# Prevalence and risk assessment of toxoplasmosis in commercial and communal sheep and goats in the North West province and occurrence in the Free State province

By

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## DECLARATION

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Prevalence and risk assessment of toxoplasmosis in commercial and communal sheep and goats in the Northwest province and occurrence in the Free State province.

I declare that the above dissertation is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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SIGNATURE DATE

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## ABSTRACT

Toxoplasmosis is one of the most widespread parasitic zoonotic diseases arising from *Toxoplasma gondii* infection. This disease significant impact on sheep and goat production; however, it sometimes goes unnoticed in the herd, leading to unexpected and inexplicable abortions and death among the new-born's deaths. This study aimed to determine the prevalence and risk factors of *T. gondii* infections in sheep and goats from commercial and communal farms in the North West, as well as its occurrence in the Free State province. Additionally, we analysed variations and phylogenetic relationships in the *T. gondii* B1 and GRA6 gene sequences from isolates deposited in GenBank (https://www.ncbi.nlm.nih.gov/) to evaluate the usefulness of the two genes as phylogenetic markers. *Toxoplasma gondii* IgG antibodies and DNA were analysed in blood samples from 439 animals (164 sheep and 285 goats), vaginal swabs, milk, sheath scrapes from the North West province, and 11 diagnostic tissue samples from the Free State province. A questionnaire was administered to farmers used to assess potential risk factors associated with animals' exposure to *T. gondii* infections. Additionally, 183 gene sequences (107 B1 and 83 GRA6 gene sequences) retrieved from GenBank from different animal species originating from different countries were analysed, and single nucleotide polymorphisms (SNP's) were present in 17% and 83%, of the B1 and GRA6 gene sequences, respectively. Of the 439 sera tested, 13.9% (95% CI: 0.00-1.00%) were positive for antibodies against *T. gondii*. It was discovered that sheep and goats had seroprevalences of 19.5% and 10.5%, respectively. *T. gondii* was not detected by PCR in any of the analysed samples (n=198). Using the Chi-Squared test or odds ratio, the main risk factors associated with *T. gondii* infections were breed, gender, species, animal origin, history of abortion, disposal of aborted material, disposal of manure, type of breeding, district, municipality, feeding system, feed storage, and presence of cats on farms. The high seroprevalence in this study suggests that *T. gondii* exposure is widespread within the farms. The absence of genetic material associated with *T. gondii* by PCR even in seropositive animals suggests the animals were at some point exposed to the pathogen, but they do not shed the parasite in their reproductive tissues. Perhaps, these animals may potentially shed the pathogen in other tissues that we did not analyse. The isolates' gene sequence analysis showed that the GRA6 gene could work as a genetic marker for *T. gondii* in population studies compared to the B1 gene. To effectively prevent and control exposure to *T. gondii* infections, the identified risk factors must be considered.

***Keywords:*** *Toxoplasma gondii*, Prevalence, PCR, ELISA, Sheep, Goats, North West, Free State, B1, GRA6, SNP, Phylogenetic Tree

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## LIST OF ACRONYMS AND ABREVIATIONS

| | |
|---|---|
| **A** | **Absorbance** |
| **ARC-OVR** | Agricultural Research Council-Onderstepoort Veterinary Research |
| **AIDS** | Acquired Immunodeficiency Syndrome |
| **bp** | Base Pair |
| **CAES** | Collage of Agriculture and Environmental Sciences |
| **CDC** | Canters for Disease Control and Prevention |
| **CI** | Confidence Interval |
| **CNS** | Central Nervous System |
| **DAFF** | Department of Agriculture, Forestry and Fisheries |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxynucleotide triphosphate |
| **DT** | Dye Test |
| **Dr** | Doctor |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **FS** | Free State |
| **HIV** | Human Immunodeficiency Virus |
| **HRM** | High Resolution Melting |
| **IFA** | Indirect Florescent Agglutination Test |
| **IFNg** | Immunity-related GTPase |
| **IgA** | Immunoglobulin A |
| **IgG** | Immunoglobulin B |
| **IgM** | Immunoglobulin M |
| **IHAT** | Indirect Haemagglutination Test |
| **LA** | Latex Agglutination Test |
| **MAT** | Modified Agglutination Test |
| **Mbp** | Million Base Pairs |
| **MLST** | Multilocus sequence typing |
| **NC** | Negative Control |
| **NCx** | Negative Control Average |
| **NW** | North West |
| **nm** | Nanometre |
| **OR** | Odd Ratio |
| **OIE** | World Health Organization for Animal Health |
| **PBS** | Phosphate Buffered Solution |
| **PCR** | Polymerase Chain Reaction |
| **PC** | Positive Control |
| **PCx** | Positive Control Average |
| **S/P** | Specificity |
| **PV** | Pariasititophorus Vacuole |
| **qPCR** | Qualitative Polymerase Reaction |
| **RAPD** | Random Amplified Polymorphic DNA |

| | |
|---|---|
| **RFLP** | Random Fragment Length Polymorphism |
| **ROP** | Rhoptry Proteins |
| **rDNA** | Ribosomal Deoxyribose Nucleic Acid |
| **rpm** | Revolutions Per Minute |
| **SA** | South Africa |
| **SNP** | Single Nucleotide Polymorphism |
| **TMB** | 50-Tetramethylbenzidine |
| **TBE** | Tris-Borate-EDTA |
| **UNISA** | University of South Africa |
| **US** | United States |
| **v** | Volts |
| **Vet** | Veterinarian |
| **WHO** | World Health Organization |
| **µl** | Microliter |
| **° C** | Degree Celsius |
| **%** | Percentage |
| ≤ | Less than or equals to |
| ≥ | Greater than or equals to |
| < | Less than |

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# CHAPTER 1

## INTRODUCTION

### 1.1 Background

Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii* (*T. gondii*), an intracellular protozoan parasite that causes widespread infections in both humans and animals (Guy, 2014). The parasite's sexual cycle is completed in its definite host (domestic cats and other felids), producing oocysts that result in contamination of pasture, soil, feed, and water (OIE, 2008b; Djurković-Djaković *et al.*, 2019), while its asexual cycle occurs in the intermediate host (all mammalians and avian species) (Robert-Gangneux and Dardé, 2012; Caldart *et al.*, 2015; Hosein *et al.*, 2016). Even though the parasite cannot induce clinical signs in the majority of animals, in other animals like sheep and goats, it induces life-threatening acute diseases (Guy, 2014). It manifests as a pregnancy disease in other animals, particularly in sheep and goats, by multiplying in the placenta and foetus (OIE, 2008b; Guy, 2014), making a diagnosis based on clinical symptoms difficult (Ishaku *et al.*, 2018).

*Toxoplasma gondii* is one of the common pathogenic parasites found to infect humans and animals with a 30% estimated worldwide human infection rate (Guy, 2014). Toxoplasmosis is therefore of veterinary public health as well as animal health significance as it also affects the development of animals (Lopes *et al.*, 2013; Pleyer *et al.*, 2019). The disease shows mild and restricted clinical symptoms in immunocompetent individuals (Khan and Khan, 2018; Hosseini *et al.*, 2019). However, in individuals with compromised immune systems and pregnant women, the parasite infection can be more severe (Hosseini *et al.*, 2019). In these individuals, serious complications like retinocortical lesions, stillbirth and miscarriage have been reported (Montoya and Liesenfeld, 2004; Klun et al., 2006; Grigg and Sundar, 2009a; Hosseini et al., 2019).

The parasite consists of three different stages of infection, namely; tachyzoites, bradyzoites and sporozoites (Hill, Chirukandoth and Dubey, 2005; Condoleo *et al.*, 2018). Infection with the parasite can be acquired by both animals and humans during the intake of raw or undercooked food, consuming fruits and drinking water and milk contaminated with oocysts, unintentional consumption of oocysts from the atmosphere and congenitally (Condoleo *et al.*, 2018; Ishaku *et al.*, 2018; Tilahun *et al.*, 2018; Oliveira *et al.*, 2019). Between 3 and 810 million

oocysts per infection are shed by cats in their faeces over an average period of 8 days, although this may last for up to three weeks; they become infectious after 24 hours and may remain infectious under environmental conditions suitable for its survival for more than a year (Areshkumar, Divya and Yasotha, 2018). These conditions include cold and hot temperatures.

Several studies have shown a significant variation in toxoplasmosis seroprevalence. Infection with *T. gondii* in domestic ruminants worldwide ranges from 3% to 92% in sheep (Tenter, 2000), 5% to 75% in goats (Tenter, 2000), and 1% to 92% in cattle (Hosein et al., 2016). In Africa, a meta-analysis performed by Tonouhewa et al. in 2017 showing reviewed data from 1969 to 2016 in African countries, found that the average approximate prevalence of the disease in camels, chickens, cattle, horses, pigs, goats was 36%, 37%, 12%, 26.1%, 26.0% and 22.9%, respectively. In South Africa (SA), the seroprevalence was reported to be 6.0 in sheep of Gauteng, 2.7% in sheep of the Free State, 6.3% in sheep of KwaZulu-Natal, 8% in sheep of the Western Cape province (Samra *et al.*, 2007; Hammond-Aryee, Van Helden and Van Helden, 2015), 15.2% from cattle in the Mnisi community in Mpumalanga province (Adesiyun *et al.*, 2020) and 20.8% from cattle in high throughput Klerksdorp and Rustenburg abattoirs of the North West province (Ndou *et al.*, 2013). A recent study conducted in the Eastern Cape province has shown an overall seroprevalence of 83.33 % in farms with sheep having the highest rate of infection of 64.46%, followed by 53.91% in goats, 33.9% in pigs, 32.11% in cats and 33.58% in chickens (Tagwireyi, Etter and Neves, 2019). There is no data for Limpopo and Northern Cape provinces.

