, reduced fertility and decreased milk production (de Figueiredo et al., 2015). Hence, the disease could have serious negative socio-economic impacts on people, especially in low-income countries, due to loss of work or income as consequence of illness and reduced profitability in the livestock sector (Benkirane, 2006).

In Iraq, the small ruminant (sheep and goats) sector is very important for sustaining the country's food security. There are presently an estimated 7-8 million sheep and 1.5-2.0 million goats in Iraq contributing a valuable source of meat and milk production, and providing income and job security to people working across the agricultural sector (Bechtol et al., 2011). An important challenge facing the small ruminant sector in Iraq is the challenging animal disease situation. Many endemic diseases are poorly managed and controlled as a consequence of the collapse of the veterinary infrastructure as a result of international economic sanctions and political and ethnic conflicts (FAO, 2009). Among the many endemic animal diseases, brucellosis continues to pose a threat to animal productivity and public health in Iraq. Jabary (2015), detected *Brucella* antibodies in 27.6% of whole blood samples ($n$= 311) from small ruminants in Al-Sulaimanya Province (north of Iraq). Turgay and Ahmed (2016), detected *Brucella spp.* in 61.24% of milk samples ($n$= 80) from sheep and goats farms in Dohuk, also in the north of Iraq. Many factors may play a role in the spread and survival of *Brucella* among animals, including altitude variation, flock or herd size, animal density, and livestock contact between flocks (Jabary, 2015; Turgay & Ahmed, 2016). The disease

incidence in humans in Iraq has been estimated to be between 52.3 cases per 100,000 person-years in a rural area to 268.8 cases per 100,000 person-years in a semi-rural area (Yacoub et al., 2006). Such wide variation in reported brucellosis incidence is evident between different province in Iraq, highlighting the need to deepen our understanding of risk factors for disease transmission at the human-animal interface.

Northern Provi Province nces of Iraq share extensive, however loose, borders with neighboring Turkey and Syria. Brucellosis control in northern Iraq is very challenging, as it demands coordinated regional control efforts with neighboring countries (Karim et al., 1979). Such coordination of control efforts is overshadowed by the political instability across the borders. For instance, Dohuk Province, at the very north of Iraq, has received a major influx of immigrants and refugees from neighboring Syria and from other parts of Iraq over the last two year (Abdul-Khaleq, 2019). This human migration also involved the movement of an estimated 100,000 sheep and goats. These livestock are often sold cheaply, grazed illegally, and not vaccinated regularly (Abdul-Khaleq, 2019). In such setting, local livestock in northern Iraq might become more vulnerable to an unprecedented exposure pressure, which might facilitate spread and persistence of many diseases. The objectives of the present study were to estimate the seroprevalence of *Brucella* among sheep and goats in Dohuk in the north of Iraq, and to identify risk factors associated with seropositivity.

## 5.5 Materials and methods

### 5.5.1 Study area and population included

Dohuk Province is located in the northern part of Iraq, and borders Syria and Turkey. The province is populated by approximately 1.2 million people, and contains about 1 million sheep and goats. Dohuk is divided into seven districts, with each district being further subdivided into two sub-districts and a number of villages (Rahman et al., 2013). The study setting included six of the seven districts of Dohuk; it was not possible to access one of the districts due to security concerns. The study was conducted between February and April 2016, preceding a *Brucella* vaccination campaign.

Dohuk contains a large area of pastures and sheep and goats are either grazed separately or together under a communal grazing system. Grazing of livestock is usually overseen by the farmers themselves or by shepherds, employed by the farmers. A shepherd may be responsible for a large number of sheep and goats belonging to different owners. In this study, only flocks managed directly by farmers were included to ensure the accuracy of the questionnaire information. Flocks with a minimum of 100 animals were eligible for inclusion in this study.

### 5.5.2 Sampling strategy

There was no sampling frame available from the local veterinary office. Hence, we used the local farmers' knowledge and network to achieve a representative sampling plan. Twelve sub-districts in Dohuk Province were included in this study (2 sub-districts from each of the 6 selected districts). Three villages from each sub-district (of the 4 to 6 villages available) were randomly selected for inclusion in this study. Local farmers were approached in the central mosque of each village, and were asked to voluntarily provide information about the farmers within the village who raised sheep and goats, and who met the inclusion criteria set for sampling (excluding shepherd-managed flocks, and excluding farms with < 100 animals). From each village, 2 farms meeting the study inclusion criteria for sampling, and whose owners agreed to participate in the study, were visited.

The sample size using an expected prevalence of 50%, a confidence level of 95% and a desired precision of 5% was 385. A total of 432 individual blood samples (335 sheep and 97 goats) were collected. The samples were distributed equally over each of the six districts (72 animals/ district). From each village, 2 farms were visited, and from each farm 6 individual animals were randomly selected for collection of blood samples. For mixed flocks, three sheep and three goats were sampled per farm. At the time of sampling a questionnaire about the flock's health and management was administered to the farmer.

### 5.5.3 Serological analyses

Serum was extracted from whole blood by centrifugation at 3000 rpm for 10 min and stored at -20°C until testing. Each serum sample was screened for anti-*Brucella* antibodies by Rose Bengal Test (VIRCELL, Spain) and using a commercial ELISA (NovaTec, Germany). The RBT was conducted according to the manufacturer's protocol. Sera from sheep and goats were tested for anti-*Brucella* IgG antibodies using ELISA kits according to the manufacturer instructions and recommended Cut-off titer level. Testing was carried out in a nationally accredited commercial laboratory in Dohuk. The serological status for a given sera was given by a parallel test interpretation of the results of the two tests. Thus, a serum was regarded as serologically positive when a positive result was recorded on one or both of the tests.

### 5.5.4 Questionnaire implementation

A questionnaire was administered directly to the selected farmers. The questionnaire was developed in English then translated into Kurdish and administered to the farmers by a native speaking Kurd. Information was gathered about the management and husbandry practices adopted, flock make-up and history of abortions in the relevant flocks (Tables 5.2 and 5.3).

### 5.5.5 Statistical analyses

The binary serological results (seronegative=0/ seropositive=1) and variables on characteristics of flock's health and management, explained in the previous section, were recorded for all animals sampled for the study. The data were entered into an Excel spreadsheet and analyzed using STATA (Version 11.2, StataCorp, College Station, Texas). To adjust for potential clustering, random-effects logistic regression models were used to assess the association between brucellosis seropositivity and predictor factors. The "farms" was assigned as the categorical variable identifying the group structure for the random effects. The analysis was conducted in two steps. Firstly, the association between putative predictor factors (independent variables) and dependent variable (*Brucella* seropositivity) was initially assessed using univariate logistic regression analysis to quantify the strength of association between the independent variables and *Brucella* seropositivity. The second stage in the analysis consisted of building a multivariable logistic regression model based on potential risk factors identified from the univariate analysis with factors with a *P*-value $\leq 0.25$ on the univariable analysis offered to the model. The most appropriate final model was selected using a backward stepwise selection approach. All pairwise interactions between the variables in the final model were examined for significance. Goodness of fit of the final model was assessed using the Hosmer-Lemeshow test. The associations between brucellosis seropositivity and putative risk factors were assessed by Odds Ratio (OR), and 95% Confidence Intervals (CIs) was considered significant at *P*-value $\leq 0.05$.

## 5.6 Ethical approval

The study had been approved by the animal and human ethics committees of Murdoch University. All procedures were explained to farmers and informed verbal consents were obtained from all participants prior to sampling and administering the questionnaire.

## 5.7 Results

The seropositivity in goats (34%, 95% CI: 24.7, 44.3) was similar to that of sheep (31.7%, 95% CI: 26.1, 36.3) (p = 0.450). When a single test was used, only 71/137 (51.8%) and 102/137 (74.4%) were respectively classified as serologically positive by RBT and indirect enzyme-linked immunosorbent assay (iELISA) (Table 5.1). When the results of the two tests were interpreted in parallel, 137/432 (31.7%, 95% confidence interval (CI): 26.1, 36.3) sera were classified as serologically positive for antibodies against *Brucella*.

**Table 5.1** ***Serological detection of antibodies to Brucella among 432 small ruminants (335 sheep and 97 goats) from Dohuk Province of Iraq using Rose Bengal test (RBT) and indirect enzyme linked immunosorbent assay (iELISA)***

| **Sheep** | | | | **Goats** | | | |
|---|---|---|---|---|---|---|---|
| | **ELISA (-)** | **ELISA (+)** | **TOTAL** | | **ELISA (-)** | **ELISA (+)** | **TOTAL** |
| **RBT (-)** | 231 | 52 | 283 | **RBT (-)** | 64 | 14 | 78 |
| **RBT (+)** | 25 | 27 | 52 | **RBT (+)** | 10 | 9 | 19 |
| | 256 | 79 | **335** | | 74 | 23 | **97** |

(+) positive; (-) negative

The results from the questionnaire and the corresponding serological results are summarized in Tables 5.2 and 5.3 for categorical and continuous variables, respectively. Based on the univariate logistic regression analysis, the factors of age, districts, number of sheep per farm, number of goats per farm, flock grazing pattern, number of aborted goats on the farm during the preceding 12 months,

farmer handling of aborted animals, source of water on the farm, cleaning of water troughs, availability of agricultural workers on the farm, number of agricultural workers on the farm, and amount of money spent in flock on the control of *Brucella* in the preceding 12 months had a *p* value <0.25, and were offered to the final random-effects multivariable logistic regression analysis (Table 5.4).

**Table 5.2.** ***Categorical risk factors associated with brucellosis seropositivity among small ruminants from Dohuk Province of Iraq using parallel interpretation of Rose Bengal test (RBT) and indirect enzyme linked immunosorbent assay (iELISA)***

| Variables | Category | n | Seropositivity |
|---|---|---|---|
| District | Aqarh | 72 | 54.1 |
| | Amadiya | 72 | 22.2 |
| | Dohuk | 72 | 20.8 |
| | Simele | 72 | 36.1 |
| | Shekhan | 72 | 26.4 |
| | Zakho | 72 | 38.9 |
| Species | Sheep | 335 | 31.0 |
| | Goats | 97 | 34.0 |
| Sex | Male | 45 | 28.1 |
| | Female | 387 | 32.0 |
| Sheep vaccinated against *Brucella* in the preceding 12 months | Yes | 211 | 31.1 |
| | No | 124 | 32.7 |
| Goats vaccinated against *Brucella* in the preceding 12 months | Yes | 53 | 30.5 |
| | No | 44 | 32.5 |
| Flock grazing pattern | Sheep and goats graze together | 252 | 33.3 |

| | | | |
|---|---|---|---|
| | Sheep and goats graze separately | 180 | 30.5 |
| Flock grazing - Mixing with flocks from different farms | Yes | 240 | 31.6 |
| | No | 192 | 31.7 |
| Abortion occurred among animals on farm in the preceding 12 months | Yes | 228 | 35.5 |
| | No | 204 | 27.4 |
| Farmer handling of aborted animals | Burn it | 6 | 50.0 |
| | Give it to dogs | 348 | 33.9 |
| | Threw it in public garbage | 42 | 21.4 |
| | Threw it in open water canal | 36 | 19.4 |
| Purchased (introduced) new animals into farm in the preceding 12 months | Yes | 120 | 33.3 |
| | No | 312 | 31.1 |
| Source of water into the farm | River | 66 | 33.3 |
| | Well | 132 | 31.8 |
| | Spring | 108 | 43.6 |
| | Tap water | 126 | 29.3 |
| Water delivered to animals through: | Concrete trough | 36 | 30.5 |
| | Metal trough | 396 | 31.8 |
| | Wooden trough | 0 | 0 |
| Cleaning of water troughs | Yes | 420 | 31.0 |
| | No | 12 | 58.3 |
| Sheep and goats share water sources with others from nearby farms | Yes | 198 | 26.3 |
| | No | 234 | 36.3 |
| Feed delivered to animals through: | Concrete trough | 36 | 22.2 |
| | Metal trough | 360 | 32.7 |

| | | | |
|---|---|---|---|
| | Wooden trough | 18 | 33.3 |
| | Foraging | 12 | 41.6 |
| | Tire | 6 | 0 |
| Electricity on farm | Yes | 204 | 29.1 |
| | No | 228 | 33.3 |
| Availability of employed agricultural workers on the farm | Yes | 33.3 | 27.1 |
| | No | 66.7 | 34.1 |

**Table 5.3.** ***Continuous risk factors associated with brucellosis seropositivity among small ruminants from Dohuk Province of Iraq using parallel interpretation of Rose Bengal test (RBT) and indirect enzyme linked immunosorbent assay (iELISA)***

| Variable | Seropositive (Mean ±SD) | Seronegative (Mean ±SD) |
|---|---|---|
| Age (months) | 4.6±1.7 | 3.5±1.4 |
| Number of sheep on farm | 403±550 | 533±504 |
| Number of goats on farm | 124±176 | 94±139 |
| If mixed with other flocks while grazing - number of external flocks | 2±1.7 | 2±1.6 |
| Number of aborted sheep on farm during the preceding 12 months | 20±32.5 | 20±33.2 |
| Number of aborted goats on farm during the preceding 12 months | 16±20.0 | 12±20.7 |
| Number of agricultural workers on the farm | 1.3±3.7 | 2±4.1 |
| Amount of money spent on feed in the preceding 12 months (in 1000 Iraqi Dinars) | 70±9.3 | 60±8.8 |
| Amount of money spent on electricity in the preceding 12 months (in 1000 Iraqi Dinars) | 10±1.9 | 16±3 |
| Amount of money spent in flock on control of *Brucella* in the preceding 12 months(in 1000 Iraqi Dinars) | 23.7±36.3 | 39.4±63.3 |

The multivariable model indicated that none of the two-way interactions were statistically significant (*P*>0.05). Hosmer–Lemeshow test showed value of 3.065 (*p*= 0.930), which indicates a good fit of the model. From the final model (Table 5.4), it could be suggested that the odds of brucellosis seropositivity were significantly higher with increasing age of animals (OR=1.7; 95% CI: 1.4, 2.1). In addition, seropositivity among animals sampled from three districts was significantly lower (Amadiya (OR=0.4; 95% CI: 0.1, 0.8), Dohuk (OR=0.3; 95% CI: 0.1, 0.7), and Shekhan (OR= OR=0.4; 95% CI: 0.1, 0.8)) as compared to those from Aqarh (Table 5.4).

**Table 5.4.** ***Multivariable logistic regression analysis of risk factors associated with brucellosis seropositivity among small ruminants from Dohuk Province of Iraq***

| **Variables** | | **OR** | **95% CI** | **S.E.** | ***P*-value** |
|---|---|---|---|---|---|
| Age (month) | | 1.7 | (1.4, 2.1) | 0.196 | <0.001 |
| District | Aqarh | 1.0 | – | – | – |
| | Amadiya | 0.4 | (0.1, 0.8) | 0.156 | 0.019 |
| | Dohuk | 0.3 | (0.1, 0.7) | 0.145 | 0.012 |
| | Simele | 0.8 | (0.4, 1.8) | 0.335 | 0.670 |
| | Shekhan | 0.4 | (0.1, 0.8) | 0.156 | 0.019 |
| | Zakho | 1.4 | (0.6, 3.2) | 0.591 | 0.331 |
| Number of sheep on farm | | 1.0 | (1.0, 1,1) | 0.003 | 0.030 |
| Flock grazing - Pattern | Sheep and goats graze separately | 1.0 | – | – | – |
| | Sheep and goats graze together | 2.0 | (1.1, 3.9) | 0.684 | 0.028 |
| Abortion among goats on farm in the preceding 12 months | No | 1.0 | – | – | – |
| | Yes | 2.2 | (1.2, 4.3) | 0.742 | 0.012 |
| Money spent (in 1000 Iraqi Dinars) on control of *Brucella* in flock in the preceding 12 months | | 0.9 | (0.8, 0.9) | 0.002 | 0.021 |

The final multivariable logistic regression model indicated that the odds of seropositivity were 2 times (95% CI: 1.1, 3.9) higher animals in flocks grazed sheep and goats together, as compared to animals from flocks not grazed sheep and goats together. Also, for goats on farms with abortions occurring in the 12 months preceding the survey the odds for brucellosis seropositivity were 2.2 times (95% CI: 1.2, 4.3) higher compared to those from farms with no reported abortion in goats in the preceding 12 months (Table 5.4).For every 1000 Iraqi Dinars (~0.85 US Dollar) spent, in the preceding 12 months to the survey, on control of *Brucella* in small ruminant flocks in the study area, the odds of brucellosis seropositivity decreased significantly (OR=0.93; 95% CI: 0.88, 0.98) (Table 5.4).

## 5.8 Discussion

In Iraq, brucellosis is considered to be one of the most important endemic animal and human diseases (Refai, 2002). The main objective of this study was to investigate risk factors associated with brucellosis seropositivity among small ruminants reared in Dohuk Province, northern Iraq. Combined results of RBT and iELISA were used as outcome variable for modeling risk factors under the study setting. The two tests used in this study are convenient and shown to be suitable for field screening. Nevertheless, none of the two tests are considered to be gold standard test (Nielsen et al., 1980). Despite being regarded as very sensitive tests, RBT and iELISA might suffer from false positive reactions due to presence of gram negative bacteria closely related to *Brucella* (Weynants et al., 1996). In this study, we combined both tests to achieve a parallel interpretation for the results. Such combination was helpful in reducing bias due to results misclassification. Our results revealed that about 25-50% of sera showing antibodies against brucellosis would have been classified as seonegative in a single testing approach either by RBT and iELISA. Several studies indicated that RBT is more suited for detecting $IgG_1$ and IgM typically produced during acute brucellosis infection. On the other hand, iELISA is more suited for detecting IgG which become dominant in chronic brucellosis cases (Weynants et al., 1996). Our results show that iELISA classified almost 25% more

sera as seropositive, suggesting the predominance of a chronic infection context. Hence, a combination of tests could improve surveillance certainty and provide more reliable results for effective diagnosis and control of brucellosis.

In our study, the multivariable model analysis identified age of animals as a risk factors associated with brucellosis seropositivity. In line with our finding, previous studies found that age has been regarded as one of the intrinsic factors to influence brucellosis seropositivity (Chimana et al., 2010; Megersa et al., 2011b). The clinical disease mainly affects the actively producing animals as compared to young animals which have not reached reproductive age (Amin et al., 2005). Furthermore, the older the animals, the higher the likelihood of contact with infected animals than younger animals.

The regression model indicated that brucellosis seropositivity was significantly higher among animals sampled from three of the districts (in Aqarh, Zakho, and Simele). Aqrah district, where 54.1% of the animals were seropositive, holds the main road for movement of animals and goods throughout the province. This led to increase of the likelihood of contact between local animals with others from different villages, and might cause increase in the risk of transmission of *Brucella*. Added to that, the three districts with significantly higher brucellosis seropositivity shared longer border with Syria, Turkey, and the Mosul Province, where most of the uncontrolled movement of animals take place, provoked by war and political instability. Uncontrolled movement and smuggling of animals across borders could contribute to spread and persistence of animal diseases in a regional context. In the neighboring country, Iran, Sharifi et al. (2015), highlighted the importance of small ruminants with an unknown history from neighboring country, mostly Syria as a significant risk factor of seropositivity to *Brucella* in small ruminants.

In our study, brucellosis seropositivity was also found to be associated with sheep flock size, with mixed grazing between sheep and goats, and with history of goat abortion on farm. The effect of flock size and mixed farming of multiple species on the risk of infections with contagious diseases

has been well documented (Salman & Meyer, 1987a). In our study area, the average size of sheep flocks was almost 4 times bigger than the average size of goats flock. The larger the flocks the higher the chances for contact between individual animals, and in particular contact with an infected animal. Added to that, the use of communal pastures allows the frequent contact between animals, and provides increased opportunity for exposure of susceptible animals with infectious materials, for instance arising from parturition. Previous studies have been reported that contact between goats and sheep at the flock level was one of the most important risk factors for infection with *Brucella* (Al-Alousi, 2008; Al-Majali et al., 2007). It has been documented that goats carry higher susceptibility to *Brucella* infection compared to sheep (Alton, 1982, 1990a). This is in line with our finding that abortion among goats on farm in the preceding 12 months was a risk factor for brucellosis seropositivity. 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 flock (Roth et al., 2003).

The regression model indicated that in the setting of Dohuk, northern Iraq, for every 1000 Iraqi Dinar (~0.85 US Dollar) spent by the farmers on control of *Brucella* in their flocks, the odds of *Brucella* seropositivity decreased significantly (OR= 0.9, *P*-value= 0.021). This finding provides indirect evidence to support the added value of investment in brucellosis control program in the area. Farmers and their families should benefit from spending on brucellosis control at farm level, for examples by reducing mortality and morbidity costs and by opening up new trade opportunities. The benefits of at farm control of brucellosis could also impact directly on consumer welfare. Consumers could either purchase more livestock products for the same level of expenditure or consume the same amount of livestock products but spend less of their disposable income. Added to that, there is an obvious human health impact from controlling brucellosis infection in livestock sector. In Mongolia, research has found that if the costs of mass vaccination of livestock against brucellosis were allocated to all sectors in proportion to the benefits, the intervention might be profitable and cost effective for

the agricultural and health sectors (Mugabi, 2012). The positive results revealed a crucial need to continue vaccination procedures annually in order to reach the lowest possible incidence rate.

Despite the results of the study, some limitations were observed. Firstly, the short period of field sampling, as this was concluded over 3 months. This was shorter than what we planned for initially, but we have been forced to conclude the field research in a shorter time due to emerging security reasons beyond our capacities. Second, our inclusion criteria to sample from larger herds (at least 100 animals) could have impacted the seropositivity estimates. However, targeting larger flock was justified by the fact that larger herds are likely responsible for the majority of animal movement within and in-between the districts. Third, microbiological cultures which would have helped to confirm the status of the serologically positive animals were not possible to carry out under the study setting conditions.

In conclusion, in this study we investigated risk factors for brucellosis seropositivity in small ruminates in Dohuk Province, northern Iraq, an area suffering from ongoing geopolitical and ethnic conflicts. This investigation revealed that age of animals, districts from which animals are reared, sheep flock size, mixed grazing between sheep and goats, and history of goat abortion on farm in the preceding 12 months were independently associated with higher brucellosis seropositivity in small ruminants in the study area. The likelihood for seropositivity decreased with increase of farmer' expenditure on control of *Brucella* in sheep flocks. Although the presence of antibodies does not necessary mean that sheep and goats are infected, these preliminary results indicate the abundant presence of brucellosis in the study setting in northern Iraq. This study should be considered as a contribution to the epidemiology of brucellosis in small ruminants in Iraq.

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# CHAPTER SIX: RISK FACTORS ASSOCIATED WITH SEROPOSITIVITY TO *TOXOPLASMA* AMONG SHEEP AND GOATS IN NORTHERN IRAQ

## ATTRIBUTION STATEMENT

The following chapter has been drafted in accordance with the journal "Veterinary Parasitology".

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The following authors contributed to this manuscript as outlined below.

| Authorship order | Contribution (%) | Concept Development | Data Collection | Data Analyses | Drafting of manuscript |
|---|---|---|---|---|---|
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| Ihab Habib | 25 | X | | X | X |
| Anne Barnes | 5 | X | | | X |
| Ian Robertson | 10 | X | | X | X |

Contribution indicates the total involvement the author has had in this project. Placing an 'X' in the remaining boxes indicates what aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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**Candidate** **Principal Supervisor**

## 6.1 In Brief

By serological screening of toxoplasmosis in sheep and goats in Dohuk Province north of Iraq Ali et al. show that the Seropositivity of *Toxoplasma* in small ruminants is 42%, the number of seropositive increase in goats that have a history of abortion in the preceding 12 months, and for farmers that give their animals prophylactic medicine, the rate of incidence abortion was decreased.

## 6.2 Highlights

- 42% of small ruminants (sheep and goats tested) were seropositive for *Toxoplasma*
- The number of abortions in goats in the 12 months before the study was strongly associated with flock seropositivity.
- Farmers who spent more money on the treatment of *Toxoplasma* in sheep and goats were more likely to have flocks with a lower level of seropositivity to *Toxoplasma*.

## 6.3 Abstract

Serum samples from 423 small ruminants (335 sheep and 97 goats) from 72 farms in 6 districts in the province of Dohuk, north Iraq were tested for the presence of antibodies against *Toxoplasma gondii* using a latex agglutinin test (Latex) and an indirect enzyme-linked immunosorbent assay (iELISA). When the test results were interpreted in parallel, 42.1% (95% confidence interval (CI): 36.7, 47.7) of sheep and 36.1% (95% CI: 26.6, 46.5) of goats were found to have antibodies against *Toxoplasma*. A multivariable logistic regression model was developed to determine the risk factors for *Toxoplasma* seropositivity in small ruminant flocks. Factors which increased the risk of infection included the presence of cats near the feed of animals (OR= 6.3; 95% CI 1.6, 24.6) and a history of abortions in sheep in the preceding 12 months (OR=13.4; 95% CI 2.1; 86.7). For every ten goats aborting in the preceding 12 months the odds of seropositivity increased significantly (OR=6.7; 95% CI 1.3; 32.9). In contrast for every 1000 Iraqi Dinars (~0.85 USD) spent by the farmers on the prophylactic treatment in their flocks the odds of *Toxoplasma* seropositivity decreased significantly (OR = 0.94; 95% CI 0.90, 0.98). This study contributes to the epidemiology of toxoplasmosis in small ruminants in northern Iraq.

## 6.4 Introduction

Toxoplasmosis, caused by the intracellular protozoan *Toxoplasma gondii*, affects all warm-blooded animals, including humans(Innes, 2010). The definitive hosts of *T. gondii* are felines, and these play an essential role in contamination of the environment with oocysts. Infection of sheep and goats can occur after consumption of feed or pasture contaminated with sporulated oocysts (Elmore et al., 2010). The primary source of infection in humans is the ingestion of uncooked meat containing tissue cysts, especially in countries where the meat from sheep and goats is regularly eaten (Kijlstra & Jongert, 2008). In livestock, toxoplasmosis mainly affects the reproductive organs resulting in abortions, fetal mummifications, stillbirths and the birth of weak offspring (Anastasia et al., 2013) and the disease has the potential to have sever negative socio-economic impacts on people, especially in low-income countries (Masala et al., 2003).

In Iraq, there are approximately 1.5 - 2.0 million goats and 7 - 8 million sheep. They are an important source of meat and milk and play an important role in the economy and food security of the nation (Bechtol et al., 2011). The veterinary infrastructure in Iraq is weak because of international sanctions and ethnic and political conflicts, and consequently, the control of endemic diseases is challenging. Antibodies to *Toxoplasma* have been reported in small ruminants, including ewes that aborted in Northern Iraq (Mikail & Al-Barwary, 2014). However, no studies have been published elucidating the risk factors for infection in either humans or other animals in the country. The main aims of the research outlined in this manuscript were to estimate the seropositivity of antibodies to *Toxoplasma* in small ruminants (sheep and goats) from Dohuk Province, north Iraq and to identify risk factors for seropositivity.

## 6.5 Materials and methods

### 6.5.1 Study area and population included

This study was conducted in Dohuk Province, which is located in the north of Iraq and borders Syria and Turkey. There are 1.2 million people and approximately 1 million sheep and goats in the province. The province is divided into seven districts, with each district containing two sub-districts and many villages (Mohammed, 2013). Flocks from six districts (12 sub-districts) were sampled between February and April 2016 in this study. It was not possible to access one of the districts due to security concerns.

Dohuk has extensive areas of pasture where sheep and goats are either grazed separately or together. The animals are usually grazed under the supervision of farmers or by employed shepherds who may supervise the grazing of stock belonging to different owners. Flocks containing more than 100 animals and both sheep and goats were eligible for inclusion in this study.

### 6.5.2 Sampling strategy

No sampling frame was available on the number and distribution of animals and flocks in the sampled districts. Consequently, a two-stage convenience sampling approach, based on a participatory approach using the local farmers' knowledge and networks, was adopted.

All of the 12 sub-districts of the 6 surveyed districts in Dohuk Province were included in this study. Local community leaders of the sub-district were approached at the central mosque, and were asked to voluntarily provide information about the farmers within the target sub-district who raised sheep and goats, and who met the inclusion criteria set for sampling (excluding farms with <100 animals). From each sub-district, all farms meeting the study inclusion criteria for sampling (typically 2-3 farms), and whose owners agreed to participate in the study, were visited. A total of 432 individual blood samples were collected from 335 sheep and 97 goats that were raised in 72 mixed flocks in six

districts of Dohuk Province. From each farm, six individual animals were randomly selected for sampling.

### 6.5.3 Serological analyses

Five ml of blood was collected from the jugular vein of each selected animal. Blood samples were allowed to clot at room temperature and then stored on ice and dispatched to the Veterinary Diagnostic Laboratory in Dohuk on the same day of collection. Serum was extracted from whole blood by centrifugation at 3000 rpm for 10 min and then stored at −20°C until testing. Each serum sample was tested for anti-*Toxoplasma* antibodies with a Toxoplasmosis Latex Test (PLASMATEC, UK) and a commercial ELISA (NovaTec, Germany) according to the manufacturer's instructions. Testing was carried out in a nationally accredited commercial laboratory in Dohuk. The serological status of individual serum (animals) was assessed by interpreting the test results in parallel where an animal was classified as serologically positive if one or both of the tests were positive.

### 6.5.4 Questionnaire implementation

At the time of sampling, a questionnaire was administered to the selected farmers using a face-to-face format. The questionnaire was initially developed in English, then translated into the local Kurdish language and administered to the farmers by a native speaking Kurd. Information was gathered about the structure of the flock, management and husbandry practices adopted, the presence of cat faeces in animal feed and water, and history of abortions in the flock (Tables 5.1 and 5. 2).

### 6.5.5 Statistical analyses

Positive flocks contained a median of 2 seropositive animals. A flock was considered positive if there were two or more seropositive animals present (≥ median). The data were entered into an Excel spreadsheet and analysed using STATA, Version 15, Software (Stata Corp LP, College Station, Texas, USA) and SPSS (Version 24, IBM).

Initially, the association between putative management/husbandry factors and flock positivity were determined with a Chi-Square test. Data that were of a continuous nature were analysed with a Kruskal-Wallis ANOVA. Factors with a P-value ≤ 0.25 were then offered to a multivariable logistic regression model to assess the association between seropositivity to *Toxoplasma* and predictor factors. A backward stepwise selection approach was used and factors with a $P < 0.05$ were retained in the final model. All pairwise interactions between the variables in the final model were examined for significance. Goodness-of-fit of the final model was assessed using the Hosmer–Lemeshow test. The associations between seropositivity and the risk factors were assessed with odds ratios (ORs) and 95% confidence intervals (CI).

## 6.6 Ethics Approval

The study had been approved by the animal and human ethics committees of Murdoch University. All procedures were explained to the farmers, and informed verbal consent was obtained from all participants before sampling and administering the questionnaire.

## 6.7 Results

Antibodies to *T. gondii* were detected in animals from all the six districts that were involved in the study. Overall, the seropositive of *Toxoplasma* in sheep (42.1%; 95%CI 36.7, 47.7) was similar to that in goats (36.1%; 95%CI 26.6, 46.5) ($P = 0.09$). There was no significant difference in the seropositivity between districts. The mean number of seropositive animals per farm was 2.43 ± 1.84. Overall the farm level prevalence (farms containing one or more seropositive animals) was 36.8% (95%CI 24.4, 50.7).

In Tables 6.1 and 6.2, the results are summarized for the univariable analyses of the categorical and continuous variables, respectively. Of the categorical variables, the present of cats near the feed of the animals, the presence of cat faeces in feed or water, the presence of abortions in sheep in the 12-month period preceding the study, using a common grazing area and grazing with other animals

had a $P \leq 0.25$ and were offered to the initial multivariable logistic regression model (Table 6.1). For the continuous variables the total number of sheep (in hundreds owned) and the number of goats which aborted (in tens) in the flock in the preceding 12-month period and the amount of money (1000 Dinars) spent on the prophylactic treatment had a $P \leq 0.25$ and were offered to the initial multivariable logistic regression model (Table 6.2).

**Table 6.1. Univariable analysis of categorical risk factors associated with flocks with a seropositivity to *Toxoplasma* ≥ median.**

| Factor | Number of flocks With the factor | Percent of flocks seropositive (95% CI) | OR (95%CI) | P-Value |
|---|---|---|---|---|
| Cats near feed | 48 | 79.17 (65.0, 89.0) | 4.49 (1.55, 13.00) | 0.004* |
| Cats not near feed | 24 | 45.83 (20.7, 72.6) | 1.0 | |
| Cat faeces in feed | 42 | 83.33 (68.6, 93.0) | 5.71 (1.93, 16.88) | 0.001* |
| No cat faeces in feed | 30 | 46.67 (23.7, 70.7) | 1.0 | |
| Cat' faeces in water | 48 | 58.33 (43.2, 72.4) | 0.20 (0.052, 0.76) | 0.012* |
| No cat' faeces in water | 24 | 87.50 (61.3, 98.5) | 1.0 | |
| Animals graze outside farm | 27 | 70.37 (49.8, 86.2) | 1.19 (0.42, 3.34) | 0.74 |
| No animals graze outside farm | 45 | 66.7 (51.0, 80.0) | 1.0 | |
| Presence of abortions in the sheep flock during the last 12 months | 60 | 75.00 (62.1, 85.3) | 6.00 (1.58, 22.8) | # 0.01 * |
| No abortions present in the sheep flock during the last 12 months | 12 | 33.33 (6.2, 72.8) | 1.0 | |
| Presence of abortions in the goat flock during the last 12 months | 37 | 72.9 (55.9, 86.2) | 1.60 (0.59, 4.33) | 0.36 |

| | | | | |
|---|---|---|---|---|
| No abortions present in the goat flock during the preceding 12 months | 35 | 62.86 (39.9, 82.3) | 1.0 | |
| Animals use a common grazing area | 42 | 73.81 (58.0, 86.1) | 1.88 (0.69, 5.12) | 0.22* |
| Animals do not have access to a common grazing area | 30 | 60.00 (35.3, 81.5) | 1.0 | |
| Livestock grazed with other Flocks | 40 | 77.50 (61.5, 89.2) | 2.68 (0.97, 7.42) | 0.05* |
| Livestock not grazed with other herds | 32 | 56.25 (32.6, 78.0) | 1.0 | |
| Water troughs not cleaned weekly | 56 | 69.64 (55.9, 81.2) | 1.38 (0.43, 4.41) | 0.59 |
| Water troughs cleaned at least weekly | 16 | 62.50 (28.7, 89.1) | 1.0 | |
| Feed troughs not cleaned weekly | 60 | 66.67 (49.3, 81.3) | 1.5 (0.37, 6.16) | # 0.74 |
| Feed troughs cleaned at least weekly | 12 | 75.00 (42.8, 94.5) | 1.0 | |
| Source of water | | | | |
| River only | 26 | 69.23 (42.2,89.1) | 1.8 (0.38, 8.53) | |
| Reticulated supply | 21 | 71.4 (47.8, 88.7) | 2.00 (0.40, 10.11) | 0.86 |
| Both rain and river | 16 | 68.75 (41.3, 89.0) | 1.76 (0.33, 9.51) | |
| Well only | 9 | 55.56 (21.2, 86.3) | 1.0 | |
| Feeder trough made from | | | | |

| | | | | |
|---|---|---|---|---|
| Metal | 23 | 69.57 (40.8, 90.3) | 1.23 (0.34, 4.42) | |
| Tyre | 18 | 66.67 (41.0, 86.7) | 1.08 (0.28, 4.13) | 0.97 |
| Wood | 11 | 72.73 (39.0, 94.0) | 1.44 (0.29, 7.21) | |
| Concrete | 20 | 65.00 (34.3, 88.6) | 1.0 | |
| District number | | | | |
| Amadiya | 11 | 91.67 (52.3, 100.0) | 11.0 (1.06, 114.09) | |
| Aqarh | 7 | 58.33 (27.7, 84.8) | 1.40 (0.28, 7.02) | |
| Zakho | 8 | 66.67 (34.9, 90.1) | 2.00 (0.38, 10.41) | 0.34 |
| Simele | 9 | 75.00 (42.8, 94.5) | 3.00 (0.53, 16.90) | |
| Shekhn | 8 | 66.67 (34.9, 90.1) | 2.00 (0.38, 10.41) | |
| Dohuk | 6 | 50.00 (21.1, 78.9) | 1.0 | |

*$p \leq 0.25$ and offered to the multivariable logistic regression model

# - Fisher's exact test

**Table 6.2. Univariable analysis of continuous risk factors associated with flocks with a seropositivity to *Toxoplasma* ≥ median.**

| Variable | Median | | P-Value |
|---|---|---|---|
| | Flocks < median prevalence | Flocks ≥ median prevalence | |
| Total number of sheep (x100) | 0.22 | 0.41 | # < 0.001 |
| Total number of goats (x100) | 0.2 | 0.51 | # <0.001 |
| Number of sheep aborting in last 12 months (x 10) | 1.4 | 4.9 | # <0.001 |
| Number of goats aborting in last 12 months (x10) | 2.1 | 5.6 | # <0.001 |
| Amount of money spent in the evidence of outbreak (x 100 U.S Dollars | 0.62 | 0.27 | # <0.001 |

*p ≤ 0.25 and offered to the multivariable logistic regression model

# - Kruskal-Wallis ANOVA

In the final multivariable logistic regression model (Table 6.3) the odds of seropositivity were significantly higher in flocks that: had cats near the feed of the animals (OR= 6.34; 95% CI 1.63, 24.58); and had a history of abortions in sheep in the preceding 12 months (OR=13.41; 95% CI 2.076; 86.71) (Table 6.3). For every ten goats which aborted on a farm in the 12 months preceding the survey, the odds of seropositivity increased by 6.65 (95% CI 1.34; 32.86). Also for every 1000 Iraqi Dinars (~0.85 US Dollar) that farmers reported that they spent on the prophylactic treatment to a lower incidence of abortion that it is also control of *Toxoplasma* in the 12 months preceding the survey, the odds of seropositivity decreased significantly (OR = 0.94; 95% CI: 0.90, 0.98) (Table 6.3).

**Table 6.3. Multivariable logistic regression model of herd-level risk factors associated with toxoplasmosis seropositivity among small ruminants from Dohuk Province (Iraq).**

| Variables | Category | OR (95% CI) | S.E. | *P*-value |
|---|---|---|---|---|
| Cats near feed | Yes | 6.34 (1.63,24.58) | 4.38 | 0.008 |
| | No | 1.0 | _ | _ |
| Abortion among sheep on farm in the preceding 12 month | Yes | 13.41 (2.08,86.71) | 12.77 | 0.006 |
| | No | 1.0 | _ | _ |
| Number of goats aborting in last 12 months (x10) | Yes | 6.66 (1.34,32.86) | 5.42 | 0.02 |

| | | | |
|---|---|---|---|
| Money spent (in 1000 Iraqi Dinars) on the prophylactic treatment in the flock in the preceding 12 months | 0.94 (0.90, 0.98) | 0.022 | 0.015 |
| Constant | - | 0.057 | 0.007 |

None of the two-way interactions of the final reduced subset model were statistically significant (all P values > 0.05). The Hosmer–Lemeshow value supported a good fit of the data in the final model (7.94; P = 0.43).