Among the evaluated risk factors found to be associated with increased seropositivity of *T. gondii* for the different species in the south-eastern region of SA were: age, animal production system, cat faecal disposal, cat feed disposal, climate, location, rodent control and seropositive cat (Tagwireyi, Etter and Neves, 2019).

## 1.2 Problem statement

Toxoplasmosis is prevalent in most areas of the world, including but not limited to South Africa, and is of veterinary and medical importance due to its ability to cause miscarriages and ocular infections in humans as well as abortions, mummification, and stillbirths in livestock, particularly sheep and goats, resulting in a sizeable socio-economic loss for the farmers (Tenter, 2000; Azimpour-Ardakan *et al.*, 2021). Despite this, it is still one of the understudied diseases

in SA with only a few studies conducted and published for toxoplasmosis in livestock. There have however been studies on seroprevalence, and associated risk factors of the disease conducted by Ndou et al., 2013 in the North West abattoirs, Twagwireyi et al, 2019 in the Eastern Cape, and Adesiyun et al, 2020 in Mnisi community Mpumalanga. Thus, there is a need to generate more widespread data for the rest of the country and identify strains that are circulating in our country to aid in prevention of future outbreaks.

There is a close link between domestic animals and the human population (rural, urban, agricultural workers, veterinarians, and butchers) and a great relationship between pets and humans which may lead to the transmission of zoonotic diseases between animals and humans (Areshkumar, Divya and Yasotha, 2018). In 1999, the Centers for Disease Control and Prevention (CDC) reported that *T. gondii* is one of three pathogens (including *Salmonella* and *Listeria*) that together account for more than 70% of all deaths because of foodborne illness in the United States (US). According to this report, *T. gondii* is responsible for approximately 24% of all deaths attributed to foodborne pathogens, with an estimated loss of 10,964 quality-adjusted life years and, 2,973 million dollars in costs due to illness, 86,686 illnesses, 4,428 hospitalizations, and 327 deaths per annum in the US (Batz, Hoffmann and Morris, 2011).

There are no recent published data on toxoplasmosis prevalence, risk factors associated with animal exposure, or knowledge of *T. gondii* strains currently circulating in the Free State (FS) and North West (NW) provinces to allow epidemiological investigations and tracing sources of infection for outbreak control in livestock and wildlife. There is therefore a need to investigate the prevalence of toxoplasmosis in communal and commercial sheep and goats in the NW province, the incidence of occurrence in the FS province, and identify risk factors associated with the disease within the animal population in the NW province, as well as generate data on the types of *T. gondii* strains currently circulating within the two provinces. Molecular characterization of Toxoplasma gondii isolates is central for understanding differences in disease transmission

Molecular characterization of *T. gondii* isolates is important for understanding differences in its transmission and manifestations. Thus, there is a need to analyse and evaluate the variation and phylogenetic relationship in the B1 and GRA6 gene sequences from the *T. gondii* isolates deposited in the GenBank, including those from South Africa in-order to assess the usefulness of the two genes as phylogenetic markers.

## 1.3 Aim and objectives

### 1.3.1 Aim

To investigate the prevalence and conduct a risk assessment of toxoplasmosis in commercial and communal sheep and goats in the NW province and the occurrence in Free State province. Furthermore, analyse variations and phylogenetic relationships in the *T. gondii* B1 and GRA6 gene sequences from the isolates deposited in GenBank to evaluate if they could be used as phylogenetic markers.

### 1.3.2 Objectives

- Investigate the prevalence of *T. gondii* in sheep and goats in commercial and communal farms across NW province using serological and molecular methods
- Assess risk factors associated with exposure and *T. gondii* transmission within the animal population using a questionnaire
- Investigate the genetic variation among the *T. gondii* B1 and GRA6 gene sequences from isolates deposited in the GenBank and their phylogenetic relationship through the construction of phylogenetic trees to assess if they could be used as phylogenetic markers
- Assess incidents of occurrence of *T. gondii* in Free State province using diagnostic samples submitted at ARC-OVR for other reproductive diseases

## 1.4 Overview of the dissertation chapters

This dissertation is made up of six chapters organised as follows:

I. **Chapter 1:** Introduction

   This chapter provides a brief overview and background on the research including, the problem statement, aim and objectives, and research justification.

II. **Chapter 2:** Literature review

   In this chapter, a literature review on the prevalence of *T. gondii* in communal and commercial sheep and goats and factors associated with exposure to the animals is discussed. It describes current and statistical data on the disease, its local and global

distribution, its effect on the veterinary public health and economy, and different methods currently used in the testing and detection of *T. gondii*.

III. **Chapter 3:** Methodology

This chapter provides details on how the study was designed, research areas, sample collection, and analysis, as well as statistical analysis of the data obtained.

IV. **Chapter 4:** Results

This chapter reports on the results obtained from the study and their interpretation.

V. **Chapter 5:** Discussion

This chapter discusses the results obtained in the study and compares them to the results obtained in other similar studies.

VI. **Chapter 6:** Conclusion and recommendation

This concluding chapter summarised the research findings based on the aim and objectives and make recommendations based on these findings.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

Toxoplasmosis is known as one of the most widespread parasitic zoonotic diseases arising from *T. gondii* infection (Lopes *et al.*, 2014). In small ruminants, *T. gondii* is one of the parasites that cause reproductive disorders (Tenter, 2000;Lopes *et al.*, 2013). This pathogen is considered an efficient parasite because it rarely causes harm to its hosts (Stover *et al.*, 1990). In 1908, Nicollae and Manceaux discovered the protozoan as an African rodent (*Chenodactylus gundi*) parasite of the spleen and other organs. This disease was differentiated from Leishmania in 1909 and was called toxoplasmosis, and there has been growing interest in its identification as a pathogen in many hosts, including humans since its discovery (Webster, 2010; Ibrahim, 2017). Most infections with the parasite show no clinical symptoms, but result in congenital toxoplasmosis, and reactivated encephalitis of toxoplasma in immunosuppressed people (transplant recipients and others) (Pleyer *et al.*, 2019).

## 2.2 Aetiology

*Toxoplasma gondii* falls under the Apicomplex phylum, Coccidia class, Eucoccidiorida order, and Sarcocystidae family, which affects humans and a large number of vertebral hosts (Montoya and Liesenfeld, 2004; Robert-Gangneux and Dardé, 2012; Lopes *et al.*, 2013). The species is the only member of the *Toxoplasma* genus. Cats are the primary hosts of *T. gondii*, but the pathogen has a large number of final hosts consisting of humans and all warm-blooded animals, including the mammalian and avian species (Guy, 2014; Ibrahim, 2017). *Toxoplasma gondii's* life cycle involves asexual replication in tissues and sexually reproducing in the cats' intestines (Tenter, Heckeroth and Weiss, 2000; Pal, Alem and Tuli, 2014; Ibrahim, 2017). Its life cycle is made up of three different stages, namely; oocysts in cat faeces, tachyzoites detected in the acute stage of the infection period in the secondary host, and bradyzoites occurring in cysts of tissues (Innes, 2010; Pal, Alem and Tuli, 2014; Ibrahim, 2017; Condoleo *et al.*, 2018). These stages are all hosts infectious and the life cycle can continue for some time by transmitting cysts of tissue between secondary hosts (even without the primary hosts) and transmitting oocysts between primary hosts (Tenter, Heckeroth and Weiss, 2000; Pal, Alem

and Tuli, 2014; Garcia *et al.*, 2017). The cyst can be found in the brain, heart muscle and striated muscle of hosts and remain in these organs and tissues for a lifetime (Irma and Nasronudin, 2015; Otranto *et al.*, 2015).

The protozoan has 13 chromosomes in its complete haploid genome with little variation among strains, which has a size of approximately 65 million base pairs (Mbp) and has been found to include more than 8300 protein-coding genes (Ajioka, Fitzpatrick and Reitter, 2001; Castro *et al.*, 2020; Xia *et al.*, 2021; Fernández-Escobar *et al.*, 2022).

### 2.3 Life cycle

*Toxoplasma gondii* has two life cycles: the sexual cycle occurring in the definitive host's enteroepithelial cells, resulting in oocytes production which are then secreted in faeces (OIE, 2008a, 2008b; Deshmukh *et al.*, 2021) and the asexual cycle occurring in the intermediate host (OIE, 2008b; Irma and Nasronudin, 2015).

The two developmental stages during the asexual cycle are the fast-growing tachyzoite and the slow-growing bradyzoites (OIE, 2008b). Tachyzoites actively infiltrate host cells in acute infection and multiply within the cells, causing them to burst and release organisms locally and into the bloodstream (OIE, 2008b; Ibrahim, 2017). As the host establishes immunity, the parasite maintains its overall size and shape but converts into bradyzoites and multiplies more gradually within tissue cysts to create a persistent infection (Ibrahim, 2017; Jasim and Ayyal, 2018). The parasite's dormant stage in the host is represented by these tissue cysts, which are commonly found in the brain and skeletal muscle (OIE, 2008b; Jasim and Ayyal, 2018). In humans, viable muscular tissue cysts (meat) are a major source of infection (OIE, 2008b; Jasim and Ayyal, 2018). Tachyzoites can be shown in ascetic fluid or lung impress, ion smears as well as in tissue sections of the liver and other organs in animals with acute infection (OIE, 2008b; Jasim and Ayyal, 2018).