## 6.8 Discussion

The current study highlights the endemic nature of antibodies to *T. gondii* in sheep and goats in Dohuk Province with a prevalence of 42.1% (95%CI 36.7, 47.7) and 36.1% (95%CI 26.6, 46.5), respectively. The overall seropositive of 40.32% was higher than that recently reported in Saudi Arabia (36.03%) (Alanazi, 2013) but lower than that reported in Sudan (52.0% ) (Atail et al., 2017).

In this study, the two tests (Latex and iELISA) used are suitable for field screening due to their high sensitivity, although they can produce false-positive results through cross-reactions with antibodies to *Anaplasma marginale* or *Neospora caninum* (Gondim et al., 2017; Rodgers et al., 1998). The Latex test detects IgG1, and IgM produced during acute cases of toxoplasmosis while the iELISA detects IgG which is the dominant immunoglobulin in chronic cases (Györke et al., 2011; Jiang et al., 2008). Consequently using these two tests

in parallel enhances the likelihood of detecting antibodies in both acute and chronic cases (Ahmed, 2010; Mouhamed et al., 2018; Parkhouse, 2013).

In this research, the presence of cats near the feed of the flocks was identified as a significant risk factor for seropositivity. Other studies have similarly shown this (Cenci-Goga et al., 2013; Cenci-Goga et al., 2011; Rêgo et al., 2016). Inspection of the feed sheds/stores in the current study found the presence of a large quantity of cats' faeces on bags and in the loose feed, most likely due to cats hunting rodents present in these locations. Consequently, it would be expected that more intensive flock management involving feeding animals may increase the likelihood of infected small ruminants. The faeces of cats can contain millions of oocysts resulting in a severely contaminated environment (Dubey, 1998c) . It is most likely that other factors favoring the contamination of feed and drinking water, such as access of cats to the shed for housing animals, water source, and feedstuff better describe the close contact between small ruminants and the excretions of cats (Skjerve et al., 1998). It has been documented that goats are more susceptible to infection with *Toxoplasma* than sheep (García-Vázquez et al., 1990). This is in line with our finding that the number of abortions in goats on the farm in the preceding 12 months was a risk factor for seropositivity.

Sheep living persistently in high endemic areas could acquire the infection before their first pregnancy and should thus be immune at conception. This is not surprising given that toxoplasmosis commonly results in abortion and environmental contamination occurs from cysts present in the placenta and aborted foetus of affected animals (Unzaga et al., 2014). Incorrect disposal of placenta or aborted foetuses can increase the opportunity for cats to be infected through consumption of this product, continuing the lifecycle and subsequently shedding oocysts (Hamilton et al., 2014) which may survive in the environment for many months (Dubey, 1998b).

In the present study, there were no significant difference in the proportion of seropositive flocks between districts. This is not surprising given the similar management practices between districts.

In this study for every 1000 Iraqi Dinar (~0.85 US Dollar) spent per farm per year on prophylactic treatment the odds of *Toxoplasma* seropositivity decreased (OR=0.94). Very little is known about the use of antibiotics in small ruminant flocks in Iraq. The use of such drugs can have a profound impact on infected animals, farmer income, and public health. In livestock production, antimicrobial are often administered on a regular basis by the farmer (Dunlop et al., 1998). A decreased incidence of abortions has been reported in farms using prophylactic antibiotic treatment (Pereyra et al., 2015).

There is a need for a complete economic analysis to be undertaken to determine the benefits of controlling the disease and the costs in achieving this control. Control of toxoplasmosis could result in reduced morbidity and mortality of small ruminants (Buxton et al., 1988), as well as reducing the risk of disease in humans (Buxton et al., 1991). It is recommended that to prevent infection in small ruminants cats should be prevented from accessing feed provided to sheep and goats, they should be prevented from entering areas where sheep/goats are housed and hygiene, particularly during parturition, should be improved including burial or burning of foetal membranes and aborted lambs/kids.

## 6.9 Conclusion

It is recommended that farmers/shepherds adopt control measures such as preventing access to cats of placental and aborted materials from their small ruminant flocks. (Abu-Dalbouh et al., 2012) reported that the proper disposal (incineration or burying) of foetuses significantly decreased the risk of seropositivity to *Toxoplasma* in sheep and goats. Farmers

could use traps or rodenticides to control rodents, keep cats out of feed-storage facilities, and provide farm cats with alternative processed food (Cruz-Vazquez et al., 1992).

The present survey confirmed the presence of antibodies to *Toxoplasma* in the Dohuk sheep and goat population. Identifying the putative risk factors can help in the development of control programs to reduce the impact of infection with *Toxoplasma*.

# CHAPTER SEVEN: SEROCONVERSION TO *BRUCELLA SPP.* AND *TOXOPLASMA GONDII* IN SHEEP AND GOATS IN DOHUK PROVINCE, IRAQ AND ITS ASSOCIATION WITH PREGNANCY LOSS

## ATTRIBUTION STATEMENT

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By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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## 7.1 In Brief

By serological screening of toxoplasmosis and brucellosis during pregnancy in sheep and goats in Dohuk Province north of Iraq, Ali et al. show that the seroconversion to *Toxoplasma* in small ruminants between 2 and 4 months of pregnancy is 7.3% and 10.6% for *Brucella;* sheep and goats that seroconverted to *Brucella* were 2.9 times more than likely to lose their pregnancy than if the animal remained negative, and there was no relationship between seroconversion to *Toxoplasma* and loss of pregnancy.

## 7.2 Highlights

- 10.6% of sheep and goats seroconverted to *Brucella* and 7.3% to *T. gondii* during a two-month period.
- Animals that seroconverted to *Brucella* were 2.9 times more likely to lose their pregnancy than animals which remained seronegative.
- There was no association between seroconversion to *Toxoplasma* and loss of pregnancy

## 7.3 Abstract

By serological screening of antibodies against of *Brucella* and *Toxoplasma* in pregnancy in sheep and goats in Dohuk Province north of Iraq Ali et al. show that the incidence risk of seroconversion to *Brucella* over the two months was 10.6% (95% CI: 6.9 - 15.3) and 7.3% (95% CI: 4.3 - 11.6) for *Toxoplasma.* Brucellosis and toxoplasmosis cause economic losses in small ruminants, notably through abortions. Both *Brucella melitensis* and *Toxoplasma gondii* are important zoonotic agents with infection of the former arising from contact with infected small ruminants or their products and the latter through ingestion of tissue cysts in undercooked/raw meat products of livestock or from oocysts of cats. In this study, sera from 240 small ruminants (192 sheep and 48 goats) belonging to 12 farms in Dohuk Province, northern Iraq, were collected on two occasions to investigate the incidence risk of seroconversion to *Brucella spp.* and *Toxoplasma gondii*. All selected animals were confirmed pregnant (approximately 2 months pregnant) by ultrasound examination at the time of the first blood collection. A second ultrasound examination and blood sampling were undertaken two months after the initial scanning/sampling. Antibodies to *Brucella* were tested using the Rose Bengal Test (RBT) and an indirect enzyme-linked immunosorbent assay (iELISA), and the results were interpreted in series. The Latex Agglutination Test (LAT) and an indirect enzyme-linked immunosorbent assay (iELISA) were also used in series to confirm the presence of antibodies to *T. gondii*. The seroprevalence for *Brucella* and *Toxoplasma* increased significantly between the two sampling times (*P*-value = 0.0003 and 0.03 in first and second sampling, respectively). The incidence risk of seroconversion to *Brucella* over the two months was 10.6% (95% CI: 6.9 -15.3) and 7.3% (95% CI: 4.3 - 11.6) for *Toxoplasma*. Animals that seroconverted to *Brucella* were 2.9 times more likely to lose their pregnancy (95% CI: 1.6 – 5.5) than animals that remained seronegative; however, seroconversion to *Toxoplasma* had no significant impact on loss of pregnancy. This study is

the first reported investigation on the influence of seroconversion to *Brucella* and *Toxoplasma* on the reproductive outcome of pregnant sheep and goats in northern Iraq. Brucellosis and toxoplasmosis continue to negatively impact small ruminants' reproductive performance and compromising food security in Iraq. It is hoped that this study will assist the development of a better-informed economic model to estimate *Brucella* and *Toxoplasma* burden in small animals in northern Iraq, and such a model could be used to validate the impact of various potential intervention programs in.

## 7.4 Introduction

In Iraq, there are an estimated 6.6 million sheep and 1.3 million goats, representing a valuable source of meat, milk, and fiber production, and providing income and job security to people working across the agricultural sector (FAO, 2018); however, the disease is a significant challenge facing the small ruminant sector in the country (AlHamada et al., 2017). Like many endemic animal diseases, Brucellosis and toxoplasmosis are poorly managed and controlled in Iraq as a consequence of poor veterinary infrastructure (FAO, 2009; Helmy et al., 2017). Infection with *Toxoplasma gondii* may result in early embryonic death and resorption or foetal mummification (Dubey, 2009b; Smith & Sherman, 2009). The infection outcome is influenced by the stage of pregnancy at which the ewe/doe becomes infected; the earlier infection occurs during the gestation period, the more severe the consequence (Dubey, 2009b). As well as the loss of offspring, there is also reduced milk production, resulting in a major economic loss to farmers and the general community (Franc et al., 2018). In small ruminants, brucellosis from infection with *Brucella melitensis* also results in economic losses through abortions, decreased milk production and infertility (Corbel, 1997). Sheep and goats are considered the primary hosts for *B. melitensis*; however, affected females usually show no clinical signs until late gestation (Akhtar & Mirza, 1995). Both *B. melitensis* and *T. gondii*

are also important zoonotic agents with infection of the former arising from contact with infected small ruminants or their products (Blasco, 1997a), and the latter through ingestion of tissue cysts in undercooked/raw meat products of livestock or from oocysts of cats (Guo et al., 2015).

In Iraq, factors which influence reproductive failure in small ruminants are mostly unknown; however, of the many endemic diseases present, brucellosis and toxoplasmosis are considered important in reducing reproductive output and productivity (Al-Dabagh et al., 2014; Mikail & Al-Barwary, 2014). Although recent studies undertaken in Iraq have highlighted a significant association between seropositivity to *Brucella* or *Toxoplasma* and abortion in sheep and goats (AlHamada et al., 2017), these were based on cross-sectional studies with their accompanying potential biases, in particular identifying whether infection as measured by a seropositive reaction occurred prior to or after pregnancy loss. This study is part of an integrated research project aiming at understanding the epidemiology of reproductive diseases in small ruminants in northern Iraq. We explored seropositivity patterns and risk factors of *Brucella* and *Toxoplasma* in sheep and goats in Dohuk Province in northern Iraq in previous work (AlHamada et al., 2017). Moving forward, we present in this work a prospective cohort study aiming to determine the incidence risk of seroconversion to *Brucella* and *Toxoplasma* and the influence of seroconversion on the reproductive outcome in pregnant sheep and goats in Dohuk. We anticipate that this study's findings will provide objective evidence that could be used for the future development of a well-informed economic modeling evaluation of *Brucella* and *Toxoplasma* burden in small animals in Iraq, and such a model could be used to validate the impact of various potential intervention programs.

## 7.5 Materials and methods

### 7.5.1 Study Area and selection criteria

Dohuk Province contains approximately 1,000,000 (one million) small ruminants (unpublished records from Dohuk Veterinary Hospital). Dohuk is the most northern province in Iraq and is located in a very sensitive area, bordering conflict and war zones in Syria and Turkey. Conducting and collecting the study samples and epidemiological data from local sheep and goats was challenging, giving security concerns and limited infrastructure in the study setting (Canada: Immigration and Refugee Board of Canada,2016). Six of the seven districts in the province were included in this study (one district could not be accessed due to security concerns). All 12 sub-districts of the six districts were included in this study. There was no complete sampling frame (structured list of farms and contacts) that could be supplied from local veterinary authorities. None formal sampling frame of farms with small ruminants was adopted in this study, where local community leaders in each district were asked to provide the names of farmers who owned sheep and/or goats in each sub-district from each sub-district, one farm was selected for inclusion in the study; these farms' owners were approached for inclusion in the study, based on whether the householder was present and willing to participate in the study. In total, 12 (one from each of the 12 sub-districts) farms representing mixed flocks of Awassi sheep and local Iraqi goats. Animals were pregnancy tested by ultrasonographic examination (as will be explained in the following section), and 20 animals that were approximately 2 months pregnant were then randomly selected from each participating flock for inclusion in the study (until a total of 240 animals were obtained; 192 sheep, 48 goats).

### 7.5.2 Ultrasonography examinations

All animals were scanned transabdominally using an ultrasound scanner equipped with a 3.5 MHz Linear array transducer (Aloka SSD-500, Aloka Co.Ltd., and Tokyo, Japan). All the selected animals were scanned at both blood collection time points (approximately 2 and 4 months of gestation). The animals were scanned in dorsal recumbency without shaving the ventral abdominal wall. The transducer was applied to both sides of the inguinal region's hairless area after the application of the coupling gel. An animal was confirmed pregnant by identifying a foetal heartbeat or visible movement of a foetus(es) during the scanning. The time spent on each animal to reach a diagnosis of pregnancy status was between 5 and 10 minutes.

### 7.5.3 Serological analyses and immunization context

In the study setting in northern Iraq, immunization policy against *Brucella* is based on using the Rev.1 vaccine; a stable live B. melitensis attenuated strain administered by the conjunctival route only (not subcutaneous). The immunization program with REV.1 vaccine in Dohuk is dedicated only to young aged small ruminants (for only lambs and kids aged between 3 to 6 months of age). The animals' ages ranged between 1 and 9 years (median age= 4 years) and were recorded and categorized as ≤ 4 and > 4 years. Animals were maintained within the flocks and managed as per standard practices by the owners/herders.

The selected pregnant animals were blood sampled on two occasions 60 days apart (between May 1, 2017, and August 29, 2017). All animals had previously been vaccinated with Rev. 1 against brucellosis, and the time between the first blood sample collection and the vaccination was confirmed (based on farm records and herders' feedback) to be more than 6 months; thus, to assure that vaccination would not interfere with the seroconversion status. Approximately 5 ml of blood was collected from each animal by jugular venepuncture and transported to the laboratory on ice within 12 hours of collection. Serum was extracted by

centrifugation at 3000 rpm for 10 min and stored at -20 ˚C until testing. Each serum sample was tested for *Brucella* antibodies using a Rose Bengal Test (RBT; VIRCELL, Granada, Spain), and an iELISA (NovaTec, Dietzenbach, Germany). Sera were also tested with a Latex Agglutination Test (LAT; Plasmatic, UK) and iELISA (NovaTec, Dietzenbach, Germany) for antibodies to *Toxoplasma*. The tests were performed according to the manufacturer's instructions in the laboratory of the Veterinary Hospital of Dohuk Province. An animal was classified as seropositive if both of the relevant tests were positive (RBT and iELISA for *Brucella,* and LAT and iELISA for *Toxoplasma*) (i.e., tests were interpreted in series).

### 7.5.4 Ethical approval

This research was approved by the Animal and Human Ethics Committees of Murdoch University (R 2805/15, 2016/002). All procedures were explained to the farmers, and informed verbal consent was obtained from all participants before sampling.

### 7.5.5 Statistical analyses

Data were analysed using STATA, Version 15 (Stata Corp LP, College Station, Texas, USA). The seroprevalence and 95% confidence intervals (95% CI) for *Brucella* and *Toxoplasma* were calculated at each sample point. The incidence risk (IR) and 95% confidence intervals (95% CI) were also calculated based on the proportion of animals seroconverting (i.e., animals that yielded a negative test on the initial blood sampling and a positive test on the subsequent test). The influence of seroconversion on pregnancy status was assessed by calculating relative risk and their 95% CI. The influence of time of sampling, species and age group on the seroprevalence and seroconversion (incidence risk) were assessed using Chi square tests for independence or Fisher's exact tests.

## 7.6 Results

At the first sampling time-point, 13 animals (5.4%; 95% CI: 2.9 – 9.1) were classified as seropositive to *Brucella* and 22 animals (9.2%; 95% CI: 5.8 – 13.5) seropositive to *T. gondii*. Three animals were seropositive to both *Brucella* and *Toxoplasma* (1.3%; 95% CI: 0.3 – 3.6) at this sampling. At the second sampling time-point, 37 animals (15.4%; 95% CI: 11.1 – 20.6) were seropositive to *Brucella* and 38 animals (15.8%; 95% CI: 11.5 – 21.1) seropositive to *T. gondii*. At this sampling point, five animals were seropositive to both *Brucella* and *Toxoplasma* (2.1%; 95% CI: 0.7 – 4.8). The seroprevalence to *Brucella* and *Toxoplasma* increased significantly between the two sampling points (*P*-value = 0.0003 and *P*-value = 0.027, respectively) (Table 7.1).

Twenty-four of the 227 animals that were seronegative to *Brucella* at the first sampling seroconverted (incidence risk - 10.6% per two months; 95% CI: 6.9 – 15.3). In contrast, 16 of 218 animals were seronegative to *T. gondii* at the first sampling seroconverted (IR - 7.3% per two months; 95% CI: 4.3 – 11.6). There was no significant difference between age groups (≤ 4 and > 4 years) for seroconversion to *Brucella* (12.4% and 7.3%, respectively; *P*-value = 0.23). However, more older animals (> 4 years) seroconverted to *Toxoplasma* (14.8%) than younger animals (≤ 4 years) (2.9%) (*P*-value = 0.002). There was no significant difference in the IR over the two-month period between sheep and goats for seroconversion to *Brucella* (sheep – 10.3%, 95% CI: 6.3 – 15.6; and goats – 11.9%, 95% CI: 4.0 – 25.6) and *Toxoplasma* (sheep – 8.1%, 95% CI: 4.5 – 13.2; and goats – 4.4%, 95% CI: 0.5 – 15.1) (*P*-values = 0.76, 0.53, respectively), consequently data for both species were combined.

During the study, 39 animals (16.3%, 95% CI: 11.8 – 21.5) (32 sheep, 7 goats) lost their pregnancy. For *Brucella* this comprised 26 (22 sheep, 4 goats) animals that were seronegative at both sampling points (12.8%, 95% CI: 8.5-18.2), 9 (7 sheep, 2 goats) animals that seroconverted (37.5%, 95% 18.8 - 59.4) and four animals that were seropositive at both

samplings (30.8%, 95% CI: 9.1- 61.4) (three sheep, one goat). Small ruminants that seroconverted to *Brucella* were 2.9 times (Relative Risk (RR))= 2.9, 95% CI: 1.6 – 5.5) more likely to lose their pregnancy than animals that remained seronegative (Table 7.1).

Seroconversion to *Toxoplasma* was not significantly associated with the number of small ruminants that lost their pregnancy (Table 7.1). Only 15 of the 39 animals that lost their pregnancy (38.5%, 95% CI: 23.4-55.4) had at least one seropositive result to *Toxoplasma* or *Brucella* (including one sheep seroconverted to both pathogens).

**Table 7.1. Serological response to *Brucella* and *Toxoplasma* in 240 pregnant sheep and goats at two sampling points and the effect on pregnancy status.**

| Initial sample | Second sample | Number of animals (%; 95% CI) | Number of animals lost their pregnancy (%; 95% CI) | Relative Risk (RR) for pregnancy loss (95% CI) |
|---|---|---|---|---|
| ***Brucella*** | | | | |
| Seronegative | Seronegative | 203 (84.6; 79.4 - 88.9) | 26 (12.8; 8.5 - 18.2) | 1.0 (Reference category) |
| Seronegative | Seropositive | 24 (10.0; 6.5 - 14.5) | 9 (37.5; 18.8 - 59.4) | 2.9 (1.6, 5.5) |
| Seropositive | Seropositive | 13 (5.4; 2.9 - 9.1) | 4 (30.8 9.1 - 61.4) | 2.4 (1.0, 5.9) |
| ***Toxoplasma*** | | | | |
| Seronegative | Seronegative | 202 (84.2; 78.9 - 88.5) | 36 (17.8; 12.8 - 23.8) | 1.0 (Reference category) |
| Seronegative | Seropositive | 16 (6.7; 3.9 - 10.6) | 1 (6.3; 0.2 - 30.2) | 0.4 (0.1, 2.4) |
| Seropositive | Seropositive | 22 (9.2; 5.8 - 13.5) | 2 (9.1 (1.1 - 29.2) | 0.5 (0.1, 2.0) |

## 7.7 Discussion

In this work, we adopted a prospective cohort study methodology to generate locally informative incidence risk parameters that could be fed into a subsequent model-based approach that informs evidence-based management of *Brucella* and *Toxoplasma* in northern Iraq. The study setting was very challenging due to security and ethnic conflicts running around the fieldwork area (Canada: Immigration and Refugee Board of Canada, 2016). The study had experienced some limitations; for instance, ultrasonography and blood collection were maintained at approximately 2 and 4 months of gestation, and it was not possible to extend the fieldwork period (due to security concerns) to identify further if the fetal losses of females could have occurred after the second blood collection. Poor record-keeping, or absence of farm records, was evident in many of the visited farms in Dohuk. Therefore, it was not practically possible to capture the exact time of pregnancy and the exact date of vaccination for some animals. As an alternative solution, we had to rely on the owners/herders' word of mouth, which could have introduced some recall bias. It is not uncommon to rely on expert locals' opinions while researching in resources limited countries, where data and records might not be available nor accessible as is the case in industrialized countries (Lupu & Michelitch, 2018). Lack of local resources and access to logistic support in the field also limited our capability to confirm some of the findings using the DNA detection of *Brucella* or *Toxoplasma* by PCR-based methods.

Despite limitations, this is the first study conducted in Iraq investigating the incidence of *Brucella* and *Toxoplasma* and the association between seroconversion to these pathogens and maintenance of pregnancy in small ruminants. Previous studies were undertaken in Iraq, and most international studies on brucellosis and toxoplasmosis have focused on cross-sectional studies (AL-Busultan et al., 2018; Khadi et al., 2009; Sharifi et al., 2015). Despite their well-

established epidemiological value, cross-sectional studies have potential biases, including biased sampling, an inability to confirm the occurrence of a disease outcome with the timing of infection or seroconversion, i.e., proving causation, and self-reporting by herders/owners of disease outcomes (Page & Higgins, 2016; Yu & Tse, 2012). Although cohort studies are more expensive and time-consuming, they overcome these biases (Euser et al., 2009; Ranschaert et al., 2019). The overall incidence of abortion in the current study (16.3%) was comparable to that (20%) reported by Al-Talafhah et al. (Al-Talafhah et al., 2003) in Awassi sheep in Jordan. However, the pregnancy losses in the group that seroconverted to brucellosis in the current study (37.5%) were significantly higher than that (13%) in the study of Al-Talafhah et al. (Al-Talafhah et al., 2003), although in the latter study infection was confirmed by culture.

In this study, seroconversion to brucellosis was associated with a higher incidence of pregnancy loss than animals that remained seronegative on both tests (RR= 2.9; 95% CI: 1.6 – 5.5). This is not surprising as one of the most commonly reported clinical signs of infection with *Brucella* in small ruminants is abortion/loss of pregnancy. Small ruminants generally abort due to infection with *Brucella* in the second half of their gestational period, with most aborting during the last third, after day 98 of gestation (Blasco & Molina-Flores, 2011; Elzer et al., 2002; Smith et al., 2009). Animals infected with *Brucella* are often seronegative for an extended period until the infection is activated by stress or other factors, including pregnancy (Grilló et al., 1997). It has been hypothesized that pregnant animals have an increased susceptibility to infection with *Brucella* due to physiological and immunological changes associated with the pregnancy (Sappenfield et al., 2013). The presence of erythritol in the placenta of small ruminants is responsible for the localization of *B. melitensis* to this site with subsequent accumulation of large numbers of bacteria, eventually leading to abortion of the foetus (Petersen et al., 2013).

Previous research has reported negative serology with the RBT four to six months after conjunctival vaccination with Rev .1 (Fensterbank et al., 1982), which is the route of vaccination adopted in small ruminants in Dohuk, and across Iraq in general, where the immunisation programme is directed towards only lambs and kids from 3 to 6 months of age and the route of vaccination is through the conjunctival (Alshwany, 2019). Vaccination of young aged small ruminants via conjunctival routes is traditionally adopted to overcome several problems experienced with subcutaneous vaccination in older animals (Blasco, 1997a; Fensterbank et al., 1987). One of the critical problem in the vaccination of adult aged small ruminants is the level of antibody responses which are induced by the vaccine, and these may stay for a long time and cause seropositivity of vaccinated animals in routine serological tests, and this causes interfering with detection of the infected ones (Blasco, 1997a; Olsen & Stoffregen, 2005; Stournara et al., 2007). This makes the simultaneous implementation of vaccination and test and slaughter impossible since vaccinated animals are falsely diagnosed as infected (Olsen & Stoffregen, 2005). Moreover, the vaccine may induce abortion, and also, the vaccine strain excretion through the milk and vaginal discharges may happen (Blasco, 1997a; Stear, 2005). When Rev.1 vaccine in sheep and goats is administered through the conjunctival route, the protection conferred is the same as that induced by the subcutaneous method, but the serological response evoked is significantly reduced (Alamian et al., 2015; Kardjadj & Benmahdi, 2014). In the present study, we tried our best to limit any bias that could be introduced by residual vaccination effect on seroconversion results. The key inclusion criterion for female animals to be included in the study sample was that the time between vaccination with REV.1 and the first blood sample was more than 6 months.

In the current study, as antibodies to *Brucella* were confirmed by testing with an RBT and an iELISA and the results interpreted in series, it is assumed that seropositivity resulted from natural infection rather than a serological response to prior vaccination. Aborted foetal and

placental material resulting from infection with *Brucella* can lead to significant contamination of the environment resulting in disease spread to other ruminants (Saxena et al., 2018). This large environmental burden may overcome the immunity induced by vaccination (Ponsart et al., 2019); however, in the current study location, it is also possible that the vaccine was not administered appropriately or was not maintained in a manner to confirm its efficacy due to poor veterinary services and infrastructure in Iraq (Al-Salihi, 2012). Furthermore, the unregulated movement of small ruminants within Iraq and neighboring countries and the lack of a nationwide mass vaccination campaign against brucellosis in Iraq are likely to result in the mixing of infected, naïve, and vaccinated animals within a flock. The practice of co-grazing of sheep and goats from different flocks in Dohuk Province further increases the likelihood of cross-infection between flocks (AlHamada et al., 2017). Unfortunately, in this study, it was impossible to culture the *Brucella* species associated with seropositivity in the small ruminants. Although it is possible that other *Brucella* species, such as *B. abortus*, may have been responsible for the observed seroconversion, other studies conducted in the region have reported that *B. melitensis* is the most important species affecting small ruminants (Al-Talafhah et al., 2003).

In contrast to brucellosis, although the prevalence of *Toxoplasma* increased during the study, there was no significant association between seroconversion and loss of pregnancy with only one animal losing its pregnancy and seroconverting to this protozoan. This may mean that the number of infected cats on the source farms is meagre. *T. gondii* infection is not commonly spread among the small ruminants in Dohuk Province, which most likely shows low transmission chance to humans through small ruminant consumption as a food of animal origin (Razzak et al., 2005). Notably, some researchers have reported that up to 20 million oocysts can be shed by infected cats (Dubey, 1995), and an infective dose of only 200 oocysts is required to induce abortion in sheep (Buxton et al., 1996; McColgan et al., 1988). Following

infection with *T. gondii*, small ruminants develop humoral and cell-mediated immune responses against the parasite that provides adequate protection against clinical disease in subsequent pregnancies (Dubey, 2009b). In the current study the proportion of *Toxoplasma* seronegative animals that lost their pregnancy (17.8%) was higher, although not significantly, than for those that seroconverted or had two positive test results. This would indicate that infection with *T. gondii* is not a significant cause of pregnancy loss in the flocks from which the animals were sourced and most likely in all flocks of Dohuk Province.

Only 15 of the 39 animals that lost their pregnancy (38.5%) had at least one positive result for *Toxoplasma* or *Brucella*, indicating that other causes, either non-infectious or infectious, were associated with these losses. Pregnancy loss is often a result of multifactorial aetiologies (Naziroglu et al., 1998), and other causes of loss in sheep and goats, such as infection with *Chlamydophila abortus*, Border Disease Virus, *Neospora caninum*, or *Coxiella burnetti* (Asadi et al., 2013; Dahhir et al., 2019; Ghattof & Faraj, 2015; Mikaeel et al., 2016), or non-infectious causes, such as deficiencies in vitamin A, E, selenium, zinc, copper, phosphorus or magnesium, may have been responsible for these losses (Naziroglu et al., 1998).

## 7.8 Conclusion

To our knowledge, this was the first prospective study to analyses the relationship between seroconversion to *Brucella* and *Toxoplasma* and loss of pregnancy in small ruminants in Iraq. Despite some limitations, the results of this work are valuable to calculate and/or model the financial impact of these two pathogens on the productivity of small ruminants and to investigate the economic value of implementing control measures, including mass vaccination programs, in the region.

# CHAPTER EIGHT: COST-BENEFIT ANALYSIS OF A MASS VACCINATION STRATEGY TO CONTROL BRUCELLOSIS IN SHEEP AND GOATS IN NORTHERN IRAQ

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Contribution indicates the total involvement the author has had in this project. Placing an 'X' in the remaining boxes indicates what aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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**Candidate** **Principal Supervisor**

## 8.1 In Brief

A cost-benefit analysis was conducted to evaluate the economic value of controlling brucellosis in small ruminants in Dohuk Province, Iraq. Al Hamada et al. show that the benefit-cost ratio was estimated to be 4.25 (95% CI: -2.71 to 11.22), with a net present value of US$ 10,564,828 (95% CI: -16,203,454 to 37,049,245) and an internal rate of return of (91.38% (95% CI:11.71 to 190.62%).

## 8.2 Highlights

- A cost benefit analysis was conducted to evaluate the financial benefit of controlling brucellosis in small ruminants in Dohuk Province, Iraq.
- Control of brucellosis through an annual mass vaccination of all females and entire males over the age of 3 months was found to be economically profitable.

  The benefit-cost ratio was estimated to be 4.25 (95% CI: -2.71 to 11.22), with a net present value of US$ 10,564,828 (95% CI: -16,203,454 to 37,049,245) and an internal rate of return of 91.38% (95% CI:11.71 to 190.62%).
- The prevalence of *Brucella* infection was predicted to decrease from 9.22% to below 0.73 % in small ruminants over a 20-year period with the mass vaccination program.

**Abstract**

Brucellosis is a major economic and production-limiting disease for livestock owners and the community in Iraq. A cost-benefit analysis was conducted to evaluate the impact of an expanded annual mass vaccination programme of sheep and goats that involves all female animals and entire males over the age of 3 months with Rev. 1 vaccine. The proposed expanded vaccination programme was compared to the current annual vaccination program, which involved only vaccinating female sheep and goats between the ages of 3 and 6 months of age with Rev. 1. The cost-benefit analysis model was developed utilizing data collected in Dohuk Province, northern Iraq. The seroprevalence in small ruminants (using Rose Bengal test and ELISA in series) was predicted to decrease from 9.22% to 0.73% after 20 years of implementing the proposed annual mass vaccination program. The net present value of the mass vaccination program was estimated to be US$ 10,564,828 (95% Confidence Interval (CI): -16,203,454 to 37,049,245), and the benefit-cost ratio was estimated to be 4.25 (95% CI: -2.71 to 11.22), and the internal rate of return was 91.38% (95% CI:11.71 to 190.62%). The proposed vaccination strategy was predicted to decrease the overall financial loss caused by brucellosis from 1.75 to 0.55 US$ per adult female animal. The results of this economic analysis highlight the benefit of implementing an annual mass vaccination program of small ruminants with Rev. 1 vaccine to reduce the prevalence of brucellosis in northern Iraq.

## 8.3 Introduction

Brucellosis is a common zoonotic disease in many countries resulting in production losses in livestock and febrile disease in humans (Beauvais et al., 2016; McDermott et al., 2013). Control has proved elusive in many countries, particularly where *Brucella melitensis*, the more pathogenic species for humans and small ruminants (sheep and goats), dominates (Atluri et al., 2011; Samadi et al., 2010). Although *B. melitensis* has been eradicated from small ruminant flocks in most industrialised countries, it remains a significant burden on small ruminants and human health in the Mediterranean region, the Middle East, Central and Southeast Asia (including India and China), sub-Saharan Africa, and certain areas in Latin America (Rossetti et al., 2017). The economic impact of brucellosis varies depending upon the prevalence and management and husbandry systems adopted, along with the local veterinary and medical services' expertise, facilities, and capacity (McDermott et al., 2013). In small ruminants, the disease can cause significant economic loss from abortions, neonatal deaths, reduced fertility and decreased milk production (Franc et al., 2018). In addition, trade restrictions on livestock and products from infected areas further the impact of the disease on a community (McDermott et al., 2013).

Brucellosis is endemic in small ruminants in most countries of the Mediterranean Basin and the Middle East (Rossetti et al., 2017). In Iraq, incidences of brucellosis in humans of between 52.3 cases per 100,000 person-years in a rural area to 268.8 cases per 100,000 person-years in a semi-rural area in the Basra region (south of Iraq) (Yacoub et al., 2006), with infection primarily arising through the handling of infected animals or consumption of unpasteurized milk or other dairy products (Jansen et al., 2019; Khan & Zahoor, 2018). Clinical symptoms in humans are generally nonspecific, leading to the potential for under-reporting the disease (Dean et al., 2012). As human infection primarily arises from infected animals or their products, controlling the disease in livestock is critical to reducing the incidence in humans

(Seleem et al., 2010). The primary control strategy adopted in animals is vaccination, in conjunction with surveillance, quarantine, culling of infected animals, and adoption of good biosecurity, husbandry, and management systems (Alshwany, 2019).

Eradication of brucellosis in small ruminants remains challenging in many developing countries (Franc et al., 2018). In many developing countries, including Iraq, brucellosis is an endemic disease in small ruminants, resulting in significant impacts on livestock productivity and the livelihood of people(Alshwany, 2019). The lack of effective control programs in Iraq exacerbates the disease's impact, and there has been a call for adopting a 'One Health' approach to solve this endemic problem (Bechtol et al., 2011). In northern Iraq, small ruminants and their products are vital to the economy of the Kurdistan Region of Iraq (KRI) and improve the quality of life for the local community (Alshwany, 2019). Of the many endemic diseases that affect sheep and goats in Dohuk Province, located in the KRI, *B. melitensis* continues to pose a threat to livestock productivity, as well as to public health (Bechtol et al., 2011). With little change in the prevalence of brucellosis in KRI, the current vaccination programme (vaccinating young animals, between three and six months of age, only), brucellosis is considered at endemic equilibrium, which calls for a change in the vaccination strategy. Where brucellosis is endemic, with a high prevalence and flocks are managed extensively, such as northern Iraq, mass vaccination is considered the best method, and frequently the only reasonable strategy, to apply in such settings (Alshwany, 2019). Hence, the current study aimed to evaluate the economic value of undertaking an expanded annual mass vaccination programme targeting all female and entire male sheep and goats older than three months with the Rev. 1 live vaccine. This study compares the alternative vaccination programme against the current programme (*status quo*) vaccinating young animals only.

## 8.4 Materials and methods

### 8.4.1 Study scope and context

In this study, a cost-benefit analysis was undertaken to compare an annual mass vaccination control programme involving vaccination of all female and entire male sheep and goats older than three months of age over a 20-year period. The time frame (20 years) simulates a long-term vaccination strategy to decrease the prevalence of brucellosis in the study setting to a level (~5%) where a test-and-slaughter control programme could be applied (Zundel et al., 1992). The study setting is Dohuk Province, located in the KRI of northern Iraq and borders Syria and Turkey. The province is populated by approximately 1.2 million people and contains about 1 million sheep and goats (Zangana et al., 2013).

## 8.5 Economic Model

### 8.5.1 Data sources and model input parameters

Inputs for the economic model (Table 8.1) were sourced from: (i) a prospective cohort study conducted in Dohuk Province, where a sample of pregnant sheep flocks was followed over time to evaluate brucellosis incidence, seroconversion, and outcome of pregnancy (AlHamada et al., 2021); (ii) a cross-sectional study conducted in Dohuk, which involved a serosurvey of sheep and goats from 72 farms (AlHamada et al., 2017); and (iii) a questionnaire conducted on the same farms to gather information on production and prices. Where no data was available in the study area, input parameters were extracted from published literature. A total of 242,405 sheep and goats were recorded by the annual report from Dohuk Veterinary Hospital in 2015 to be owned by the surveyed farmers in that study (AlHamada et al., 2017), of which 70.7% (171,336) were adult females (over one year of age), 3.3% (8,064) were adult males older than one year, and 26% (63,005) were lambs or kids less than one year of age.

The cost-benefit model in this study was developed in Microsoft Excel 2010 with the add-on package @Risk (version 7.5, Palisade Corporation, New York, U.S.A.). A Monte-Carlo simulation with Latin-Hypercube sampling was carried out to simulate the distribution depicting some of the model variables (Table 8.1) and to account for variability and uncertainty associated with such variables. The PERT distribution was used with data from the literature to specify the minimum, mode (most likely), and maximum values. The model was run for 10,000 iterations. The model inputs and parameters are summarised in Table 8.1. A discount rate of 6.0% was applied as the reported discount rate used by the central bank of Iraq (KRG Ministry of Planning, 2015) and a sensitivity analysis using 5% and 10% was conducted.

The average price of a lamb or kid was estimated at US$50 (data from questionnaire gathered at the study site). The cost of the vaccine was included at US$ 0.10 per head in this study (personal communication of Dohuk Veterinary Hospital, Dohuk). The true prevalence was calculated from the apparent prevalence (AP) of brucellosis in female sheep and goats using RBT and ELISA in series (AlHamada et al., 2017), together with published values about test characteristics (Sensitivity (Se) and specificity (Sp)) (Table 8.1) (EFSA, 2006; Nielsen et al., 2004). The attributable risk of pregnancy loss in new (incidence) cases and in existing (prevalence) cases in sheep and goats, was 25% and 18%, respectively (AlHamada et al., 2021).

### 8.5.2 Model assumptions and simplifications

Several simplifying assumptions are made here to render the modeling and analysis more tractable while retaining adequate accuracy to ensure meaningful results:

Implementation cost: It was assumed that 24 vaccinator teams would be required, with each team comprising one veterinarian, two veterinary nurses, and one driver. These teams would work for the Veterinary Medical Centres in the districts and sub-districts for 30 days each year

to ensure 100% vaccination coverage would be achieved (personal experience, as recommended by the Director of Dohuk Veterinary Hospital). It was assumed that the teams could vaccinate between 1,000 and 3,000 small ruminants a day (average 1,400). The direct costs for the vaccination program included the salaries of the vaccination team members, transport costs of the vaccine, transportation of the team members to the vaccination sites (flocks), consumables, and materials to keep the vaccines chilled (cold chain). Based on local setting (informed by the first author in this study), US$20 per day was estimated for general consumables, meetings, and transport (including ice for packaging vaccines, fuel, syringes, personal protective equipment and waste disposal, expenses for sub-district meetings and administration) per team. Thus, total implementation cost (based on the above description) = US$ 20 × 30 (days - period of vaccination programme) × 24 (numbers of teams) = US$ 14,400.