Figure 2.1 shows a toxoplasmosis life cycle, in which unsporulated oocysts are shed in the feces of the cat. Normally, large quantities of oocysts are shed for 1–2 weeks only (Areshkumar, Divya and Yasotha, 2018). Oocysts sporulate in the atmosphere for 1–5 days and become infective, depending on aeration, humidity and temperature (OIE, 2008b). Susceptible animals are infected after ingestion of sporulated oocysts, sporozoites are then released to penetrate the intestinal lining, which transforms into tachyzoites and causes

infection (OIE, 2008b; Ibrahim, 2017). Tachyzoites are present in the neural and muscle tissue and grow into bradyzoites of the tissue cyst (Tenter, 2000; Ibrahim, 2017).

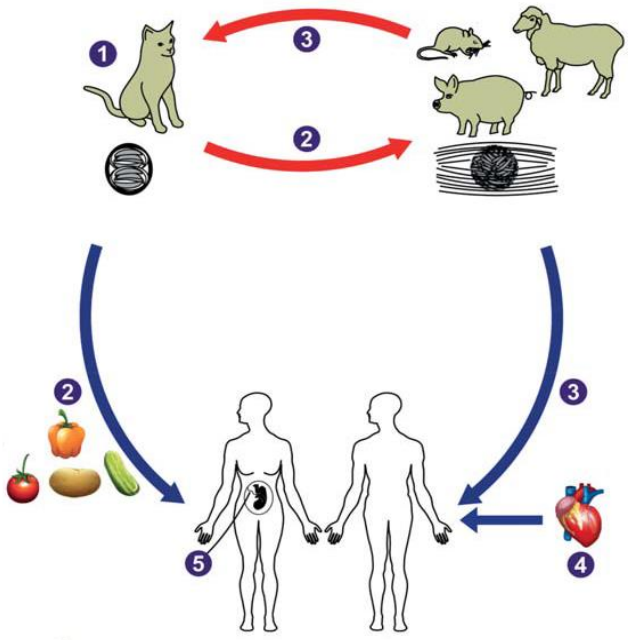


**Figure 2.1:** Life cycle of *T. gondii.* Infective pathogens are spread in the form of oocysts (spores) shed in the faeces of infected cats (1) *T. gondii* is transmitted by contaminated food to domestic animals and rodents as well as to humans. (2) Cats acquire the disease while feeding, for example, infected rodents that contain tissue cysts. (3) The same occurs to humans when undercooked food from infected animals is eaten, (4) through infected tissue during transplantation. (5) and congenital placental transmission (adapted from Pleyer *et al.*, 2019).

## 2.4 Epidemiology

*Toxoplasma gondii* is spread worldwide, but the spreading varies across different geographic locations of a region, across different geographic locations within the socio-economy, with no specificity in warm-blooded animals (Tenter, 2000). The degree of the protozoan's natural spread is determined by the environmental conditions. The parasite is most common in warm and low-lying climates than in cold ones and areas with mountains and wetlands than in dry areas (Dubey, 1991; Pal, Alem and Tuli, 2014; Ibrahim, 2017). Sporulated oocysts can survive for months and years under mild environmental conditions in moist soil, (Dubey, 2004; Hill, Chirukandoth and Dubey, 2005; Dubey and Lindsay, 2006).

### 2.4.1 In animals

Since felines are widespread and produce large quantities of oocytes, domestic cats are likely the main source of contamination of feed, plants, soil and water (Hill, Chirukandoth and Dubey, 2005). The rate of infection in naturally infected cats is measured through the infection of native rodent and avian populations as cats mostly acquire the infection by preying on such animals through different serological and molecular detection methods (Hill, Chirukandoth and Dubey, 2005). The more oocytes present in the environment, the greater the likelihood of infection of prey animals, resulting in an increased degree of infection in cats (Hill, Chirukandoth and Dubey, 2005). Seroprevalence in wild felids is frequently very high, reaching up to 100% in some cases.(Robert-Gangneux and Dardé, 2012).

Some marine species (sea otters, dolphins, seals, and walruses) have been reported to be infected, with prevalence ranging from 47 to 100 percent (Robert-Gangneux and Dardé, 2012). These marine animals act as sentinels for oocytes pollution of the environment through freshwater flow into the marine ecosystem (Conrad *et al.*, 2005).

Prevalence in poultry can differ significantly depending on the production system (Robert-Gangneux and Dardé, 2012). *Toxoplasma gondii* infection is almost non-existent in industrialized poultry farms, although seroprevalence in free-range or backyard birds is typically high and can be up to 100 percent (Robert-Gangneux and Dardé, 2012).

In Southern European countries, sheep are the main source of infected meat with the seroprevalence ranging from 17 to 22 percent in lambs to 5 to 89 percent in adults (Dubey, 2009b; Robert-Gangneux and Dardé, 2012). Seropositivity rates in goats range from 4 to 77 percent (Dubey *et al.*, 2008). Calves show higher rates of infection than adult cattle's during their initial grazing season, indicating that they become infected after being exposed to *Toxoplasma gondii* on pastures (Opsteegh *et al.*, 2011).

In Africa, several studies conducted on animal toxoplasmosis have shown the variability in the level of infection within different animal species and areas, with high prevalence being observed in chickens and low prevalence in cattle (Tonouhewa et al., 2017). Seroprevalence studies conducted in SA have reported the *T. gondii* to be 5.6% in sheep (Samra *et al.*, 2007), 8% in sheep of the Western Cape province (Hammond-Aryee, Van Helden and Van Helden, 2015) and 83.33 % in farms of the south-eastern region with sheep in the farms having the highest rate of infection of 64.46%, followed 53.91 % in goats (Tagwireyi, Etter and Neves, 2019). In the US, cattle infections are less prevalent when compared to sheep and pigs,

however, reviews conducted in European countries using serological and molecular assays to detect the parasite have shown negligible rates of infection in pigs and horses when compared to cattle and sheep (Tenter, 2000).

The largest non-feline reservoir of *T. gondii* is sheep and goats, especially pregnant or perinatal ewes (Areshkumar, Divya and Yasotha, 2018). Pigs usually become infected through the ingestion of oocytes from polluted soil and ingesting tissues from infected animals or by the prenatal transmission of the parasite transplacental (Dubey, 2009a).

### 2.4.2 In humans

Veterinarians, abattoir workers and cat owners have a high rate of infection (Ibrahim, 2017). A report by the CDC shows that an estimate of 11 percent of the 6-year-old population has been infected with the parasite in the US and different locations all over the world. It is estimated that *T. gondii* infects 2 billion people worldwide with the majority of affected individuals remaining asymptomatic, making it the most widespread parasite of humans and animals (Fern, 2019; Pleyer *et al.*, 2019). The World Health Organization (WHO) reports that 20 percent of the risk of food-borne disease in Europe results from *T. gondii* infection.

The first serological survey of toxoplasmosis from SA was first conducted in 1974 in the Transvaal and reported 37% seroprevalence (Monika and Paul, 2014). The survey found incidences of infections among Indians, Coloureds Whites, and Blacks to be 58%, 43%, 33% and 29% respectively (Mason, Jacobs and Fripp, 1974). A more recent study on the seroprevalence of the disease was conducted in Gauteng and reported a seroprevalence of 9.8% (Kistiah *et al.*, 2011). In Africa (Benin, Burkina Faso, Cameroon, DR Congo, Ethiopia, Ivory Coast, Nigeria, Rwanda, Tanzania and Zambia), a systematic review and meta-analysis on toxoplasmosis infection reported an overall prevalence of 51.01% (Dasa *et al.*, 2021).

## 2.5 Genotyping and genotypes

Genotyping provides information on the genotypes that are common throughout various geographic areas, which is helpful for phylogenetic and molecular epidemiology studies to identify the origin of infections and outbreak investigations to establish a link between genotypes and clinical forms of the disease (Tibayrenc *et al.*, 2002; Gebremedhin *et al.*, 2014).

The genotypes of *T. gondii* isolates can be determined using some genetic markers, including as the surface antigens SAG1 to SAG4, MAG1, 850, L328, 62, BSR4 and SRS1 to 3, and excretory-secretory antigens GRA1 to GRA4, GRA6 and ROP1 (Howe and Sibley, 1995; Maryam *et al.*, 2016; Lachkhem *et al.*, 2021a). Dense granule antigens, also known as GRA proteins, are one of the most well-known markers (Maryam *et al.*, 2016). They are typically produced in tachyzoites, but they are also present in encysted stages and bradyzoites (Maryam *et al.*, 2016).

The first genotyping studies on *T. gondii* strains used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on a small number of laboratory strains and isolates, mostly from France and the US, and they resulted in the description of a clonal population structure with three main lineages, type I, II, and III (Howe and Sibley, 1995; Montoya and Liesenfeld, 2004; Lachkhem *et al.*, 2021a). All these linages are related to virulence in mice with pathogenicity in mice and variability in certain genetic markers, such as the B1 and SAG genes used to classify strains during microsatellite analysis, multilocus sequence typing (MLST), PCR-RFLP, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), and high-resolution melting (HRM) analysis (Howe and Sibley, 1995; Miller *et al.*, 2004; Montoya and Liesenfeld, 2004; Dardé, 2008; Abd *et al.*, 2015; Lachkhem *et al.*, 2021b). The three dominant *T. gondii* genotypes (type I, II, and III) emerged from one or more historically recent events of sexual recombination between two separate *T. gondii* ancestral lineages among felid definitive hosts, according to studies using SAGE 1, SAGE 2, ROP1, 850, L328, and 62 markers (Howe and Sibley, 1995; Su *et al.*, 2003; Montoya and Liesenfeld, 2004).

Strains of *T. gondii* show diverse virulence or relative pathogenicity in a single species, and this may change with host adaptation (Su *et al.*, 2010). Although the difference between the three genotypes is less than 1% at the genome sequence level, they have completely diverse virulence phenotypes (Dubey *et al.*, 2007; Dardé, 2008). Virulence in one species does not necessarily correlate with virulence and genotypes in another species and different parts of the world (Grigg and Sundar, 2009).

A study on the genetic characterization of *T. gondii* discovered another clonal lineage (genotype 12) using PCR-RFLP in the wildlife of Northern America with types that were previously identified as types A and X of sea otter breeds using PCR-RFLP method (Dubey *et al.*, 2011; Rajendran, Su and Dubey, 2012). Type II and type III strains are more prevalent in

Europe and North Africa, whereas types II, type III, and type 12 are dominant in wildlife in North America, while type I found in China is predominant in East Asia (Rajendran, Su and Dubey, 2012; Saraf *et al.*, 2017). The types found in Africa and South America have more genetic diversity compared to the ones found in North America and Europe (Rajendran, Su and Dubey, 2012; Zheng *et al.*, 2016). There is no available information about the strains (genotypes) prevalent in South Africa. Table 2.1 shows a summary of the geographic distribution of *T. gondii* genotypes as determined by microsatellite genotyping and PCR-RFLP and its probable link to human toxoplasmosis.

**Table 2.1:** Geographic distribution of *T. gondii* genotypes (Robert-Gangneux and Dardé, 2012)

| Geographic distribution | Genotypes |
|---|---|
| **Africa** | African 1, 2, and 3 (haplogroup 6); type III (haplogroup 3); type II. |
| **Asia** | Type III (haplogroup 3), a common haplogroup found across the continent, has less genotypic variation than the ones in South America. |
| **Europe** | Type II (haplogroup 2) is the most common; type III is more common in South Europe, and other genotypes are seen infrequently. |
| **North America** | Type II (haplogroup 2), haplogroup 12, type III (haplogroup 3). |
| **South and Central America** | High genotypic diversity; some haplogroups are shared with Africa (haplogroup 6); type II occurs infrequently; type I is uncommon; very atypical genotypes in the Amazonian Forest |

## 2.6 Virulence

*Toxoplasma gondii* virulence is described as the number of tachyzoites needed to kill a mouse after intraperitoneal injection using different strains for complete virulence ranges. This can differ from maximum virulence (lethal one single tachyzoite) to avirulence (killing no matter what the dose) (Keane *et al.*, 2011; Dubremetz and Lebrun, 2012; Hassan *et al.*, 2019). The fact that this parasite is so widespread that it may infect an extensive spectrum of hosts, with the sole constraint being coldblooded animals, and this makes defining *T. gondii* pathogenicity difficult (Dubremetz and Lebrun, 2012; Li *et al.*, 2014). The range of hosts, susceptibility to infection and the acute form of the disease, is quite diverse. Mice can die in a few days and rats

can be completely resistive, demonstrating that virulence is influenced by both parasite and host variables (Dubremetz and Lebrun, 2012).

Because the mouse is the most common laboratory host for *T. gondii*, many studies have focused on this model, and *T. gondii* virulence is mostly defined in terms of mouse infection, leading to a lot of uncertainty when defining virulence factors, because other hosts, particularly humans, may behave quite differently than rodents (Dubremetz and Lebrun, 2012; Robert-Gangneux and Dardé, 2012; Hassan *et al.*, 2019).

Type I and III genotypes have different patterns of virulence in mice, with type I being highly virulent at a 100 percent lethal dose in mice following parasite dosage (Taniguchi *et al.*, 2018). At low doses of infection, genotypes (type II, III, and African 1, 2, 3 types) that are prevalent in Europe, Asia, North America and North Africa, are not lethal to mice, while large quantities of *T. gondii* in South America's strains are very virulent and lethal to mice (Shwab *et al.*, 2016).

### 2.6.1 Virulence factors

Virulence is known as the ability of a protozoan to cause disease in the host (Batt, 2016). It is therefore vital to define the virulence factors for *T. gondii* to provide possible therapeutic targets as well as to shed light on the general biology and evolution of Apicomplexans (Weilhammer and Rasley, 2011). Given that some strains of *T. gondii* are inherently considerably more virulent than others in mouse models, this parasite offers an intriguing framework for the analysis of virulence mechanisms (Howe and Sibley, 1995; Weilhammer and Rasley, 2011). *Toxoplasma gondii* has effectors that modify host cells in different ways, either leading to better parasite conditions, intracellular resistance to innate immune defence, or homeostasis modulation of the host immune system to control secondary response (Dubremetz and Lebrun, 2012).

Most of these factors are secreted during the invasion and passed to the cytosol host cell where they interfere with the work of host cells (Dubremetz and Lebrun, 2012). This process involves the folding of the host plasma membrane into a parasitophorous vacuole (PV), where the parasite undergoes numerous rounds of replication (Pernas and Boothroyd, 2011; Robert-Gangneux and Dardé, 2012). The PV membrane (PVM), which forms the actual interface between the parasite and the host cell is a highly specialized, unique membrane that typically lacks important host cell proteins but is greatly modified by secreted *T. gondii* proteins from rhoptries and dense granules, distinct secretory organelles that release their contents during invasion (Bradley and Sibley, 2007; Robert-Gangneux and Dardé, 2012).

These rhoptry proteins (ROP) are among more than 8300 genes encoded in *T. gondii* genome, and the kinase homologues lacking the catalytic triad needed for enzymatic action were the first to be described (Dubremetz and Lebrun, 2012). Their role was mysterious for close to 20 years until instigations on genomic and proteomic levels showed that some family members were true kinases, which led to more interest in their study, revealing that they are transferred to the host cell at the time of invasion (Dubremetz and Lebrun, 2012). The current knowledge on the interaction of *T. gondii* and these proteins is essential to the control of inflammation at multiple levels depending on the ROP protein concerned (Dubremetz and Lebrun, 2012). Their interference leads to a huge variance in virulence between strains as the genetic difference between these proteins has emerged as a major factor in the outcome of an infection, which can act at two main levels leading to infection (Dubremetz and Lebrun, 2012). At the first level, the Toll-like receptors (TLRs) stimulate antigen-presenting cells (APCs), resulting in NFkB activation and nuclear translocation, which activates the transcription of proinflammatory cytokines like IL12 and 18, which activates the production of interferon-gamma (IFNg) by T lymphocytes and NK cells (Dubremetz and Lebrun, 2012). In the second level, IFNg-activated infected cells will activate Interferon Regulated GTPases, which are capable of destroying the previously invulnerable PV, and virulent parasites will employ this second line of defence (Dubremetz and Lebrun, 2012).

According to reports, certain polymorphic rhoptry proteins including ROP18, ROP5, ROP16, and ROP17 are responsible for *T. gondii* virulence and that virulence-associated to ROP18 and ROP5 is the allelic forms (Dubremetz and Lebrun, 2012; Shwab *et al.*, 2016). Immunity-related GTPases (IRGs) are disrupted by this pair of effectors (Behnke *et al.*, 2012; Rêgo *et al.*, 2017). In mice IRGs allow IFN-γ to regulate toxoplasmosis (Taylor, Feng and Sher, 2007; Howard, Hunn and Steinfeldt, 2011). *Toxoplasma gondii* pathogenicity is mediated by ROP16 and ROP17 (Taniguchi *et al.*, 2018). ROP16 is essential for the regulation of the host's innate immune response by STAT3/6 activation, while ROP17 aids in preventing the clearance of the parasite by host cells (Etheridge *et al.*, 2014).

The genes of the *T. gondii* virulence in mice have a strong bias towards variations in these effectors, as at least three of the effectors (ROP 5,16 and 18) have been identified through genetic crosses and the mapping of major virulence genes in the progeny (Fentress and Sibley, 2011; Dubremetz and Lebrun, 2012; Saeij *et al.*, 2014). Because mice are a natural host in the parasite life cycle, the link between some of these factors and IRGs that are extensively expressed in mice but not in other species or humans may be biologically important

(Dubremetz and Lebrun, 2012). As a result, the potential influence of these virulence factors on other species and human toxoplasmosis infections is limited (Dubremetz and Lebrun, 2012).

## 2.7 Host range susceptibility

*Toxoplasma gondii* normally parasites the host without causing clinical illness (Hill, Chirukandoth and Dubey, 2005). Toxoplasma occurs in two forms: the free proliferative form present during acute infections and the cyst form which is connected to antibody production in the host (Mcgirr, 1968). The free form of the cyst is well accommodated by the host and may stay dormant in animal tissues for life (Robert-Gangneux and Dardé, 2012)

Cats play an important role in the epidemiology of the disease and the disease has not been shown to be virtually present in areas with no cats (Sarvi *et al.*, 2015; Ibrahim, 2017). For instance, studies in the USA indicate that 60the per cent of cats were serologically positive for toxoplasma antigen, with the rest being infected through hunting (Ibrahim, 2017).

Toxoplasmosis occurs in domestic animals, wildlife, and poultry worldwide. However, prevalence varies in species. Seroprevalence in cattle is doubtful since cattle are not a good host for the parasite, though they may be infected (Mcgirr, 1968; Smith, 1991; Dubey, 2000; Hill and Dubey, 2013). The parasite is maintained in the environment by other wildlife, especially wild cats, through tissue cysts, which serve as a source of infection for predators and scavengers, as well as transmission to offspring (VanWormer *et al.*, 2013). Domestic cats and hunting dogs can be infected by a variety of wild small animals, including rodents and birds which are the source of infection (VanWormer *et al.*, 2013).