Budget for salaries: Was allocated as US$ 3,200 salary per team per month. Thus, total budgeted salaries = 24 (numbers of teams) × 3,200 = US$ 76,800

Budget for vaccine allocation: It was estimated that the *Brucella* vaccine would cost US$ 0.10 per dose, and the total number of small ruminants requiring vaccination was 1,000,000 (According to the most recent annual report of Dohuk Veterinary Hospital). Thus, the total estimated budget for vaccine cost = US$ 100,000.

The total costs of the mass vaccination control program per year of the program was estimated at US$ 191,200 = US$ 14,400 (implementation) + US$ 76,800 (salaries) + US$ 100,000 (vaccine allocation).

### 8.5.3 Productivity and reproduction impacts

The Awassi is the leading sheep breed in Iraq (Hailat, 2005), and data on their fertility and milk production were sourced from several existing reports (Table 8.1). As there is a lack of available data regarding the productivity of local goats in Iraq, we used the same values for sheep and goats (Table 8.1). The economic impact of the disease in sheep and goats was

assessed based on its influence only on milk production (per liter) and abortions, as these are considered to be among the most relevant impacts arising from the disease (Franc et al., 2018). This study assessed the impact of brucellosis on reproduction and productivity according to the formulas listed below (Seleem et al., 2010). Cases of mastitis, hygromas, orchitis, and epididymitis were not factored into this analysis as little data were available regarding these features of the disease in sheep or goats in Iraq, and given the fact that the significant impact of the disease is attributed to abortion, infertility and reduced milk yield (Seleem et al., 2010).

Production losses = **A** + **M**

Where: **A:** is the cost of an abortion [A = (Number of prevalent infected females * attributable risk (for prevalent cases) * Fertility rate * Average price of one lamb or kid (US$)) + (Number of newly infected females * attributable risk (for new cases) * Fertility rate * Average price of one lamb or kid (US$))]. **M:** is the cost of reduced milk production [M = (Number of prevalent infected females * attributable risk (in existing (prevalence) cases) * Fertility rate * Milk production per adult female per year * Average price of milk per liter (US$)) + (Number of newly infected females * attributable risk (for new cases) * Fertility rate * Milk production per adult female per year * Average price of milk per liter (US$))].

Annual loss per seropositive animal = **T** ÷ **I**

Where: **T**: is the total loss due to brucellosis in sheep and goats in Dohuk Province. **I**: is the number of seropositive animals (number of infected animals).

### 8.5.4 Strategy and control of the disease

Cost-benefit analysis is a method that is commonly used to determine whether the benefit from a control programme exceeds the costs of conducting it (Marsh, 1999). The Net Present Value (NPV), Benefit-Cost Ratio (BCR), and Internal Rate Return (I.R.R) were calculated (Marsh, 1999). The median values and their 95% confidence intervals were calculated for NPV, BCR, and I.R.R using@ Risk software (version 7.5, Palisade Corporation, New York, U.S.A.).

This study assumed that 60% vaccination coverage of all female and entire male sheep and goats older than three months was achieved using a standard dose of Rev. 1 administered in the conjunctiva [15]. It was assumed that the number of female sheep and goats kept each year for replacement purposes was equal to the number of female sheep and goats that died or were culled each year, i.e., the overall population size was stable. It was also assumed that brucellosis in Dohuk Province was at an endemic equilibrium at the start of the study where the number of newly infected animals produced by one infected animal (sheep or goat) during its infectious period (effective reproduction number, $R_e$) was 1. The calculation of the number of new cases of brucellosis in each year was derived from the formula as follows (Hegazy et al., 2009):

$$R_e = R_0 \times s$$

Where: $\mathbf{R_e}$: is the effective reproduction number, which is the average number of secondary cases that result from an infectious individual in a particular population. $\mathbf{R_0}$: is the basic reproduction ratio (the mean number of secondary cases arising from one infectious individual in an entirely susceptible population). s: is the proportion of susceptible animals in the total population.

The economic value of the mass vaccination programme were calculated as the financial savings arising from the reduced number of infected female sheep and goats (increased milk

production and reduced abortions) and the extra costs associated with a mass vaccination program compared with the current vaccination programme (vaccination of young sheep and goats only).

### 8.5.5 Sensitivity analysis

A sensitivity analysis was conducted (in @Risk), using the Spearman rank correlation coefficient (*r*), to determine the impact of variability and uncertainty for the input parameters on the predicted model output. A sensitivity analysis was carried out to assess the effect of the protection rate from vaccination on the NPV, BCR, and I.R.R from the mass vaccination strategy. The protection rate was based on both the efficacy of the vaccine and the vaccination coverage achieved.

**Table 8.1.** Input parameters included in the cost-benefit analysis model comparing two different vaccination strategies to control brucellosis in sheep and goats in Dohuk, northern Iraq.

| Parameters | Description | Value | Reference/source |
|---|---|---|---|
| Annual milk production | litre per adult female (ewe/doe) per year | Pert distribution (Min=60; Mode=109; Max= 134) | Pacinovski et al. (2016) |
| Fertility rate | Per year, for adult female % | Pert distribution (Min=0.76; Mode=0.85; Max= 0.95) | Galal et al. (2008) |
| Average price of milk* | Per kg | Pert distribution (Min=0.6317; Mode=0.8421; Max= 1.0528) | Questionnaire data |
| Average price of a lamb or kid | Per a lamb or kid | Pert distribution (Min=25; Mode=50; Max= 75) | Questionnaire data |
| Percentage of adult females | Out of the total population | 70.7% | Questionnaire data |
| Percentage of lambs/kids | Out of the total population | 26% | Questionnaire data |
| Attributable risk | For new (incidence) cases | Pert distribution (Min=0.05; Mode= 0.25; Max= 0.45) | AlHamada et al. (2021) |
| Attributable risk | For existing (prevalence) cases | Pert distribution (Min=0; Mode=0.18; Max= 0.34) | AlHamada et al. (2021) |
| TP | True prevalence (TP) = $\frac{(AP+Sp-1)}{Se+Sp-1}$ | 9.22% | Calculated |
| AP | Apparent prevalence (AP) | 8.33% | AlHamada et al. (2017) |
| Se | Sensitivity (series testing) | 90.22% | EFSA (2006) and Nielsen et al. (2004) |

| Parameters | Description | Value | Reference/source |
|---|---|---|---|
| Sp | Specificity (series testing) | 99.99% | EFSA (2006) and Nielsen et al. (2004) |
| s | Proportion of susceptible animals [t=0] | 0.46 | Calculation (s = 1 – TP – Pr) |
| Pr | Proportion of protected animals [t=0] | 0.45 | Calculation (Pr = Vc × Ve) |
| Vc | Vaccination coverage | 60% | Directorate of Dohuk Veterinary Hospital |
| Ve | Vaccine efficacy | 75% | (Benkirane et al., 2014) |
| N | Number of adult female sheep and goats in Dohuk Province | 706,800 | Total number of animals × percentage of adult females |
| P | Number of protected females in Dohuk Province (year 0) | 299,115 | Calculated (P = (N × Pr)) |
| Re | Effective reproduction number | 1 | assumption of endemic equilibrium |
| R0 | Basic reproduction number $R_0 = Re \div s$ | 2.18 | Hegazy et al. (2009) |
| D | Duration of overall managed breeding before culling | Five years | Calculation (1 ÷ u) |
| U | Replacement sheep per year (Cull rate) | 20% | Director of the vet. Services in Dohuk city |
| beta | Transmission coefficient | $6.18 \times 10^{-7}$ | Calculated beta = $R_e \div (N \times D \times s)$ |

| Parameters | Description | Value | Reference/source |
|---|---|---|---|
| Rev. 1 vaccine | Price per dose | US$0.10 | Directorate of Dohuk Veterinary in Dohuk city |

* Prices expressed in US$ ((United States of America dollar, with US$1 = 1,190 Iraqi Dinar) (https://www.mataf.net/)

## 8.6 Results

The prevalence of brucellosis in sheep and goats in Dohuk was predicted to decline over the 20-year period from 9.22% to 0.73% (Figure 8.1a). The analysis predicted that, after adopting a mass vaccination control program for 20 years, the annual losses from pregnancy loss and decreased milk production had decreased to US$115,299 (US$0.16 per adult female). The total economic impact of brucellosis per year in sheep and goats at the start of the control program in Dohuk Province (2015) was estimated to be US$1,524,151 (US$2.16 per adult female).

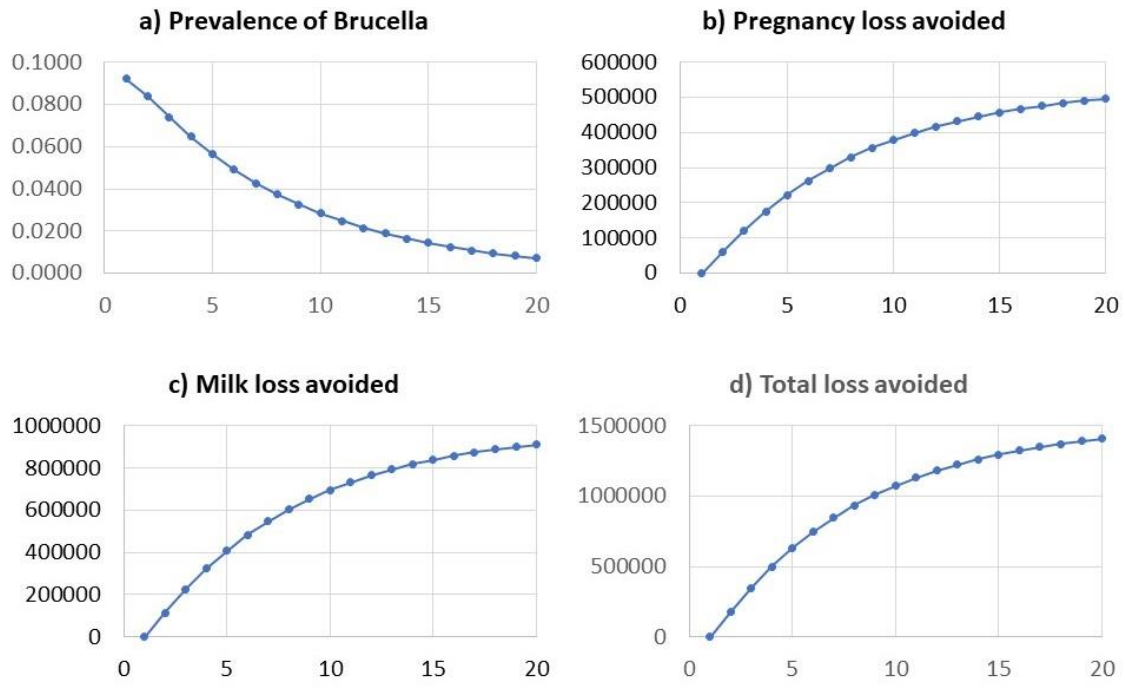


**Figure 8.1. Estimated decline in the prevalence of *Brucella* (a) when implementing a mass vaccination programme over twenty years (horizontal axis). The figure depicts the avoided pregnancy loss (b), avoided milk loss (c), and the predicted total loss avoided (c) over twenty years of application of the proposed mass vaccination.**

The results of the cost-benefit analysis are presented in Tables 8.2 and 8.3. The median total benefit in present day dollars was estimated at US$ 13,813,524 (95% CI: - 12,964,774 – 40,290,145). For the mass vaccination control programme compared to the existing control programme using 4%

interest rate, the median NPV was US$ 10,564,828 (95% CI: -16,203,454 – 37,049,245), and the median BCR was 4.255 (95% CI: -2.71 – 11.22). The median of IRR was 91.38% (95% CI: 11.7-190.6%). The median total costs of the mass vaccination program over the 20-year period was estimated at US$ $ 3,241,685 (95% CI: 2,971,912 – 3,515,547) (Table 8.3).

**Table 8.2. Summary of the results of the benefit-cost analysis comparing an expanded vaccination control programme compared with a continuation of the current control programme.**

| Years | Future Benefits | Future Costs | Future Value | PV of Benefits | PV of Costs | NPV |
|---|---|---|---|---|---|---|
| 1 | $0 | $191,200 | -$191,200 | $0 | $179,728 | -$179,728 |
| 2 | $176,770 | $191,200 | -$14,430 | $156,194 | $168,944 | -$12,750 |
| 3 | $347,854 | $191,200 | $156,654 | $288,922 | $158,808 | $130,114 |
| 4 | $499,649 | $191,200 | $308,449 | $390,101 | $149,279 | $240,821 |
| 5 | $632,108 | $191,200 | $440,908 | $463,907 | $140,322 | $323,584 |
| 6 | $747,260 | $191,200 | $556,060 | $515,512 | $131,903 | $383,609 |
| 7 | $847,307 | $191,200 | $656,107 | $549,460 | $123,989 | $425,471 |
| 8 | $934,257 | $191,200 | $743,057 | $569,494 | $116,550 | $452,944 |
| 9 | $1,009,865 | $191,200 | $818,665 | $578,647 | $109,557 | $469,091 |
| 10 | $1,075,649 | $191,200 | $884,449 | $579,361 | $102,983 | $476,377 |
| 11 | $1,132,914 | $191,200 | $941,714 | $573,592 | $96,804 | $476,788 |
| 12 | $1,182,789 | $191,200 | $991,589 | $562,913 | $90,996 | $471,917 |
| 13 | $1,226,245 | $191,200 | $1,035,045 | $548,579 | $85,536 | $463,043 |
| 14 | $1,264,123 | $191,200 | $1,072,923 | $531,593 | $80,404 | $451,189 |
| 15 | $1,297,150 | $191,200 | $1,105,950 | $512,753 | $75,580 | $437,173 |
| 16 | $1,325,955 | $191,200 | $1,134,755 | $492,691 | $71,045 | $421,646 |
| 17 | $1,351,084 | $191,200 | $1,159,884 | $471,906 | $66,782 | $405,124 |
| 18 | $1,373,011 | $191,200 | $1,181,811 | $450,791 | $62,775 | $388,016 |
| 19 | $1,392,148 | $191,200 | $1,200,948 | $429,650 | $59,009 | $370,641 |
| 20 | $1,408,853 | $191,200 | $1,217,653 | $408,717 | $55,468 | $353,249 |
| Total | $19,224,992 | $3,824,000 | $15,400,992 | $9,074,783 | $2,126,463 | $6,948,320 |

NPV: net present value; PV: present value

**Table 8.3. Summary of a cost-benefit analysis of a mass vaccination program applied for twenty years to control brucellosis in small ruminants in Dohuk Province, Iraq.**

| | Benefits (median and 95% CI) |
|---|---|
| **PV Benefits** | US$ 13,813,524 (95% CI: - 12,964,774 – 40,290,145) |
| **PV Costs** | US$ 3,241,685 (95% CI: 2,971,912 – 3,515,547) |
| **NPV** | US$ 10,564,828 (95% CI: -16,203,454 – 37,049,245) |
| **BCR** | 4.25 (95% CI: -2.71 – 11.22) |
| **IRR** | 91.38% (95% CI: 11.7– 190.6%) |

PV: present value; NPV: net present value; BCR: benefit-cost ratio; IRR: internal rate of return.

In the sensitivity analysis (Figure 8.2), the attributable risk of pregnancy loss (prevalent cases) (regression coefficient = 0.75) had the most considerable positive effect on the outcome, followed by the average price of one lamb or kid (0.57), and the attributable risk of incidence loss of pregnancy (regression coefficient = 0.27). All other factors had minimal impact on the control program (low coefficients) (Figure 8.2).

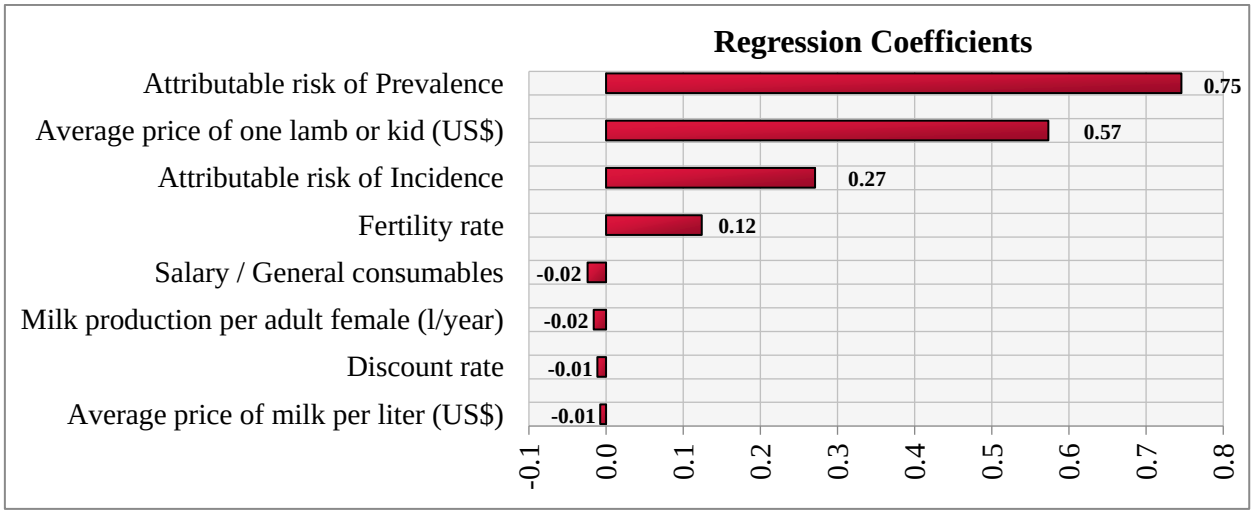

**Figure 8.2. Regression coefficients of the sensitivity analysis for Net Present Value (NPV) of the mass vaccination control programme.**

## 8.7 Discussion

The current study was oriented on analysis of the economic losses occurring due to brucellosis in small ruminants in Iraq. This analysis focused on impacts arising from loss of pregnancy and reduced milk yield, as these have been reported to be among the most significant outcomes of infection (Alves et al., 2015; Seleem et al., 2010). In this study, we populated the cost-benefit analysis model based on integrated data from our previous research on brucellosis in small ruminants in the study area; key model parameters we informed based on a recent cohort study (AlHamada et al., 2021) and a former cross-sectional serological survey done by our group in Dohuk (AlHamada et al., 2017).

This study found that the proposed mass vaccination programme had an NPV greater than zero and a BCR greater than one, indicating that the programme was economically beneficial. It is likely that the benefits in this study and the corresponding BCR and NPV would have been higher if data were included in the model on all potential losses in animals along with the impact of the disease in humans. The present cost-benefit analysis predicted that the losses decreased from US$ 1.75 to US$0.55 per adult female over the 20-year period, and the seroprevalence decreased from 9.22% to 0.73%. Compared to our study, another study in Kurdistan region in Iraq found that the loss in animals was decreased over ten years from US$2.56 to US$0.76 per adult female based on seroprevalence 4.9% (Alshwany, 2019). In addition, the IRR in our study was estimated to be 91.38%, which was slightly higher than another recent study conducted in the Kurdistan region of Iraq, where an IRR of 67.9% was reported (Alshwany, 2019). Although the losses resulting from infection with *B. melitensis* have not been investigated widely in developing countries, some studies have been undertaken in middle-income countries. In Malaysia, based on a reported seroprevalence of 2.9% using the

complement fixation test, it was estimated that annually the economic impact of caprine brucellosis was US$ 50,391.13 in 15 herds containing a total of 12,499 goats. This resulted in an average loss of US$ 162.55 for the 310 infected animals (Bamaiyi et al., 2015). In another study in India, the annual economic losses per animal were estimated at US$ 21.58 and US$ 38.80 per goat (Sulima & Venkataraman, 2010).