The epizootic disease in pigs has been studied in the USA, signs, lesions and the pathogen have been found in the lungs, lymph nodes, liver, and kidneys of piglets, and toxoplasma were recovered after mouse inoculation with material from the brain of the piglets' mother (Ibrahim, 2017).

Sheep and goats are *T. gondii's* primary non-feline reservoir, particularly pregnant or perinatal ewes, and their unpasteurized milk or milk-derived cheese may be contaminated by the organism (Areshkumar, Divya and Yasotha, 2018). Lopez et al 2013's study on sexual transmission of toxoplasma found that ewes negative for all reproductive pathogens became infected with the parasite after mating with seropositive male sheep, proving that sheep semen can also be a source of infection for sheep (Lopes *et al.*, 2013). Pregnant sheep and goats that

become infected mostly during the intensive feeding cycle before lambing, processed food polluted with cat faeces containing oocysts and congenitally (Lopes *et al.*, 2013; Ibrahim, 2017). After orally infecting seronegative males with *T. gondii* oocytes and allowing them to naturally mate with seropositive breeder female goats, Santana et al. 2013 were able to prove that male goats can sexually transmit *T. gondii*. The presence of antibodies against *T. gondii* was tested after mating using the ELISA test specific antibodies against *T. gondii* after mating and PCR analysis of semen samples, female foetal tissues, and the placenta revealed that ten of the twelve females utilized in the study had the parasite in their tissues (Santana *et al.*, 2013).

## 2.8 Source of infection and transmission

### 2.8.1 Source of infection

Cat faeces serve as the sole source of infection for cattle, goats, sheep, and horses while cats become infected from ingesting tissues of the intermediate hosts (rodents and birds) infected with *T. gondii* as demonstrated in figure 2.2 (VanWormer *et al.*, 2013; Ibrahim, 2017). Cats are infected through ingestion of intermediate hosts infected tissues with rodents and small birds being the most common, but all animals can also be intermediate hosts of the parasite (VanWormer *et al.*, 2013). Rodents remain a source of infection for a long period (Ibrahim, 2017).

Following the development of sexual forms in the cat's intestinal epithelium (the only species in which this occurs), oocysts are shed in cats' faeces but are not infective for about 72 hours (Hill, Chirukandoth and Dubey, 2005; Markey *et al.*, 2013). Faecal oocysts are shed in large quantities, particularly by young cats, and are highly resistant to climate conditions with the potential of causing infections in animals and humans (Markey *et al.*, 2013; Ibrahim, 2017). Tissue cysts containing bradyzoites are the form most frequently seen in the tissues of animals, but in acute toxoplasmosis, tachyzoites may also be present (Markey *et al.*, 2013).

Another potential source of *T. gondii* infection in humans is tissue cysts from game meat and other wild animal meat (Tenter, 2000). During evisceration and game handling, hunters and their families may also become contaminated (Dubey, 1991).

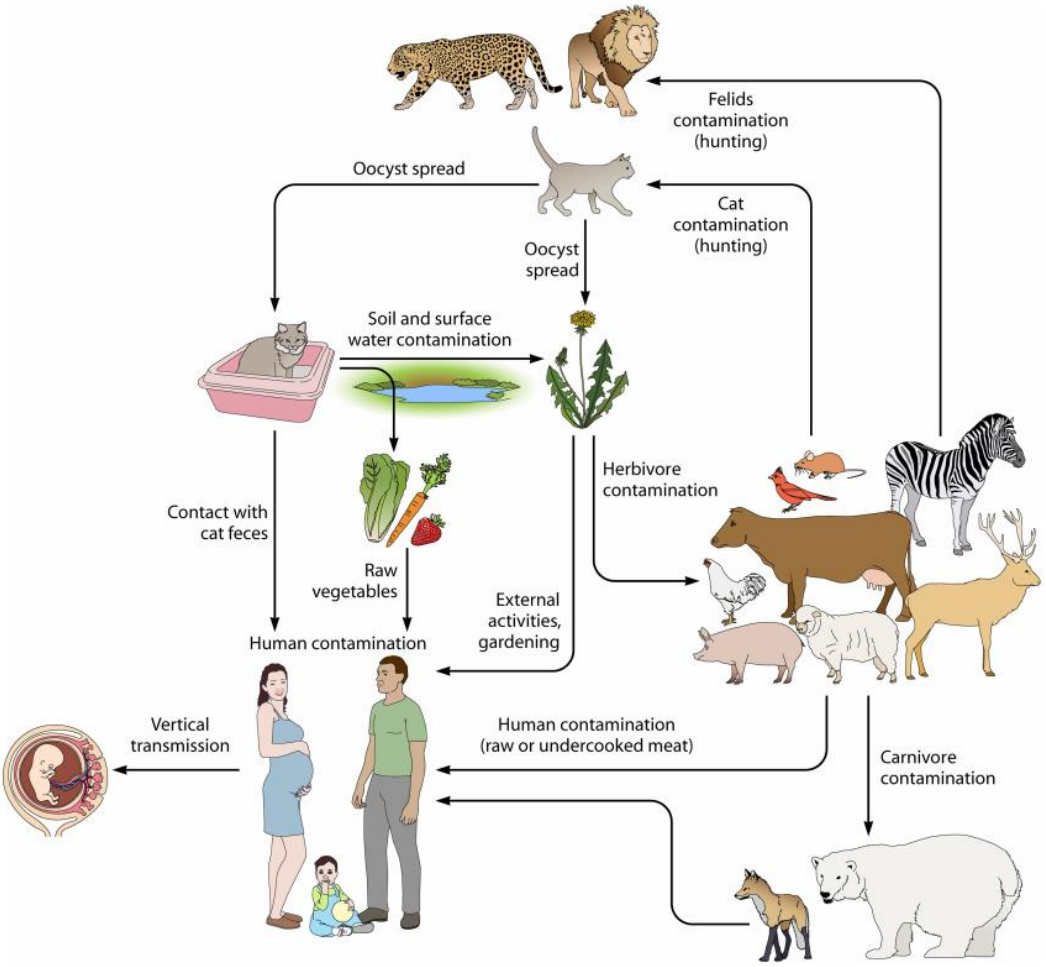


**Figure 2.2:** Sources of *T. gondii* infection (adapted from Robert-Gangneux and Dardé, 2012).

### 2.8.2 Transmission

Unlike most Apicomplex parasites, *T. gondii* can be transmitted horizontally through carnivorism or vertically from the mother to foetus between secondary hosts as demonstrated in Figure 2.3 (Blader and Saeij, 2009).

#### 2.8.2.1 In animals

##### 2.8.2.1.1 Transmission in cats

Cats play an important role in the transmission of toxoplasmosis. According to epidemiological data, the majority of cats acquire the infection in nature immediately after weaning, either by eating raw pet food or sharing food brought by the dam, thus *T. gondii* infections are higher in feral cats than in domestic cats (Dubey, 1991).

Cats often defecate on the soil and in the hay, barns, food bins, gardens, and flowerpots (Ibrahim, 2017). Cat faeces are usually hard and can stay limited to the region where defecation occurred for a longer period unless they are ill, few or no faeces stick to their anal area because of their leaching (grooming) (Dubey, 1991). The risk of transmission to humans through touching or caring for a cat is therefore low (Dubey, 1991).

#### 2.8.2.1.2 Transmission in livestock

Intermediate hosts can get *T. gondii* infection through ingestion of feed and drinking water contaminated with sporulated oocytes from the environment (Elsheikha *et al.*, 2009; Shwab *et al.*, 2016; Dubey *et al.*, 2020).

Experimental studies indicate that viable cysts in tissues can persist for life in animals (Dubey, 1991). In sheep, goats, pigs, and rabbits, tissue cysts are more common than in cattle, horses, and commercially raised fowl (Dubey, 1991). Cattle and buffaloes have an innate resistance to *T. gondii* and can eliminate tissue cysts from their tissues (Dubey, 1991).

#### 2.8.2.1.3 Transmission in wildlife

*Toxoplasma gondii* infection and clinical toxoplasmosis can affect a wide range of wildlife animal species (Wyrosdick and Schaefer, 2015). The white-tailed deer is a species of importance as a sentinel host for domestic herbivores because of its proclivity to graze alongside livestock and its widespread distribution (Wyrosdick and Schaefer, 2015). Because of several factors, including poor DNA material from naturally infected wildlife due to the low density of *T. gondii* in tissues of asymptomatic animals, and difficulties in preserving and transporting tissue samples from remote areas, isolation of *T. gondii* from wildlife is difficult and time-consuming (Dubey *et al.*, 2011; Vitaliano *et al.*, 2014).

#### 2.8.2.1.4 Vertical transmission

A study conducted by Franco et al., 2011 checked vertical transmission of *T. gondii* in mice and showed that vertical transmission occurs when females are infected primarily during pregnancy. In the study, females were infected with the *T. gondii* cyst before pregnancy and were also re-infected on the first day of pregnancy (Franco *et al.*, 2011). Then animals were killed and placenta and embryos were collected and processed on the 19th day of pregnancy for morphological investigation, immunohistochemistry, and parasite detection using PCR and a mouse bioassay (Franco *et al.*, 2011). Only placental tissues were shown to have parasites, according to morphological and immunohistochemical investigations (Franco *et al.*, 2011).

Only mice inoculated with placental material demonstrated seroconversion in the mouse bioassay and *T.gondii* DNA was also only found in placental samples (Franco *et al.*, 2011).

#### 2.8.2.2 Transmission in humans

##### 2.8.2.2.1 Horizontal transmission

Intermediate hosts acquire *T. gondii* infection horizontally through ingestion of infected animal tissues, consumption of food, water, or milk contaminated with sporulated oocytes or soil and tissue cysts in undercooked meat or meat by-products (Elsheikha *et al.*, 2009; Guo *et al.*, 2015; Deshmukh *et al.*, 2021; Fazel *et al.*, 2021).