In this study, the proposed expanded vaccination program in Dohuk Province was estimated to cost in PV US$9.1 million over the 20-year period. Although the infection was predicted to remain after 20 years of vaccination, the true prevalence reduced to 0.73%. Others have similarly shown that mass vaccination programs over ten years can significantly reduce the prevalence and transmission of brucellosis between animals (Roth et al., 2003). In another study, mass vaccination with the Rev. 1 vaccine over four and a half years reduced the prevalence of *B. melitensis* in Kuwait's small ruminant population from 5.8% in 1993 to 2.02% in 1997 (Scharp et al., 1999). Although vaccination does not eliminate infection, the model predicted a significant reduction in prevalence after 20 years, at which time it might be possible to evaluate adopting eradication programs, such as test and slaughter programs (Corbel, 2006). Nevertheless, implementing a test and culling programme will be challenging unless a formal compensation system is developed for the culled animals. Raising awareness of the farming community regarding biosecurity and preventative measures is critical for any disease control program and should be a vital component of any official disease control program in the north of Iraq (AlHamada et al., 2017).

The results of this and other studies indicate that mass vaccination with Rev. 1 is a crucial strategy to reduce the prevalence of brucellosis arising from infection with *B. melitensis* in small ruminants, mainly when the prevalence is initially high (Blasco & Molina-Flores, 2011; Blasco Martínez, 2010). Our scenario assumed that the disease had reached an endemic equilibrium with the current vaccination programme involving the vaccination of sheep and goats 3 to 6 months of age. Brucellosis–positive animals were still present in Dohuk Province, presumably due to a lack of collaboration by farmers in vaccination time, absence of communication of the presence of young

animals eligible for Rev. 1 vaccination. In addition, the illegal movement of animals of unknown disease status from Syria and Iran to Iraq and specifically to the KRI and the uncontrolled movement of domestic animals within the region are likely responsible for the wide dissemination of brucellosis throughout northern Iraq.

Others have similarly shown the impact of vaccination with Rev. 1 on reducing disease prevalence in sheep and goats in countries where the disease is endemic, including Mexico (Montiel et al., 2015) and India (Singh et al., 2018b). In a study in Greece, vaccination of sheep and goats with Rev. 1 vaccine administered subcutaneously was shown to decrease abortion in small ruminants and the incidence of brucellosis in humans (Minas et al., 2004). Abortion is the primary clinical outcome of brucellosis infection in sheep and goats and typically occurs in the last two months of pregnancy (Perrett et al., 2004). Not surprisingly, the attributable risk of pregnancy loss in prevalent cases had the most significant influence on the sensitivity analysis in this study. The mass vaccination scenario proposed should reduce the number of abortions, reducing potential environmental contamination (Singh et al., 2018b). Although Rev. 1 vaccine induces effective immunity against brucellosis in sheep and goats (Blasco, 1997b), it has disadvantages, including the potential to result in abortions when administered to pregnant females due to the live vaccine containing mutants with residual virulence (Saeedzadeh et al., 2013). Therefore, it is recommended that female sheep and goats are vaccinated before the breeding season commences and at milking season.

Although annual mass vaccination was predicted not to eliminate *Brucella* infection over 20 years, the program predicted a sizeable reduction in the prevalence, allowing for implementing a test and slaughter programme, similar to that used in other countries (McDermott et al., 2013). Furthermore, because infected animals and their products are the primary sources of human infections, control of brucellosis in animals is an essential step to mitigating the disease in humans (Seleem et al., 2010).

The findings of this study have to be seen in the light of some limitations. This study only focused on vaccination in small ruminants; however, brucellosis can be transmitted between small

ruminants and cattle, particularly in co-grazing situations – which could be common in some regions in Iraq (Al-Majali et al., 2009; Montiel, 2014), and this is an area open for investigation in the context of Iraq in future research. Some assumptions and extrapolations of other studies were required due to a lack of local data. Future studies are hoped to be undertaken to collect local data to ensure the model is as accurate as possible for the situation in Dohuk and Iraq. Inclusion in the economic modelling of other costs and benefits associated with milk production and fertility rate due to brucellosis with brucellosis control will also help generate a more accurate model.

It is recommended that an expanded vaccination program is implemented in Iraq in conjunction with a program to increase the awareness of the local farmers about the disease, its impact, and how it is spread through implementing educational campaigns by the regional Department of Veterinary Services. It is critical to focus on vaccination and concurrently build a robust disease surveillance system involving monitoring all abortion cases by the provincial veterinary system to prevent the spread of disease or reinfection. In addition, developing a solid quarantine system and control on the illegal movement of animals from neighbouring countries, especially Syria and Iran, are essential for the ongoing control of brucellosis (and other diseases) in Dohuk Province. The purpose of controlling brucellosis in small ruminants is essential to reduce the economic impact of the disease in sheep and goats and lessen the infection burden in humans (Blasco & Molina-Flores, 2011).

## Conclusions

Adoption of a mass vaccination programme involving vaccination of all females and entire males older than three months of age with Rev. 1 in Dohuk Province, Iraq, was predicted to lead to a reduction in the prevalence of brucellosis from 9.22% to 0.73% after 20 years of implementing the program. The program was shown to be economically advantageous, with a median NPV of U$ 10,564,828 and a median BCR of 4.25. It is concluded that the Iraqi government should plan and implement an evidence-based vaccination program in Dohuk, and other areas of Iraq, for the control

and future eradication of brucellosis in sheep and goats based on initially reducing the prevalence through this vaccination campaign.

# CHAPTER NINE: GENERAL DISCUSSION

## 9.1 Introduction

Historically, there has been a lack of support from the Kurdistan Regional Government in Iraq for the control of diseases of sheep and goats in Dohuk Province, and this, combined with a lack of information about brucellosis and toxoplasmosis in sheep and goats in the province, has resulted insufficient progress in the control of these two diseases. In addition, veterinary staff in Iraq are suffering from a lack of infrastructure, material and funds, inadequate security, lack/unreliability of electrical supply, problematic logistics and transport (Alshwany, 2019). The war in Iraq causes significant challenges for the surveillance and control of communicable disease (Valenciano et al., 2003). Until now, lack of security continues to be a barrier for effective public health surveillance and response in Iraq (Valenciano et al., 2003). As Maxwell and Bill (2008) mentioned, prior to the research reported in this thesis, most surveys conducted on brucellosis and toxoplasmosis in small ruminants in Iraq were not undertaken by using sound scientific epidemiological methods, and therefore their results are questionable.

The studies outlined in this thesis were designed to improve our understanding about the epidemiology of brucellosis and toxoplasmosis in sheep and goats in Dohuk Province to advocate for resources to develop and implement a scientifically sound control programme. This initially involved undertaking a cross-sectional seroprevalence study (Chapter 5 and 6), followed by research to assess putative risk factors for both the diseases. Further work was undertaken to determine the incidence of seroconversion to *Brucella* and *Toxoplasma*; performing a cohort study to examine the relationship between seroconversion to these pathogens and maintenance of pregnancy in sheep and goats in Dohuk Province (Chapter 7).

The epidemiology and economic impact of brucellosis is context specific and varies according to the geographical location, livestock management systems utilised, and the socio-political environment. In Dohuk Province little was known about the distribution and impact of brucellosis in small ruminants prior to this study. Therefore, using results gathered in the serological studies, there was an evaluation of the economic impact of brucellosis in small ruminants and the potential economic value of implementing a mass vaccination control programme (Chapter 8).

## 9.2 Seropositivity and risk factors of *Brucella melitensis*

Brucellosis is considered an important public health risk in most Middle East countries, due to similar environmental conditions, livestock management and husbandry systems, and cultural practices in countries within the region (Gwida et al., 2010; Refai, 2002). The burden of brucellosis in Kurdistan Province of Iran was estimated to be 2.93 disability-adjusted life years (DALYs) per 1000 population in 2014 and 248 per 1000 in 2015 (Piroozi et al., 2019), and 0.27 DALYs per 1000 population in the Kurdistan Region of northern Iraq (Alshwany, 2019). The majority of patients suffering from brucellosis need medical attention as the disease is often painful and debilitating (Nicoletti, 2001)..

Brucellosis is also responsible for significant economic losses to the small ruminants industry because of abortion, reduced milk production, premature birth, and decreased reproduction rate of affected animals (Ganter, 2015). Therefore, it is critical to human and animal health, and to the economy, to effectively manage the disease.

In this study (Chapter 5), the seroprevalence of *Brucella* in one region of Iraq was investigated in small ruminants, along with a survey to determine risk factors that could lead to recommendations for effective management of disease. Two tests (RBT and iELISA) were utilised to confirm infection

with *Brucella* spp. with the results interpreted in parallel to improve the sensitivity of detection (Sanogo et al., 2012).

In this study, older animals were more likely to be seropositive than younger animals (OR =1.7, 95%CI: 1.4 -2.2). A higher seroprevalence has been found in animals originating from the three districts of Aqrah, Zakho and Simele than in Dohuk, Amadiya and Shekhan, the highest seropositivity of the animals has been found in the Aqrah district (54.1% of the animals positive). This may be largely due to the main road passing through the district used for the illegal movement of animals.

Herd size has been also found to be associated with brucellosis seropositivity; and larger herd might be suspected to be related with intensive management practices, which are typically more difficult to control and allow for closer between animals and their environment. It increases the potential exposure to infectious excretions, which favour the easy spread of the disease among animals (Coelho et al., 2007; Coelho et al., 2008; Solorio-Rivera et al., 2007). The odds of *Brucella* seropositivity in small ruminants was 2.2 higher (95% CI: 1.2; 4.3) for animals originating from farms with a history of goat abortion in the preceding 12 months. The survival of *Brucella* to subsist outside mammalian hosts is relatively persistent as compared to other non-sporulating pathogenic bacteria in similar circumstances (Garin-Bastuji, 1993). Therefore, the cohort study aims to investigate more about different aspects of brucellosis. The odds of *Brucella* seropositivity in flocks, where sheep and goats grazed together was 2.0 times higher (95% CI: 1.08; 3.9) as compared to flocks, where sheep and goats grazed separately. Considering the contagious nature of *Brucella* species, sharing gazing and drinking water between sheep and goats facilitate the transmission of the disease (Reviriego et al., 2000). Aune et al. (2012) found that, *Brucella* could persist on soil or vegetation and foetal tissue for 21-81 days, depending on the month, temperature, and exposure to sunlight. Environmental contamination caused by an aborted foetus, birth tissue, faeces, or vaginal fluids can persist on soil, and vegetation might transmit the bacteria to female sheep and goats graze, these pastures during the pregnancy period, and causes seroconvert of serological results.

The amount of money spent on animal health, was shown to reduce the seroprevalence to brucellosis (Chapter 5), and this agreed with results of other researchers and they found that, lack of money to pay for livestock health services plays a significant effect on the increase of the brucellosis infection (Mwinyi, 2017).

### 9.3 Seropositivity and risk factors of *Toxoplasma gondii*

Toxoplasmosis is also considered a significant health problem in sheep and goats resulting in economic losses for producers (Hill & Dubey, 2013). Improving the awareness of farmers about the ways of transmission and prevention of infection with toxoplasmosis should be increased via education. Further study should be conducted to explore the effects of disease on food animal production (Henneb et al., 2019; Tilahun et al., 2018).

In general, *T. gondii* infection is common and widespread in small ruminants of the study area. The prevalence of infection in small ruminants indicates the potential transmission to humans, when people consume products ( undercooked meat) from these animals (Gazzonis et al., 2015).

In this study (Chapter 6), the present of cats on the farm increases the seropositivity of *T. gondii* in sheep and goats flocks (OR= 6.3, 95%CI 1.6 –24.6), and it is in agreement with another study (Zhang et al., 2016a). The consumption of feed and pasture contaminated with oocysts of affected cats is considered the principal method of infection for sheep and goats (Dubey, 1998b); however, congenital infection is also possible (Chiebao et al., 2019). In contrast to other studies, they found that, the presence of cats is not a risk factor for increasing seropositivity of *T. gondii* in infection in small ruminants (Dahmane et al., 2020; Tzanidakis et al., 2012).

In the current study, goats had higher seropositivity than sheep, even though they were sampled in the same region and often in the same flock. These findings agree with another study (Anastasia et al., 2013) that hypothesised that, goats were more susceptible to infection with *T. gondii* than sheep due to differences in their immune systems. However, it is possible that, these differences are not

indicative of true species differences but may be associated with management practices, implemented for the two species. However, the co-grazing of sheep and goats and the similar management in mixed flocks should result in similar seroprevalences. Furthermore, given the browsing nature of goats, it could be expected that, goats would have a lower seroprevalence to *Toxoplasma* than sheep (Rahman et al., 2014). These findings further support the belief that, there are differences in susceptibility to *Toxoplasma* between sheep and goats, potentially associated with different immunological responses to infection. In another study, they found the highest *T. gondii* seroprevalence in sheep than goats (Gazzonis et al., 2015).

The results presented in Chapter 6 support the belief that, *T. gondii* is endemic in Dohuk Province, with infection widely distributed in sheep and goats.

## 9.4 Seroconversion to *Brucella* and *Toxoplasma gondii* during pregnancy and its association with loss of pregnancy in small ruminants in Dohuk Province, Iraq

Reproductive failure has a negative impact on animal production, health and welfare and ultimately rural economies (Gebremedhin et al., 2013). Brucellosis and toxoplasmosis are reported to have detrimental effects on pregnancy outcomes in the Middle East as well as being potential zoonotic diseases (Ortega-Mora, 2007; Smith & Sherman, 2009). Most studies investigating the diseases have relied upon cross-sectional studies. The results of such studies can be questioned, especially with respect to causation and temporal events, because the cross- sectional studies are observational studies that analysis data from population at a single point in time. Prior to the study outlined in Chapter 7, the influence of seroconversion on the reproductive outcome of sheep and goats had not been investigated using prospective studies in Iraq. Although prospective or cohort studies are time consuming and costly, they offer the significant advantage that more confidence is obtained on the validity of disease outcome (Grimes & Schulz, 2002).

Small ruminants that seroconverted to *Brucella* during the two-month prospective study were 2.9 times more likely to lose their pregnancy than animals that did not seroconvert. Mailybayeva et al. (2017) revealed that, the presence of erythritol in the placenta has been identified as an important growth factor for *Brucella* (Petersen et al., 2013); therefore, it is not surprising that, pregnant sheep and goats which are carriers of *Brucella* would seroconvert during their pregnancy, as *Brucella* considered as facultative intracellular parasites of sheep and goats, typically infecting lymphoid as well as reproductive organs (Rittig et al., 2001).

In the current study, only 37.5 % of abortions were associated with seroconversion to *Brucella* or *Toxoplasma* indicating that potentially other infectious or non-infectious factors are associated with abortions in small ruminants in Iraq. Further studies need to be undertaken needed to identify these causes in Dohuk Province and should involve a more thorough sampling protocol of aborted material and from ewes and does that abort. To diagnose the aetiology of abortion events/storms in sheep and goats, samples are required from multiple cases and multiple sites, including the aborted foetuses, placentas, and sera from the dam, so the causes of abortion can be diagnosed (Rowe, 2019).

The effect of toxoplasmosis in pregnancy sheep and goats with seroconversion and loss of pregnancy are low compared to brucellosis. Following infection, small ruminants develop immunity against toxoplasmosis which plays an important role to protect them against toxoplasmosis in subsequent pregnancy (Innes et al., 2009a).

### 9.5 Economic assessment

Brucellosis caused by *Brucella* spp. is an infectious zoonotic disease with reportedly significant economic impacts on both the livestock industry and public health (Rossetti et al., 2017). Although the economic effect of brucellosis in small ruminants has been evaluated in other countries (Montiel et al., 2015; Singh et al., 2015), a lack of available data in Iraq has meant previous evaluation of economic losses from this disease has been challenging. Using data sourced from the cross-sectional and longitudinal studies conducted as part of the research reported in this thesis chapter (5,6, and 7),

and from local and regional studies, it was possible to investigate the economic impact of brucellosis through pregnancy losses, reduction of milk yield in sheep and goats in Iraq. This study found that the annual financial loss decreased because of mass vaccination at approximately US$ 1.75 to US$ 0.55 per head of population. In this study, the proposed expanded vaccination program in Dohuk Governorate was estimated to cost in PV US$9.1 million over the 20-year period . This study found that the mass vaccination programme proposed (vaccinating all females and entire males older than three months of age) was financially viable (positive NPV and BCR > 1). However, the 95% confidence intervals for the NPV included 0 and the BCR included 1, therefore increased precision on the input parameters are needed before policy recommendations can be made. Others have also reported the financial benefit of controlling brucellosis by implementing a vaccination programme in small ruminants (Alshwany, 2019; Singh et al., 2018b). Control of brucellosis in livestock also benefits humans by reducing infections (Alshwany, 2019; Singh et al., 2018a). From our study, mass vaccination was predicted to decrease the seroprevalence in small ruminants from 9.22% to 0.73% after 20 years of implementing of the proposed mass vaccination programme. This highlights the potential benefit of implementing a mass vaccination programme (with e.g. Rev. 1) for reducing the prevalence of brucellosis in Dohuk Province.

The sensitivity analysis in this study indicated that the uncertainty of the input variables (i) attributable risk of pregnancy loss in prevalent cases (SRCC = 0.75); and (ii) average price of lamb or kid (SRCC = 0.57) were considered to have a strong impact of the precision of the NPV, with the lower precision interval less than 0. In another similar study conducted in Kurdistan, Iraq Alshwany (2019), the abortion rate also had the largest effect on a cost-benefit analysis; with the average price of lambs or kids had the second-highest effect. These studies highlight that the uncertainty in the proportion of pregnancy loss attributable to brucellosis has a major impact on the outcome of the analysis. It is often stated that higher seroprevalence results in greater losses in productivity with seropositive animals having increased rates of abortions, perinatal mortality and infertility, also reduce milk yields and growth (McDermott et al., 2013). However, in Chapter 7 no association

between prevalent infection (animals that were seropositive at 2 and 4 months of pregnancy) and pregnancy loss was found. This demonstrates the need for well-designed cohort studies of pregnancy loss, with veterinary and laboratory investigation into potential nutritional, environmental and infectious causes simultaneously.

The prevalence of brucellosis in small ruminants was estimated to be 4.9% in 2015 and annual economic losses were approximately US$ 6.14 Million (Alshwany, 2019). Control of brucellosis in animals varies between countries, which results in different impact of the disease on profitability (Godfroid et al., 2011). In Nigeria, the annual economic losses caused by brucellosis in small ruminants were US $ 3.2 million (Brisibe et al., 1996); in Peninsula Malaysia the loss is estimated at US $ 2.56 million for caprine brucellosis (Bamaiyi et al., 2015). Treatment of infected animals is not attempted due to the intracellular localization of *Brucella* and its ability to adapt the environmental conditions encountered in the macrophage host cell (Köhler et al., 2002).

## 9.6 Limitations of the present study

There are several limitations to the current study. The use of values for parameters taken from other countries or from expert opinions may over or under-estimate values for Dohuk Province. These could result in an over or underestimate of the economic value of mass vaccination presented in Chapter 7. Although the questionnaires used in the current study (Chapters 4 to 6) played an essential role in identifying the risk factors of brucellosis and its economic impact, several factors may have potentially affected the results of the economic study for brucellosis. Firstly, the current government policy is to support farmers through the supply of animal feed at a discounted price; however, the amount provided is determined by the farmer's herd size. Consequently, some farmers may have overstated the number of livestock owned to gain extra benefits. Secondly, reluctant interviewees may have introduced information bias; this is a limitation of the study and results in bias whereby having an official present during the questionnaire in the study may have resulted in over or

underestimating economic losses and providing dubious associations between factors and seropositivity.