##### 2.8.2.2.2 Vertical transmission

If primary infection occurs during pregnancy, the congenital transmission may occur from mother to foetus (Blader and Saeij, 2009; Guo *et al.*, 2015). After the maternal infection, the parasite enters the foetal bloodstream through placental penetration (Montoya and Liesenfeld, 2004; Colf *et al.*, 2020). Before pregnancy, the maternal infection presents little or no threat to the foetus except in women who become infected a few months before conception (Tenter, 2000; Santana *et al.*, 2013; Lopes *et al.*, 2013; Colf *et al.*, 2020). Only a small amount of *T. gondii* infections in adult human populations are acquired vertically (Tenter, 2000).

The frequency of congenital transmission varies depending on the time the mother was infected during gestation (Montoya and Liesenfeld, 2004). Transmission rate and disease severity are inversely related (Montoya and Liesenfeld, 2004). Infection acquired during the first and second trimesters has the potential to be transmitted to the foetus, resulting in severe congenital toxoplasmosis leading to abnormality, abortion and foetal death. (Montoya and Liesenfeld, 2004; Lopes *et al.*, 2013; Guy, 2014; Guo *et al.*, 2015).

#### 2.8.2.3 Organ transplants

Seropositive donors may transmit the disease to seronegative recipients of organ transplants (Montoya and Liesenfeld, 2004). *Toxoplasma gondii* may also be transmitted by immunocompromised donors through blood or leucocytes, but these transmission modes are less common than cyst and oocyte transmission (Dubey, 1991; Irma and Nasronudin, 2015).

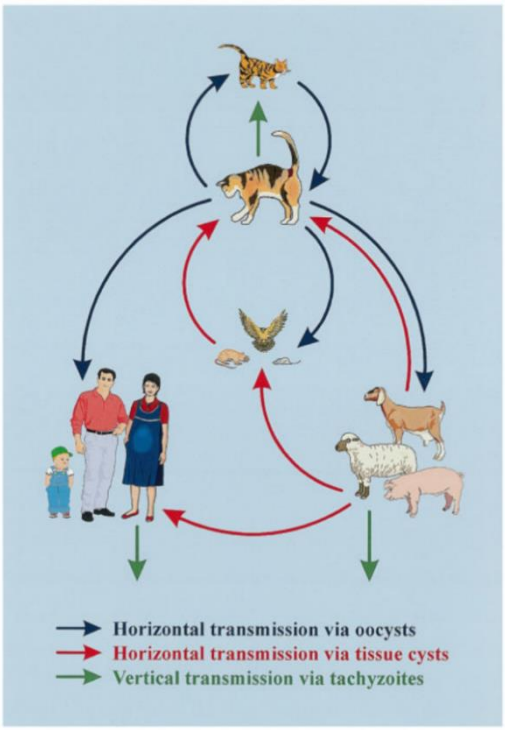


**Figure 2.3:** Major routes of *T. gondii* transmission (sourced from Tenter, 2000).

## 2.9 Risk factors

The great majority of factors (age, gender, environmental and management practices, animal production system, climate and feed storage, water source, and presence of cats on the farm) have been discovered to pose a risk for the spread and transmission of *T. gondii* within the animal population (Deng *et al.*, 2016; Tagwireyi, Etter and Neves, 2019). With pathogen risk factors, there are no tests that can distinguish the route of infection between oocysts and tissue cyst ingestion (Pal, Alem and Tuli, 2014). The available proof of oocysts' route of infection is based on epidemiological surveys (Pal, Alem and Tuli, 2014).

### 2.9.1 Pathogen risk factors

Oocysts are very resistant to outside conditions such as short periods of cold and dehydration, which enables their environmental survival for at least one year and can also survive from 2 to 4.5 years in marine and freshwater (VanWormer *et al.*, 2013; Ibrahim, 2017; Areshkumar, Divya and Yasotha, 2018). They can however be destroyed by exposure to temperatures between 90 º C for 30 seconds and 50 º C for 2.5 minutes (Ibrahim, 2017). Although tissue cysts are less resistant to external conditions than oocytes, they are more resistant to temperature changes and can survive for up to three weeks in chilled (1–4 °C) carcasses or

minced meat (Dubey *et al.*, 1990; Tenter, 2000; Dubey, 2009b; Ibrahim, 2017). Tissue cysts may also withstand freezing for more than a week at temperatures between 1 and 8°C (Tenter, Heckeroth and Weiss, 2000; Mirza Alizadeh *et al.*, 2018).

### 2.9.2 Farm environment and management risk factors

Farm environment such as bedding, contaminated water source and control have an impact on the spread of infection as they may be contaminated with oocysts (Samra *et al.*, 2007; Andrade *et al.*, 2013). Some studies indicate the cat's presence on the farms and direct contact with farm animals as the principal cause of the infection as cats can shed oocysts in their feces which results in the horizontal transmission of *T. gondii* in intermediate hosts (Pinheiro *et al.*, 2009). An increased prevalence has been found in animals who drink water from the municipal water source than in a pond and mixed water sources (Tilahun *et al.*, 2018).

The protozoan is mostly found at lower altitudes and in warm, hot and humid climates (Hammond-Aryee, Van Helden and Van Helden, 2015). A high infection rate has been shown in sheep as a result of high rainfall, which is a favourable environment for longer survival of oocytes on pasture (Ibrahim, 2017).

### 2.9.3 Human risk factors

Risk factors associated with contracting toxoplasmosis in humans arise from new-borns whose mothers are diagnosed with toxoplasmosis during pregnancy and people who are immunocompromised such as those with HIV/AIDS (Grigg and Sundar, 2009; Julie *et al.*, 2019).

### 2.9.4 Age and gender of animals

Studies conducted by Tagwireyi et, al, (2019) and Deng et al, (2016) found that the age of the animals are a risk factor as adult animals are more susceptible to getting infected than young animals who are younger than 1 year (Deng *et al.*, 2016; Tagwireyi, Etter and Neves, 2019). This might have to do with the fact that younger animals are more immune to infections than adult animals (Samra *et al.*, 2007). Other studies conducted on the seroprevalence of *T. gondii* in ruminants noted a higher prevalence in female than male animals (Guimarães *et al.*, 2013a; Tegegne *et al.*, 2016; Tilahun *et al.*, 2018). Females' higher sensitivity may be linked to their poorer immunologic resistance during stages of their lives when they experience hormonal changes and imbalances (Alexander and Stimson, 1988; Van Der Puije *et al.*, 2000; Guimarães *et al.*, 2013b).

### 2.9.5 Breed and species

The prevalence of *T. gondii* is higher in sheep and goats than in all the other animal species and different breeds within the species, proving that the type of breed is also a risk factor for the spread and transmission of *T. gondii* (Carneiro *et al.*, 2009; Sarvi *et al.*, 2015; Tagwireyi, Etter and Neves, 2019; Chaklu *et al.*, 2020).

## 2.10 Pathogenesis

The number, genetic diversity and immune adaptation of the pathogen play a vital role in its pathogenesis. The parasite infection is acquired through carnivorism, oocytic and congenitally (Ajioka, Fitzpatrick and Reitter, 2001; Wang *et al.*, 2013; Kadle, 2014).

Upon ingestion of uncooked meat containing tissue cysts or feed infected with cat faeces containing oocytes in warm-blooded animals, these cysts walls are digested inside the host stomach and release bradyzoites that are immune to gastric peptidases and eventually invade the small intestine and initiate extra-intestinal replication. (Tenter, 2000; Blader and Saeij, 2009; Ibrahim, 2017). Bradyzoites and sporozoites are released respectively and infect intestinal epithelium (Ibrahim, 2017). They then convert into tachyzoites after several rounds of epithelial replication, and then rapidly grow the disease-causing form that spread via the bloodstream and the lymph (Tenter, 2000; Blader and Saeij, 2009; Ibrahim, 2017). Tachyzoites invade and infect tissue throughout the body and replicate intracellularly until the cells burst (Tenter, 2000; Ibrahim, 2017). Young and immunocompromised animals will give in to generalized toxoplasmosis at this point, while older animals grow a strong cell-mediated immune response to tachyzoites (Ibrahim, 2017). Tissue cysts are usually seen in neural and muscle tissues following the development of bradyzoites and are typically located in the central nervous system (CNS), the brain, and skeletal and cardiac muscles. (Tenter, 2000; Ibrahim, 2017). The tissue cysts can live in the host for a lifetime with no apparent clinical signs in healthy animals (Ajioka, Fitzpatrick and Reitter, 2001; Otranto *et al.*, 2015).

## 2.11 Clinical signs and symptoms

Toxoplasmosis occurs in four forms; subclinical, sub-acute, acute and chronic, depending mostly on the host's immune system (Guy, 2014; Ibrahim, 2017; Areshkumar, Divya and

Yasotha, 2018). Most cases of exposure do not cause clinical signs (Innes *et al.*, 2019; Pleyer *et al.*, 2019). The sub-acute infections are the ones with few or no visible clinical signs and lead to sudden death. The acute form is the result of tachyzoite tissue infection and tissue reactions (Ajioka, Fitzpatrick and Reitter, 2001).

*Toxoplasma gondii* affects different tissues and the clinical signs are dependent on the tissue involved (Ibrahim, 2017). Lungs, liver, brain, lungs, placenta, ears, spleen, lymph nodes, and adrenal glands are the most frequently affected tissues during the acute phase (Ibrahim, 2017). The chronic form is associated with the presence of parasite tissue cysts that contain bradyzoites that divide slowly (Ajioka, Fitzpatrick and Reitter, 2001). Bradyzoites remain inactive within cysts and do not cause tissue reactions (Ibrahim, 2017). The majority of infections are acquired through the gastrointestinal tract (Ibrahim, 2017). The clinical condition and the cause of toxoplasmosis differ between species and age groups (Montoya and Liesenfeld, 2004; Ibrahim, 2017).

### 2.11.1 In animals

*Toxoplasma gondii* infections in cats are mostly asymptomatic and rarely occur through vertical transmissions (Pal, Alem and Tuli, 2014). Dormant *T. gondii* infections are popular worldwide in domestic cats and wild felines (Tenter, 2000).

In cattle, the disease usually takes an acute course which results in fever, dyspnoea, and early nervous symptoms, accompanied by severe laziness and stillbirth, but it is not substantially involved in causing bovine abortion (Ibrahim, 2017). Pigs are highly sensitive and can be influenced by all ages (Ibrahim, 2017). The major symptoms of sheep and goats are foetal resorption, abortion, mummified lambs and death (Dubey, 2009b; Ibrahim, 2017; Ishaku *et al.*, 2018). In horses, the disease is rare however, subclinical infections occur accompanied by atypical clinical symptoms such as ataxia, fever, encephalomyelitis, retinal degeneration, as well as abortion or stillbirth in pregnant horses (Miao *et al.*, 2013; Ibrahim, 2017). Natural fowl outbreaks have been reported and as the result, the parasite was transmitted to mice (Ibrahim, 2017).

### 2.11.2 In humans

Although most human infections are asymptomatic, some result in clinical signs such as mild fever and swelling of lymph nodes which may continue for 1 to 12 weeks (Montoya and Liesenfeld, 2004). Pregnant women are at high risk of acquiring clinical toxoplasmosis as *T.*

*gondii* can present a danger to the foetus if they become infected for the first time at the early stages of pregnancy, leading to chorioretinitis, microcephaly, hydrocephalus and stillbirth (Guy, 2014; Areshkumar, Divya and Yasotha, 2018). Immunocompromised individuals, such as those living with HIV/AIDS, can develop significant illnesses ranging from diarrhoea, pneumonia, and liver disease to weight loss and central nervous system infection, and even death in severe cases (Shah *et al.*, 2013; VanWormer *et al.*, 2013).

## 2.12 Diagnosis

### 2.12.1 Histopathology

During a *T. gondii* infection, tachyzoites are converted into bradyzoites with the initiation of an immune response which slowly replicates in the cells to produce tissue cysts. There are often difficulties in finding *T. gondii* in aborting cows, goats and pigs; however, they might be seen in brain and placenta sections (OIE, 2008b). An autopsy may be performed in dead animals and aborted foetus to check for signs of toxoplasmosis (OIE, 2008a). Immunoperoxsidase staining technique in tissue parts or infected body fluids may reveal tachyzoites formation (Irma and Nasronudin, 2015). Confirmation for the identification of structures that looks like *T. gondii* in tissue sections from autopsies and the acute form of toxoplasmosis may be accomplished by immunohistochemistry which marks intact *T. gondii* (OIE, 2008b). This method is practical and sensitive as it may also be used for decomposed fixed tissues that cannot be used for isolation (OIE, 2008b).

### 2.12.2 Microscopic examination

*Toxoplasma gondii*'s identification has historically relied on microscope analysis in faecal, soil, environmental and tissue specimens to distinguish the cyst of the parasite (Liu *et al.*, 2015). Light microscopy detection alone, however, is less sensitive and inaccurate (Liu *et al.*, 2015).

### 2.12.3 Faecal floatation

The faecal flotation method is performed on cat faeces to detect oocytes however, this method is not practical for the detection of *T. gondii* oocytes in cat faeces (Pal, Alem and Tuli, 2014). In fresh faeces unsporulated oocytes measure between 10 to 12 µm (Ibrahim, 2017). The existence of cysts in cat faeces does not indicate a relationship to clinical disease, as cysts can

be present in acute and chronic infections (OIE, 2008b). An active infection indicated by the presence of tachyzoites is in blood or body fluids (OIE, 2008b).

### 2.12.4 Mouse inoculation

Mouse neutralization assay may be used as a definitive diagnosis by inoculating suspicious substances into mice followed by an examination of exudates, tissues, or organs for the presence of tachyzoites or bradyzoites (Irma and Nasronudin, 2015; Ibrahim, 2017). The foetal brain and placental cotyledons are the best tissues for inoculation (OIE, 2008b). This test is regarded as a gold standard as it is highly sensitive and it can use larger tissue volumes, however, it is costly, time-consuming and has ethical problems (Sharma *et al.*, 2019).

### 2.12.5 Serological assays

For the detection of groups of *T. gondii* antibodies or antigens, many serological assays are available. The dye test (DT) and Indirect fluorescent agglutination test (IFA) microscope can show the colour of tachyzoites (OIE, 2008b). Other serological assays that are dependent on the principle of toxoplasma tachyzoites agglutination with red blood cells or latex particles includes; a direct agglutination test (DAT), modified agglutination test (MAT), an indirect haemagglutination test (IHAT) and latex agglutination (LA) test (Liu *et al.*, 2015). In an enzyme-linked immunosorbent assay (ELISA), the intensity of colour change defines the number of specific antibodies in a given solution in (OIE, 2008b).

IgM antibodies can be identified approximately 1 week after infection in the host and remain in the host for many months or years, which is why their detection without another test is inadequate for acute infection (Ibrahim, 2017). IgA antibodies are regarded in acute infections that occur before the production of IgM antibodies and can remain in the host for months (Guy, 2014; Ibrahim, 2017). The shorter IgE cycle may provide a higher indicator of an infection that is current. IgG antibodies indicate the presence of infection, but it does not give details on the nature of the infection. (Liu *et al.*, 2015).

#### 2.12.5.1 Dye test

The Dye test is regarded as a gold standard although it is clear and sensitive for humans and not for other species (OIE, 2008b). The test is hazardous as it uses live parasites, requires a high level of technical expertise and is therefore carried out only in reference laboratories (OIE, 2008b; Liu *et al.*, 2015).

#### 2.12.5.2 Indirect fluorescent antibody test

This assay is easy to perform and commonly employed as a tool for detecting IgG and IgM toxoplasma antibodies. Diluted serum samples are incubated with killed toxoplasma tachyzoites with sufficient anti-species fluorescent serum and a fluorescent microscope is then used to view the results (OIE, 2008b; Liu *et al.*, 2015). Fluorescent-labelled antibodies and IFAT kits are being sold for various animals, and the procedure is affordable (OIE, 2008b). A fluorescence microscope is required to read the results, however, they are read virtually and individual variability can occur (Liu *et al.*, 2015). Many species-specific conjugates may be difficult to find and potential cross-reactivity with rheumatoid and antinuclear antibodies can occur (OIE, 2008b).

#### 2.12.5.3 Direct agglutination test

The direct agglutination test is sensitive as well as precise. Formalin-treated toxoplasma tachyzoites are incorporated in the well-microtiter plate in U-shaped form and diluted with a sera sample (OIE, 2008b). Samples will produce agglutination of various strengths if they are positive and precipitation of tachyzoites at the well's base if they are negative (OIE, 2008b).

#### 2.12.5.4 Modified agglutination test

This test is extensively used to detect *T. gondii* from sera of all animal species. In a U-shaped microtiter plate, toxoplasma tachyzoites fixed in formalin are inserted and diluted with the sample sera. Production of a thin agglutination MAT will indicate positive serum samples, while negative samples at the bottom of the plate will produce a compact pellet of precipitated tachyzoites (Liu *et al.*, 2015). The MAT may produce false-negative results in the early stages of infection or canine sera (OIE, 2008b).

#### 2.12.5.5 Latex agglutination test

The latex agglutination test (LAT) is often used for screening in the epidemiologic survey due to the simplicity of performance; however, positive result requires further serological confirmation (Tp, 2010). In LAT, the soluble antigen is coated on latex particles, and agglutination is observed after the addition of positive serum (OIE, 2008b).

#### 2.12.5.6 Enzyme-linked immunosorbent assay

The Enzyme-linked immunosorbent assay (ELISA) usually comprises a solid phase antigen or antibody, an enzyme-labelled antigen or antibody, and the enzyme reaction substratum that can

be changed to test antibodies and antigens (Liu *et al.*, 2015). Clinically, acute infections need to be differentiated from chronic infections (OIE, 2008b). With ELISA, toxoplasma-specific IgG and IgM antibodies together with IgA can allow for a degree of discrimination between acute and chronic toxoplasmosis (OIE, 2008b; Liu *et al.*, 2015; Khan and Khan, 2018).

### 2.12.6 Molecular assays

Several methods of detection of *T. gondii* are based on polymerase chain reactions (PCR) assay from tissue, body fluids, soil, water, and faecal nucleic acids have been developed. These assays are used for the diagnosis of toxoplasmosis in contrast to traditional serological methods and their findings are not based on the patient's immunological status (Su *et al.*, 2010). Normally, conventional methods are not deceptive, but they are restricted to prenatal cases or patients with an immunocompromised system (Su *et al.*, 2010). The advantages of using molecular methods include the need for small genetic material, the lack of confusing effects of environmental conditions and host, the examination of many samples in a short period and their high sensitivity (Azizi *et al.*, 2014).