Usually, brucellosis is diagnosed by serological assays; however, no test is 100% accurate (Nielsen & Yu, 2010). In Chapter 4, two tests were utilised. For brucellosis, all samples were tested with the RBT and iELISA and the results interpreted in parallel (animals were classified as seropositive if at least one of the serological assays was positive). The iELISA depends on the detecting of antibodies in the serum of infected or vaccinated animals (Hobbs, 1985). For the RBT, the agglutination intensity is affected by many factors, including the amount of antigen, the temperature at which the test is run, the duration between adding the antigen and reading (interpreting the result), the experience and visual acuity of the test interpreter and potential cross-reactions with LPS of other bacteria (Cho et al., 2010). In the current study, isolating (culturing) and identifying the infecting *Brucella* species was not undertaken because of time and funding constraints.

For toxoplasmosis (Chapter 5), sera was tested for anti-*T.gondii* IgG antibodies by using two commercial serological tests (iELISA and LAT). The latex test detects IgG1, and IgM produced during acute cases of toxoplasmosis while the iELISA detects IgG, which is the dominant immunoglobulin in chronic cases therefore we used combination of serologic tests (LAT and iELISA) to establish whether an individual has been most likely chronically infected or has been recently infected. To better understand the epidemiology of toxoplasmosis in sheep and goats, using more accurate tests such as PCR and better protect small ruminants from toxoplasmosis, it is necessary to validate the commonly used serological tests in this species.

## 9.7 Recommendations

Control measures for a disease should be designed based on the epidemiological features of the disease of interest in the specific locality, as it is rare that one programme can be universally adopted in all the areas or countries (Robertson, 2020). The development and implementation of a programme must be appropriate for the specific country, region or area. Firstly, it is important to specify whether

the goal of the programme is the control or eradication of the disease, and this mainly depends upon the financial situation within the country, the economic impact of the disease, predicted benefits of control or eradication, and political will. Secondly, it is critical to have accurate information about the factors associated with the disease (Al-Rawahi, 2015). 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 and their willingness to cooperate in a control programme. Once the epidemiological information of the disease is known, the country may adopt a specific strategy or a combination of strategies to control the disease (Chen et al., 2016; Seleem et al., 2010; Senein & Abdelgadir, 2012).

Many *Brucella*-free countries have policies in case there is the introduction of disease by implementing strict border control, quarantine and slaughtering policies against infected and in-contact susceptible animals, as well as imposing strong restrictions on the movement of animals and vehicles from and around potentially infected premises. After slaughter, the carcasses are either burnt or buried in the infected premises, and the building thoroughly washed and disinfected, movement restrictions applied and the affected farms quarantined. Test and slaughter programme could be adopted in situations, where the prevalence is less than 5% and surveillance and laboratory have excellent facilities (Zamri-Saad & Kamarudin, 2016). However, a test and slaughter programme is not suitable, when there is a high prevalence or in countries that have limited financial resources. Therefore, a confirmation process using more accurate methods such as PCR for aborted material and isolation *Brucella* by agriculture and different controlling methods has been adopted in many countries (Nielsen & Yu, 2010).

Some farms are at high risk of infection with *Brucella* spp. through adopting the practices such as co-grazing of sheep and goats where small ruminant's flocks that co-grazed were 2.0 times more likely to be positive for brucellosis than those that grazed separately (Chapter 5). Education is a key aspect of disease control (Tilahun et al., 2018). In the current situation, it is recommended that, educational material is developed and administered to producers focusing on the need for improved

biosecurity and ensuring that animals are tested for brucellosis prior to introduction into their flocks. All purchased animals should undergo a quarantine period for one month prior to mixing with the existing flock to minimise the likelihood of transmission of brucellosis (Giasuddin et al., 2018). If purchased animals are pregnant, they should be isolated from the flock until they have given birth. It is recommended that, the importation of ruminants into Dohuk Province be restricted to animals that are seronegative and have originated only from brucellosis-free flocks. Implementing and enforcing such importation protocols or completely banning of importation of small ruminants from neighbour countries or provinces would be prudent to reduce the risk of introducing new biotypes of *Brucella* or reintroducing infection to a brucellosis-free flock. Mass conjunctival vaccination of sheep and goats with test and slaughter programme, in addition to the adoption of improved hygiene and biosecurity practices such as flocks health management, environmental management, farm management, and animal welfare, is recommended as a control strategy in Dohuk. Ideally, the Kurdistan government should be more involved in the control of brucellosis in small ruminants. It should consider paying a premium for milk from brucellosis-free flocks to minimise the public health impact of the disease. To increase awareness and encourage the adoption of better health behaviours, the government should conduct the public education programmes related to the disease in schools and communicates in rural areas. Information should be provided on the clinical signs, transmission routes and preventive and control measures for the disease. The educational message may be provided through various routes, including television, radio, warning signs, post and newspapers (Chen et al., 2016). Farmers should also seek veterinary advice on the brucellosis status of their animals by testing their animals. Furthermore, as brucellosis reduces flock productivity through decreased production of milk, there is a financial benefit of producers being involved in a certified-free flock system. Moreover, apply penalties for the infected flock, so it is producer driven by restricting movement of animals from infected flocks, with the only allowed movement to slaughter /abattoirs. The government should conduct an annual public education programme on the brucellosis and toxoplasmosis in schools and communities in rural area. An educational campaign for the public could

be conducted through advertisements advising of certified free flocks providing fresh milk, and awareness programme and health education, especially for the rural population by utilizing a wide variety of media technologies, and education of all personnel involved are essential.

There is also a need to improve the diagnostic capacity of both veterinary and district hospital laboratories by a confirmatory process by using more accurate tests and different controlling method. Capacity building and training for improving the quality of the veterinary services and appropriate diagnostic laboratories on the basis of adopted standards of the International Organization for Animal Health (OIE), including standardization and quality control of diagnostic kits or reagents and vaccines, are necessary.

In developing areas such as Dohuk Province, the implementation of effective test-and-slaughter policies is hindered by a lack of financial resources to compensate farmers whose animals are slaughtered (Godfroid et al., 2011; McDermott & Arimi, 2002). Test-and-slaughter policies can also paradoxically contribute to the spread of infection when identified seropositive animals are sold instead of being slaughtered (Renukaradhya et al., 2002). Therefore, after slaughter positive animals the carcasses should burnt or buried on the infected premises and put the affected farms under quarantined. Consequently, vaccination is considered the best way to initially control the disease in the region as the vaccine, Rev-1, is efficacious in adult animals, as well as animals 3 to 6 months of age, and induces a high and durable immune response (Alton & Elberg, 1967; Blasco, 1997a). However, surprisingly, the seroprevalence in the current study was still high (31.7%) even though the kids and lambs are vaccinated by the conjunctival route, when they are between 3 and 6 months of age. This high seroprevalence is unlikely to associate with false-positive reactions arising from the vaccination , this could be false –positive *Brucella* antibody test results can be caused by cross –reactivity of antibodies to *Esherichia coli O 157: H7* and *Yersinia enterocolitica O:9* (Bonfini et al., 2018; Saxena et al., 2018). However, it could be due to a low vaccine efficacy associated with inappropriate storage and transport of the vaccine in and to the field (poor cold chain) (Kornspan et al., 2019). It is important to follow the instructions shared by the vaccine manufacturers and provide

formal training of the vaccinators by experienced veterinarians working for the Kurdistan government. The cost-benefit analysis outlined in Chapter 8 revealed that, vaccination of all entire animals over the age of three months was a viable method to reduce the prevalence of the disease over time and it is recommended that such a mass vaccination programme be implemented throughout the Kurdistan region. Prevent illegal movement of animals between Iraq and other neighbour countries, apply mass vaccination programme may have on the brucellosis control programme and potentially would reduce the prevalence of brucellosis in small ruminants. All imported animals need to have a certification that they have been vaccinated, along with a negative test and be sourced from certified flocks free from disease.

Development and implementation of appropriate veterinary legislation and animal health policies, as well as the adoption of appropriate control and elimination programmes are essential. Compulsory vaccination of all young female lamb/kids between 3-6 months, and vaccination other adult female at least six months before starting the breeding season with Rev .1 via the conjunctiva, this lead to reduce the prevalence of brucellosis. Compulsory tagging all animals should be introduced. Each property was allocated a property identification colour, to monitoring the disease in Dohuk Province. Allowing individuals to be traced back to their property of origin. It was a legal requirement that all animals were identified by different tags before sale or inspection at abattoirs. Trace back and trace forward of test positive sheep or goats were the integral part to the success of disease checking.

As there is no facilities in Dohuk rovince, milk ring test was selected for long term surveillance of brucellosis in sheep and goats flocks. Each district or state in Iraq should conduct milk ring tests on each sheep and goats flock at least three times a year throughout the eradication phase, and for five years after becoming brucellosis free.

All infected flocks should be tested at six months' intervals and all positive animals should be removed for slaughter in order to decrease the prevalence of the disease. The abattoir also should be under monitoring each mature breeding sheep and goats that were sent to abattoirs for slaughter were

sampled and tested. All cases of abortion are reported to authorities and it has been investigated to dismiss the possibility of brucellosis infection.

The incidence of abortion due to toxoplasmosis was very low in Dohuk Province, and the aim of this thesis to estimate the prevalence of the *T. gondii* antibodies in small ruminants as toxoplasmosis considered as one of the major causes of infectious reproductive failure of sheep and goats in many countries.

Prevention of the spread of toxoplasmosis through strengthening farm biosecurity measures is essential. It is recommended that to prevent infection in small ruminants, cats should be vaccinated by zona pellucida (ZP) and this vaccine causes a tendency towards an increased incidence of unsuccessful mating or pregnancy (this will causes decrease the number of cats in the farm and this will lead to decrease the oocyst from the infected cats and then the number of infected sheep and goats will decrease) (Eade, 2007). Control and prophylactic measures must be adopted to improve the rearing system and the implementation of health promoting programmes in a joint effort between sheep and goats farmers, farmers associations and veterinarians to inform about the means of transmission of the infection and for a better understanding of toxoplasmosis. These programmes should cover methods of disposal of foetal membranes and aborted lambs/kids through deep burial or burning (Williams & O'Donovan, 2009) . Measures to prevent infection of food animals include keeping the animals indoors; keeping cats away from farms, feed, and bedding production and storage; providing clean drinking water and blocking access to surface water; implementing strict rodent control. In addition, control strategies for the stray cat population must be implemented.

The methodology adopted in the seroconversion study for both pathogens brucellosis and toxoplasmosis should be repeated in a larger population of small ruminants and continued until birth in Dohuk to confirm the findings of the current study and to establish the impact of toxoplasmosis and brucellosis on the reproduction and productivity of sheep and goats in Dohuk Province.

Adopting a one-health approach is recommended where the veterinary and medical professionals work together to minimise the impacts of toxoplasmosis and brucellosis on the human and livestock populations. However, for such studies to succeed a better veterinary services unit including infrastructure and expertise is required in the province.

## 9.8 Future research

Isolation of *Brucella* is the gold standard in the laboratory diagnosis of brucellosis. It is necessary to determine the serotypes and species circulating in Dohuk Province, and is a worthwhile investment to provide the basis for the designing and implementation of control strategies in this area. Culture of *Brucella* is being accepted for brucellosis diagnosis for many reasons such as time consuming, insensitive, previous antibiotic, because of bacteriological culture for *Brucella* was not performed in many provinces in Iraq; other tests are used to diagnosis brucellosis. Also *Brucella* cannot be culture from patient's samples as it is need higher many facilities which are not available in the laboratory diagnosis in Iraq. In Italy, analysis of field circulating strain of *Brucella* explores more knowledge on the epidemiology of brucellosis. It is also useful for formulating the policies and strategies for the control and eradication of the brucellosis in animal population, as there is inferred evidence of the predominance of *B. melitensis* and other types of brucellosis such as *B. abortus , B. canis,* and *B. ovice etc.* (De Massis et al., 2019). Therefore, use blood culture to identify the specific *Brucella* species circulating in the country (Tekle et al., 2019).

As brucellosis is a transboundary animal disease, information on the current prevalence in different livestock should be shared between neighbouring countries in order to improve the understanding and effectiveness of the regional control of this disease. Several countries in Central Asia are making good progress in reducing brucellosis prevalence in small ruminants by continuous vaccination (Rozstalnyy, 2013). In Middle East countries, there are many things to protect the investment already made, the neighbouring countries need to enhance control measures within their borders, regular intercountry veterinary meeting, regional control of the disease, accepted and

documented movement controls, need to understand all livestock movements both inter and intercountry, traditional and trade movements.

Brucellosis has been reported in a variety of terrestrial wildlife species throughout the world (Godfroid, 2002). Consequently, the risk of transporting brucellosis to small ruminants in Dohuk Province will increase. In addition, aborted and parturient material in the environment can also be dispersed by carnivorous animals such as dogs, foxes and wolves (Godfroid, 2002; Simpson et al., 2021), potentially resulting in exposure of small ruminants to *Brucella* (Al-Rawahi, 2014). However, reducing the prevalence of brucellosis in wildlife reservoirs is complicated and costly (Olsen, 2010). Conversely, wildlife species could be a spill over host, infected after exposure to the bacteria from domesticated livestock. In Iraq, wild ruminants, such as wild goats (*Capran aegagrus*) and Persian fallow deer (*Dama dama mesopotamica* ), may be found grazing alongside domesticated livestock, so implementing a control programme in livestock could minimise transmission to wildlife species and prevent the establishment of a potential wildlife reservoir of infection. To understand the role of wildlife species in the spread and maintenance of brucellosis in Iraq, sampling studies should be undertaken of wild species, particularly in regions where there wild and domesticated ruminants are in close contact.

Further validation of serological assays and molecular characterization studies are required to find phenotypes differences as a way to better understand the biology of the different *T. gondii strain* may provide further evidence on the public health risk of *Toxoplasma* from small ruminants in Dohuk Province. For better understanding of the risk factors associated with *T. gondii* infection in this region, further studies are necessary to provide detailed information on risk factors associated with infection with *T. gondii*. These are such as production system, small ruminants welfare, food and water source by using more developed tools including PCR, environmental sampling as an indicator of oocyst contamination and the number of cats (Stelzer et al., 2019; Su et al., 2003). Control of feline carriers remains a challenging problem. Cooking meat to prevent infection, the tissue cysts become non-viable by cooking meat to an internal temperature of 66 $^{\circ}$C (Dubey, 2016). Serological tests on blood samples

from cats should be performed to diagnosis the seroprevalence of *T.gondii* in cats from Dohuk Province. Analysis of hospital records in order to provide data into seroprevalence rates of selected pathogens that can be utilised to guide screening and diagnostic laboratory testing. Relevant seroprevalence data for endemic pathogens in a given region provide into population susceptibility to acute infection or risk for reactivation disease.

Further studies should be conducted on sheep and goats to investigate other potential causes of abortion in the Kurdistan Region of northern Iraq. Although there was evidence that toxoplasmosis is widespread in this study; there was little evidence to suggest an association between seroconversion to *T. gondii* and the outcome of pregnancy. An expanded comprehensive study is required involving sampling small ruminants from birth to clarify when animals are seroconverting and what impact that has on productivity. Such a study will improve the understanding of the epidemiology and impact of toxoplasmosis on the local animal industry and the potential risk of transmission to humans through consumption of meat or milk from small ruminants (Boughattas, 2017). Also, as toxoplasmosis have zoonotic potential; further studies should include a wide range of hosts such as humans, companion animals, small ruminants and cattle to understand their transmission dynamics in Dohuk Province.

## 9.9 Conclusions

In conclusion, the work described in this thesis highlighted that animals that seroconverted to *Brucella* were more likely to suffer pregnancy loss than those that did not seroconvert. In contrast, seroconversion to *Toxoplasma* did not significantly influence the outcome of pregnancy. Based on the findings of this study, it is recommended that, to control the effect of brucellosis resulting in improved the low income for local farmers and less disease in the human population, an integrated approach should be implemented including adopting risk-based control measures, education and mass vaccination. An integrated approach should be developed and implemented, including mass vaccination for brucellosis, education of the farmers and livestock workers. From the findings presented in this thesis, it was confirmed that, the epidemiology of brucellosis and toxoplasmosis was

influenced by several factors, including such as size flock, livestock production type, and interaction with the economy. From the research done in this thesis, indicated that density, size, ag flocke of animals, reduced veterinary service like vaccination programmes are associated with high *Brucella* prevalence and the presence of cats, a large flock size, and the method of disposing of aborted foetuses are potential risk factors for *Toxoplasma* prevalence in sheep and goats. The evidence gathered and results presented in the thesis highlight how effectively control brucellosis and toxoplasmosis resulting in improved income for local farmers, an integrated approach should be implemented for both diseases including adopting risk-based control measures and education.

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**Appendix 1:**

An epidemiological study of the impact of *Toxoplasma gondii* and *Brucella melitensis* on reproduction in sheep and goats in Dohuk Province, Iraq

Questionnaire

District: Sub-district: _____

Village:

Record number: Date:

1 How many sheep do you have in your flock and what are their ages? ( )

2 How many goats do you have in your flock and what are their ages? ( )

3 Do you own any other animals? Yes ( ) No ( )

4 If yes what types and numbers of other animals do you own?

| | Goats | | Sheep | |
|---|---|---|---|---|
| Age | ≤ 6 months | > 6 months | ≤ 6 months | > 6 months |
| Number of females | | | | |
| Number of males | | | | |
| Any other animals | | | | |
| Total | | | | |

| Any other animals | Yes | No | Type | Number |
|---|---|---|---|---|

5 Do your sheep and goats graze together on your farm, or do they graze separately?

Together ( ) Separately ( )

6 Do your sheep and goats graze with other flocks of sheep or goats?

Yes ( ) No ( )

7 If yes – approximately how many flocks does your flock graze with? ( )

8 If yes – how long does your flock graze with other flocks each day? ( )

9 Approximately how long does your flock graze, in total, each day? ( )

10 In the last two years have your sheep and goats grazed outside your farm?

Yes () No ( )

11 How many live born lambs and kids have been produced in the last 12 months?

Sheep: Male () Female () Unsure ()

Goats: Male () Female () Unsure ()

12 Did any of your sheep abort during the last 12 months?

No () Not Sure () Yes ( ) If yes, how many? ( )

13 If yes, at which stage of pregnancy did the abortion occur?

A- Early pregnancy: conception < 40 days ().

B- Mid – pregnancy: 40 – 105 days ().

C- Late- pregnancy: 105 days to birth ().

D- Not sure ().

14 Did any of your goats abort during the last 12 months?

No ( ) Not Sure ( ) Yes ( ) If yes, how many ( )

15 If yes, at which stage of pregnancy did the abortion occur?

A- Early pregnancy: conception < 40 days ( ).

B- Mid – pregnancy: 40 – 105 days ( ).

C- Late- pregnancy: 105 days to birth ( ).

D- Not sure ( ).

16 Did all the abortions result in the birth of dead foetuses, or did some survive for a period of time? All died ( ) Some survived ( )

17 How did you dispose of the aborted foetuses?

Burnt ( ) Gave to dogs ( ) Threw away ( ) If other, please specify ( )

18 Have you sold any sheep from your flock during the last year?

No ( ) Yes ( ) If yes, how many? ( )

19 Have you sold any goats from your flock during the last year?

No ( ) Yes ( ) If yes, how many? ( )

20 Have you purchased any sheep for your flock during the last year?

No ( ) Yes ( ) If yes, how many? ( )

21 If yes, where did they come from?

Same village ( ) Different village, same sub-district ( )

Different village, different sub-district ( ) Different village, different district ( )

22 Have you purchased any goats for your flock during the last year?

No ( ) Yes ( ) If yes, how many? ( )

23 If yes, where did they come from?

Same village ( ) Different village, same sub-district ( )

Different village, different sub-district ( ) Different village, different district ( )

24 Do you have any pet cats on your farm?

No ( ) Yes ( ) If yes, how many? ( )

25 Are there any stray cats on your farm?

Not sure ( ) No ( ) Yes ( ) If yes, how many? ( )

26 Have you seen cats near the feed you give your animals?

No ( ) Yes ( )

27 If yes, have you ever seen the droppings of cats in your animal's feed?

Yes ( ) No ( )

28 Do you know if the droppings of cats have ever contaminated the water stock drink from? No ( ) Yes ( )

29 What is the primary source of water for your sheep and goats?

River ( ) Well ( ) spring ( ) If other, please specify ( )

30 How is the water provided to your animals?

Concrete trough ( ) Metal trough ( ) Wooden trough ( ) other ( )

31 Do you clean the water troughs? Yes ( ) No ( )

32 Do your sheep and goats share these water sources with other nearby goat and sheep farms? Yes ( ) No ( ) Not sure ( )

33 If yes how often? ( )

34 What kind of flooring is the pen/enclosure for sheep and goats?

Cement/Stone ( ) Ground/dirt ( ) Mixed ( ) other ( )

35 What style of feeding container do you use on your farm?

Concrete trough ( ) Metal trough ( ) Wooden trough ( )

Foraging ( ) other ( )

36 Do you clean the troughs used for feeding the animals? Yes ( ) No ( )

37 If yes how often? ( )

38 Do you clean the area where your animals live? Yes ( ) No ( )

39 If yes how often? ( )

40 Approximately how much did you spend on food last year for your sheep? ( )

41 Approximately how much did you spend on food last year for your goats? ( )

42 About how much did you spend to treat sick sheep in the past year? ( )

43 About how much did you spend to treat sick goats in the past year? ( )

44 What was the main disease/condition you treated your sheep for? ( )

45 What was the main disease/condition you treated your goats for? ( )

46 Do you have electricity on your farm?

Yes ( ) No ( )

47 Approximately how much did you spend on electricity for your sheep within the last 12 months? ( ) Not sure ( )

48 Approximately how much did you spend on electricity for your goats within the last 12 months? ( ) Not sure ( )

49 Are there any paid agricultural workers on your farm?

No ( ) Yes ( ) If yes, how many ( )

50 Approximately, what is the annual cost of these workers?

Cost ( ) Not sure ( )

51 Have any of your sheep been vaccinated against brucellosis in the last year?

No ( ) Not sure ( ) Yes ( )

52 If yes, how many times were they vaccinated in the last 12 months? ( )

53 If yes, how many were vaccinated in the last 12 months? ( )

54 If yes – in what month were they last vaccinated? ( )

55 Have any of your goats been vaccinated against brucellosis within the past year?

No ( ) Not sure ( ) Yes ( )

56 If yes, how many times were they vaccinated in the past 12 months? ( )

57 If yes, how many were vaccinated in the past 12 months? ( )

58 If yes – in what month were they last vaccinated? ( )

59 In your opinion what have been the three most important health problems in your sheep over the last 12 months?

1.

2.

3.

60 In your opinion what have been the three most important health problems in your goats over the last 12 months?

1.

2.

3.

61 Do you know if your sheep are infected with *Brucella*?

Yes ( ) No ( ) don't know ( )

62 If yes, how do you know your sheep are infected? ( )

63 Do you know if your sheep are infected with *Toxoplasma*?

Yes ( ) No ( ) don't know ( )

64 If yes, how do you know your sheep are infected? ( )

65 Do you know if your goats are infected with *Brucella*?

Yes ( ) No ( ) don't know ( )

66 If yes, how do you know your goats are infected? ( )

67 Do you know if your goats are infected with *Toxoplasma*?

Yes ( ) No ( ) don't know ( )

68 If yes, how do you know your goats are infected? ( )

69 Has brucellosis ever been diagnosed in your sheep or goat flock?

Yes ( ) No ( ) Not sure ( )

70 If yes, how many sheep and goats were infected?

Sheep ( ) Goats ( ) don't know ( )

71 Have any of your sheep or goats ever been diagnosed with Toxoplasmosis?

Yes ( ) No ( ) Not sure ( )

72 If yes, how many sheep and goats were infected?

Sheep ( ) Goats ( ) don't know ( )

73 Have you spent any money treating or controlling brucellosis in your sheep during the last year? Yes ( ) No ( )

74 If yes – how much did you spend? ($ )

75 Have you spent any money treating or controlling Toxoplasmosis in your sheep during the last year? Yes ( ) No ( )

76 If yes – how much did you spend? ($ )

77 Have you spent any money treating or controlling brucellosis in your goats during the last year? Yes ( ) No ( )

78 If yes – how much did you spend? ($ )

79 Have you spent any money treating or controlling Toxoplasmosis in your goats during the last year? Yes ( ) No ( )

80 If yes – how much did you spend? ($ )

81 Sheep

A- How much did you earn from the sale of sheep's milk in the last 12 months?

Income ($ ) Not sure ( )

B- How much did you earn from the sale of sheep wool in the last 12 months?

82 Goats

A- How much did you earn from the sale of goat's milk in the last 12 months?

Income ($ ) Not sure ( )

B- How much did you earn from the sale of goat hair in the last 12 months?

Income ($ ) Not sure ( )

83 How much did you earn from the sale of sheep in the last 12 months?

Income ($ ) Not sure ( )

84 How much did you earn from the sale of goats in the last 12 months?

Income ($ ) Not sure ( )

**Procedure for the Rose Bengal plate test**

All sera samples were screened using RBPT antigen (VLA Weybridge, UK) and the test methodology recommended by (Alton et al., 1988), as follows:

**Qualitative method**

1. Allow the reagents and samples to reach room temperature; however, the sensitivity of the test may be reduced at low temperatures.

2. 50 µL of the sample and one drop of each of the positive and negative controls are placed into separate circles on the slide test.

3. The Rose Bengal reagent is mixed vigorously before it is used and one drop added to the sample to be tested.

4. The slide is placed on a mechanical rotator at 80-100 r.p.m. for 4 minutes. False positive results can appear if the test is interpreted later than two minutes.

**Procedure for conducting the Brucella ELISA IgM**

The enzyme linked immunoabsorbent assay (ELISA) technique was adopted against Brucella in the qualitative immune enzymatic determination of the IgM-class antibodies. The Brucella antigens were pre-coated on the microtiter strip wells, thus binding the specimen antibodies. The wells were washed, and horseradish peroxide labelled anti-animal IgM conjugate was then added, which in turn binded the captured Brucella antibodies. To view the conjugate formed, tetramethylbenzidine (TMB) substrate was added, and the presence of the conjugate was visualized when a blue reaction product was given. The more intense the blue product was, the greater the number of Brucella-specific IgM antibodies in the specimen and vice versa. The reaction was halted by adding sulphuric acid, which produced a yellow endpoint colour. The ELISA micro-well plate reader showed absorbance at 450 nm.

**Materials**

**Reagents supplied**

Brucella Coated Wells (IgM): 8 well snap-off strips coated with Brucella antigen (12 break-apart) in resealable aluminium foil

IgM Sample Diluent ***: 100 ml bottle; pH 7.2 ± 0.2; coloured green; ready to use; white cap.

Stop Solution: 15 ml sulphuric acid, 0.2 mol/l; ready to use; Red cap

Washing Solution (20x conc)*: 50 ml of a 20-fold buffer with pH 7.2 ± 0.2; white cap

Brucella anti-IgM Conjugate**: 20 ml of peroxidase labelled rabbit antibody to animal IgM; coloured red, ready to use; black cap

TMB Substrate Solution: 15 ml tetramethylbenzidine (TMB); ready to use; yellow cap.

Brucella IgM Positive Control***: 2 ml; coloured yellow; ready to use; red cap.

Brucella IgM Cut-off Control***: 3 ml; coloured yellow; ready to use; green cap.

Brucella IgM Negative Control***: 2 ml; coloured yellow; ready to use; blue cap.

Note:* contains 0.1% Bronidox L after dilution

** contains 0.2% Bronidox L

*** contains 0.1% Kathon

**Materials supplied**

1 Strip holder

1 Cover foil

1 Test protocol

One distribution and identification plan

Materials and Equipment

An ELISA micro-well plate reader that is able to measure absorbance at 450/620 nm

37 °C Incubator

Rinsing wells equipment

Pipettes of volumes 10–1000 µl

Deionised/freshly distilled water

Disposable tubes

Vortex tube mixer

Timer

**Storage and Stability**

The reagents are stable to the indicated expiry dates if stored at 2–8 °C.

**Preparation of Reagents**

All samples, reagents and controls were brought to room temperature (20–25 °C).

For coated snap-off strips, storage was at 2–8 °C to expiry date even after resealing. Aluminium foil and the supplied desiccant should be used to reseal the remaining strips after the removal of some strips.

The *Brucella* anti-IgM conjugate was ready to use as provided by the manufacturer. After opening the bottle, storage should be maintained at 2–8 °C to ensure stability until the expiry date.

Positive, cut-off and negative controls were ready to use. They were stored at 2–8 °C to ensure stability to the expiry date.

The IgM Sample Diluent was used for the dilution of the patient specimen. The solution was ready for use and was stable to the expiry date if kept at 2–8 °C.

For the washing solution (20x conc), 10 ml was added to 190 ml redistilled water that was fresh and sterile. The diluted buffer is stable for only five days when kept at room temperature. The concentrate is valid to the expiry date after it is first opened.

The tetramethylbenzidine (TBM)/hydrogen peroxide system was ready for use. It is colourless or sometimes has a blue tinge. A blue substrate is an indication of contamination; hence, it should not be used. The solution is stable after opening to the expiry date if it is kept away from light at 2–8 °C.

The stop solution (15 ml 0.2 M sulphuric acid solution) was ready for use and stored at 2–8 °C.

**Specimen Collection and Preparation**

The animal serum/plasma (citrate) was made using this assay. The specimen was aliquoted and deep frozen at -70 to -20 °C if the assay was performed 5 days after the sample collection. However, if it was performed within 5 days of the sample collection, it could be stored at 2–8 °C. Heat inactivation, repeat freezing and thawing of samples are not advised.

IgM Sample Diluent at 1+ 100 was used to dilute the samples before assaying; 10 µl of the sample and 1 ml of IgM Sample Diluent were dispensed in tubes to obtain a 1+100 dilution and then were mixed thoroughly using a Vortex.

**Assay procedure**

**Test preparation**

Because the reliability of the results depended on the adherence to protocol, the test protocol was read closely. The procedure that was undertaken was only valid for manual procedures. Tests on ELISA were recommended to be automatic in order to avoid the effects of washing. The specimens and controls were established based on the result sheet provided in the kit.

The required number of wells was selected and inserted into the holder.

The selection of wells was carried out as follows:

Substrate blank, one well (e.g., A1)

Cut-off control, two wells (e.g., C1+D1)

Negative control, one well (e.g., B1)

Positive control, one well (e.g., E1)

All assay steps were performed in the right order and with the correct timing. Each control and sample was dispensed using a clean disposable tip. The temperature of the incubator was 37° ± 1 °C. The 100 µl controls and diluted samples were dispensed into their respective wells. Well A1 is left for the substrate blank.

The wells were then covered with foil and incubated for 1 hour ± 5 min at 37±1 °C. The foil was removed after incubation; the contents were aspirated and washed with 300 µl of the washing solution. The soaking time between wash cycles exceeded 5 seconds. The remaining fluid was removed by carefully tapping the strips on tissue paper.

100 µl of Brucella anti-IgM Conjugate was dispensed into wells other than the blank well and covered with foil. The wells were incubated for 30 minutes away from direct sunlight at room temperature. The foil removal procedure was repeated. All wells were dispensed with 100 µl of TMB Substrate Solution and incubated for 15 minutes at room temperature in the dark. 100 µl of Stop Solution was

dispensed into every well at an even rate with the TMB Substrate Solution. The blue colour that developed during incubation turned yellow.

The absorbance of the specimen was measured at 450/620 nm within 30 minutes after the addition of the stop solution.

**Measurement**

The blank in well A1 was used to adjust the ELISA microwell plate to zero. If not, the well A1 readings were the benchmarks for the absorbance readings. The absorbance of all wells was measured at 450 nm, and the absorbance values were recorded. The reference for dual wavelength reading was 620 nm. The mean absorbance values of all duplicates were calculated where feasible.

**Procedure for the *Toxoplasma* ELISA IGM**

The enzyme linked immunoabsorbent assay (ELISA) technique was adopted against Toxoplasma gondii in the qualitative immune enzymatic determination of the IgM-class antibodies. The anti-animal IgM-class antibodies were pre-coated on the microtiter strip wells, thus binding the specimen antibodies. The wells were washed, and horseradish peroxide labelled anti-animal IgM conjugate was added, which in turn binded the captured Toxoplasma-specific IgM antibodies. To view the conjugate formed, tetramethylmbenzidine (TMB) substrate was added, and the presence of the conjugate wass visualized when a blue reaction product was given. The more intense the blue product was, the greater the number of Toxoplasma gondii-specific IgM antibodies in the specimen and vice versa. The reaction was halted by adding sulphuric acid, which produced a yellow endpoint colour. Using the ELISA microwell plate reader, absorbance was given at 450 nm.

**Materials**

**Supplied Reagents**

Microtiter plate (IgM): 8-well snap-off strips (12 break-apart) with anti-human IgM-class antibodies coating in resealable aluminium foil

Diluent Sample**: 100 ml of ready-to-use buffer for sample dilution; pH 7.2 ± 0.2; coloured yellow.

Stop Solution: 15 ml ready to use sulphuric acid, 0.2 mol/l; red cap

Washing Solution (20x conc.)*: 50 ml of a 20-fold concentrated buffer; pH7.2 ± 0.2, white cap.

T. gondii Conjugate Lyoph: 1 vial containing lyophilized conjugate comprising of a complex of Toxoplasma antigen monoclonal antibody anti-P30 conjugated with horseradish peroxidase.

T. gondii Diluent Conjugate: 14 ml of a ready to use buffer; coloured red, white cap (pH 7.2 ± 0.2)

TMB Substrate: 15 ml -tetramethylbenzidine; ready to use, yellow cap

T. gondii IgM Cut-Off Control**: 3.0 ml vial; coloured yellow; ready to use; green cap

T. gondii IgM Positive Control**: 2.0 ml vial; coloured yellow; ready to use; red cap

T. gondii IgM Negative Control**: 2.0 ml vial; coloured yellow; ready to use; blue cap.

Note:

* contains 0.1 % Bronidox L after dilution

** contains 0.1 % Kathon

**Supplied Materials**

Three labelled vials (white, empty with white cap)

1 Strip holder

1 Cover foil

1 Test protocol

One distribution and identification plan

**Materials and Equipment**

ELISA microplate reader that is able to measure 450/620 nm absorbance

37 °C Incubator

Rinsing wells automatic/manual equipment

Pipettes of volumes 10–1000 µl

Vortex tube mixer

Deionized or (freshly) distilled water

Disposable tubes

Timer

**Stability and Storage**

The reagents are stable to the indicated expiry dates if stored at 2–8 °C.

**Reagent Preparation**

All samples, reagents and controls were brought to room temperature (20–25 °C). For coated snap-off strips, storage was at 2–8 °C to expiry date even after resealing. Aluminium foil and the supplied desiccant should be used to reseal the remaining strips after the removal of some strips.

For Toxoplasma gondii Conjugate, 5 ml of Conjugate Diluent was used to reconstitute the lyophilized vial. The solution was transferred into the Conjugate Diluent l. After the first use, the conjugate was aliquoted into vials and stored at -20 °C. Storage was possible in polypropylene (PP) vials at -20 °C to maintain stability to the date of expiry.

The positive, cut-off and negative controls were ready to use. They were stored at 2–8 °C for stability to the expiry date.

The IgM Sample Diluent was used for the dilution of the patient specimen. The solution was ready for use and stable to the expiry date if it was kept at 2–8 °C.

For the washing solution (20x conc), 10 ml was added to 190 ml redistilled water that was fresh and germ free. The diluted buffer was stable for only five days when kept at room temperature. The concentrate was valid to the expiry date after it was first opened.

The tetramethylbenzidine (TBM)/hydrogen peroxide system was ready for use. It was colourless or sometimes had a blue tinge. A blue substrate indicated contamination and would not have been used. The solution was stable to the expiry date after opening if it was kept away from light at 2–8 °C.

The stop solution (15 ml 0.2 M sulphuric acid solution) was ready for use and was stored at 2–8 °C.

**Specimen Collection and Preparation**

Animal serum/plasma (citrate) was used with this assay. The specimen was aliquoted and deep frozen at -70 to -20 °C if the assay was performed after 5 days of the sample collection. However, if it was performed within 5 days of the sample collection, it was stored at 2–8 °C. Heat inactivation, repeat freezing and thawing of samples was not advised.

Before assaying, the samples were diluted with 1+ 100 Sample Diluent; 10 µl sample and 1 ml Sample Diluent were dispensed in tubes to obtain a 1+100 dilution and then were mixed thoroughly using a Vortex.

**Assay Procedure**

**Test Preparation**

Because the reliability of the results depends on adherence to the protocol, the test protocol was read closely. The procedure to be undertaken was only valid for manual procedures. The ELISA tests were automatic in order to avoid the effects of washing. The specimens and controls were established on the result sheet present in the kit. The required number of wells was selected and inserted into the holder. The necessary volumes of washing solution and T. gondii Conjugate were prepared.

The selection of wells was carried out as follows:

Substrate blank, one well (e.g., A1)

Cut-off control, two wells (e.g., C1+D1)

Negative control, one well (e.g., B1)

Positive control, one well (e.g., E1)

All assay steps were performed in the right order and correct timing. Each control and a patient sample was determined in duplicate. The temperature of the incubator needs to be 37° ± 1 °C. 100 µl controls and diluted samples were dispensed into their respective wells. Well A1 was left for the substrate blank.

The wells were then covered with foil and incubated for 1 hour ± 5 min at 37±1 °C.

The foil was removed after incubation, and the contents aspirated and washed with 300 µl of washing solution. The soaking time between wash cycles exceeded 5 seconds. The remaining fluid was removed by carefully tapping the strips on tissue paper. 100 µl of T. gondii Conjugate was dispensed into the wells, except the blank well, and covered with foil. The wells were incubated for 1 hour ± 5 min at 37±1 °C away from direct sunlight. The foil removal procedure was repeated.

All wells were dispensed with 100 µl TMB Substrate Solution and incubated in the dark for 30 minutes at room temperature; 100 µl Stop Solution was dispensed into the wells at the same rate as he TMB Substrate Solution. The blue colour that developed during incubation turned yellow. The absorbance of the specimen was measured at 450/620 nm within 30 minutes after the addition of the stop solution.

**Measurement**

The blank in well A1 was used to adjust the ELISA Microwell Plate to zero. If not, the well A1 readings were then used as the benchmarks for the absorbance readings. The absorbance of all wells was measured at 450 nm, and the absorbance values were recorded. The reference for dual wavelength reading is 620 nm. The mean absorbance values of all duplicates were calculated where feasible.

**Procedure for the latex test**

All sera samples were screened using rapid later kit and the test methodology recommended by Lorne laboratories, as follows:

**Qualitative method**

1. place in separate test circle of the same slide one drop of undiluted serum, one drop of negative control and one drop of positive control using the provided disposable pipettes.

2. Next to each test circle add one of LE latex reagent

3. Specimen over entire area of the teste circle and using the broad end of pipette spread the latex reagent

4. Gently tilt agglutination slide forwards and backwards for 3 minutes whilst observing for agglutination

Thank you for taking the time to answer these questions. The results from this survey will help further our understanding of brucellosis and toxoplasmosis in sheep and goats in Dohuk