#### 2.12.6.1 Conventional PCR

The PCR is based on the same principles for copying DNA as those found in nature. PCR is an active method for enzyme amplification that enables accurate DNA amplification of starting material in a short time from minute quantities (Su *et al.*, 2010; Liu *et al.*, 2015; Hanafiah *et al.*, 2018). Many multicopy targeting genes are typically used to detect *T. gondii* to achieve high sensitivity in biological samples, including the B1 gene, the 529 bp repeat element and the ITS-1 or 18S rDNA sequence (Su *et al.*, 2010; Liu *et al.*, 2015). Also used as PCR markers, are other single-copy genes including SAG1, SAG2, and GRA1 (Liu *et al.*, 2015). Copies of specific DNA fragments are usually analysed using an agarose gel and can be viewed using ethidium bromide staining and ultraviolet light illumination.

#### 2.12.6.2 Real-Time PCR

Real-Time-PCR can identify low target DNA concentrations and quantify copies of similar template DNA starting copies. It integrates amplification steps and PCR material detection in one cycle, increasing the turnaround time to less than 4 hours from 24 to 48 hours (Pal, Alem and Tuli, 2014). The amplification product of the DNA amplification product is calculated during cycles using a probe or dyes during each cycle and can be measured by a known concentration standard (Liu *et al.*, 2015).

### 2.12.7 Molecular genotyping

Molecular typing methods play an important role in the identification of *T. gondii* genotypes responsible for infections during epidemiological studies as it helps with keeping track of the source of their origin (Su *et al.*, 2010; Castro *et al.*, 2020). Several molecular technologies have been developed for *T. gondii* genotyping, including microsatellite analysis, multilocus sequence typing (MLST), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and high-resolution melting (HRM) analysis (de Melo Ferreira *et al.*, 2004; Wang *et al.*, 2013; Can *et al.*, 2014; Zhang *et al.*, 2015; Murphy, Stewart and Taylor, 2018).

#### 2.12.7.1 Microsatellite analysis

This assay uses the length polymorphism of short nucleotide tandem repeats of a DNA (Su *et al.*, 2010). In a population, the number of repeat units varies, generating multiple alleles at a microsatellite locus (Liu *et al.*, 2015). *Toxoplasma gondii* microsatellite markers (TUB-2, W35, TgM-A, B18, B17, M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, N83) are detected using a single multiplex PCR assay during the analysis (Can *et al.*, 2014).

#### 2.12.7.2 Multilocus sequence typing

The MLST is focused on sequence polymorphisms like single nucleotide polymorphisms (SNPs), deletion and addition of nucleotides in the sequence, and has the highest resolution among all the typing methods when there is sufficient genomic DNA (Su *et al.*, 2010; Liu *et al.*, 2015). When typing, three polymorphic genes; SAG3, GRA6, and GRA7 are targeted using SAG3, GRA6 and GRA7 markers (Fernández-Escobar *et al.*, 2020). This method, however, is not a good option for clinical samples because it requires high quantities of genomic DNA (Liu *et al.*, 2015).

#### 2.12.7.3 Polymerase chain reaction-restriction fragment length polymorphism

This method relies on the ability of endonucleases to recognize SNPs, the digestion of PCR products and the presence of distinct DNA patterns on the agarose gel after electrophoresis (Howe and Sibley, 1995). The traditional multilocus PCR-RFLP relies on single-copy polymorphic DNA sequences and normally needs a high *T. gondii* DNA concentration, thus making it hard to genotype *T. gondii* in clinical samples because of the small *T. gondii* DNA available (Liu *et al.*, 2015). To reduce this disadvantage, a multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) assay that uses 10 genetic markers namely: SAG1, SAG2, SAG3,

BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico (Su *et al.*, 2010; Rajendran, Su and Dubey, 2012). Before typing, all markers are amplified in a single reaction using multiplex PCR with external primers, and the products are used as templates to amplify individual markers by nested PCR (Liu *et al.*, 2015). The benefit of this approach is that only a small number of individual samples are required and it is very useful if only small quantities of samples are available (Su *et al.*, 2010).

#### 2.12.7.4 Random amplified polymorphic DNA-PCR (RPD-PCR)

This assay is PCR-based and can be used without predetermined genetic data to classify DNA polymorphisms (Liu *et al.*, 2015). It amplifies DNA under low-stringency conditions using single short arbitrary primers (de Melo Ferreira *et al.*, 2004; Liu *et al.*, 2015; Zhang *et al.*, 2015). The assay has been used to detect the genetic differentiation of closely related organisms and in the identification of the *T. gondii* genotypes (Guo, Gross and Johnson, 1997). *T. gondii* can be divided into virulent and avirulent strains by making use of arbitrary primers based on RAPD-PCR murine virulence, and some of these primers are useful in identifying markers of virulence (Guo, Gross and Johnson, 1997). RAPD markers are DNA fragments obtained by PCR amplification of random regions of genomic DNA using a single primer of any nucleotide sequence (de Melo Ferreira *et al.*, 2004). This assay is quick, easy and effective, but it can be difficult to replicate RAPD band profiles when laboratories, workers, equipment or conditions are changed (Liu *et al.*, 2015).

#### 2.12.7.5 Variable number tandem repeats

Tandem repeat variations, often known as VNTRs (varying number of tandem repeats), are loci where the population's internal copy count varies (Gelfand *et al.*, 2014). Variable Number Tandem Repeat (VNTR) sequences have become important genotyping markers for a variety of organisms, including *T gondii*. Due to their high polymorphism about the number of tandem repetitions at a specific VNTR locus, VNTRs were initially used as markers for linkage mapping (Gelfand *et al.*, 2014). Molecular markers (Table 2.2) have been developed to distinguish between genetic variants found in each clonal lineage and/or haplogroup (Table 2.2) (Moretta *et al.*, 2018). The two main benefits of this method are: One is that variation results through DNA polymerase slippage and is independent of the parasite's sexual reproduction, and two is that nearby areas are typically conserved as a result of negative selective pressure (Moretta *et al.*, 2018). This genotyping method primarily depends on PCR amplification using primers designed for the flanking regions of the VNTRs and on measuring

the sizes of the amplicons following electrophoretic migration (Moretta *et al.*, 2018; Bakhtiari *et al.*, 2021). These sizes correspond to the number of amplified VNTR copies since the length of the repeat units is known and the outcome is a numeric code that represents each VNTR locus's repeat count (Gelfand *et al.*, 2014).

**Table 2.2:** Molecular markers and target genes on VTNR genotyping

| Target gene/ locus | Molecular marker (s) |
|---|---|
| Cwf21 protein | 282140 |
| Uncharacterized protein | 225090; 231200; 225830; 316650 |
| Folate-binding protein YgfZ protein | 267560 |
| Serine/threonine specific protein phosphatase | 223985 |
| RNA pseudouridine synthase superfamily protein | 202640 |

## 2.13 Differential diagnosis

Toxoplasmosis is rarely considered in a primary diagnostic list other than with problems of abortion and associated neonatal mortality (Ibrahim, 2017). The differential diagnosis for abortion in cattle, sheep and goats is associated with brucellosis, while in pigs it is associated with leptospirosis (Ibrahim, 2017). The cause of encephalitis in animals is not seen as a sign of toxoplasmosis in the animals because is linked to viral infections, bacteria, and verminous encephalomyelitis due to parasitic organisms with somatic movement larva migration (Ibrahim, 2017).

## 2.14 Treatment

### 2.14.1 In animals

For exotic ruminants, there is a shortage of clearly established toxoplasmosis treatments (Ibrahim, 2017). Treatment with a combination of sulfamethazine and pyrimethamine is effective in reducing the effects in pregnant ewes of experimentally induced toxoplasmosis (Dunay *et al.*, 2009; Ibrahim, 2017). Treatment is given for 3 periods with an interval of 5 days over 3 days (Ibrahim, 2017). Sulphadiazine chemotherapy (60 mg/kg/day) every 4-6 h and pyrimethamine (0.5-1 mg/kg/day) as a single dose restrict the spread of infection before immunity is gained from the host (Ibrahim, 2017).

### 2.14.2 In humans

Women contract the diseases during pregnancy for the first time and those infected with congenital ocular toxoplasmosis need to be treated, as well as the ones with the weak immune system (those with HIV/AIDS or neoplastic disease) and transplant recipients with active or reactivated infection (Pleyer *et al.*, 2019).

Conventional treatment for clinical toxoplasmosis is normally made up of a mixture of pyrimethamine and sulphonamides, but is discouraged for use by pregnant women due to its effects on the foetus (Montoya and Liesenfeld, 2004; Ibrahim, 2017).

## 2.15 Prevention and control

### 2.15.1 In animals

On farms, toxoplasmosis prevention is more difficult, but animal feed should be protected where possible to exclude cats and prevent them from being exposed to insects (Ibrahim, 2017). Infection in cats can be prevented by ensuring that they are not fed raw or undercooked meat and unpasteurized milk and by keeping them indoors (Ross, Jones and Lynch, 2006; Robert-Gangneux, 2014). Dead animals should be properly disposed of to prevent them from being eaten by pigs and cats (Dubey, 1991; Ibrahim, 2017). An aborted ewe as a result of toxoplasmosis does not normally have recurring abortions due to toxoplasmosis, and can, therefore, be used for breeding in the future (Dubey, 1991).

In mid-pregnancy, monensin and decoquinate are given to ewes as an attempt to control abortion due to toxoplasmosis (Ibrahim, 2017). A live vaccine only for sheep consisting of attenuated tachyzoites that are approved for use in sheep to help prevent *T. gondii*-related abortion is commercially available in the United Kingdom, France and New Zealand (Dubey, 2009b; Ibrahim, 2017; Innes *et al.*, 2019). There are plans to develop more vaccines for a one health approach to combat toxoplasmosis in cats, humans and food-producing animals (Innes *et al.*, 2019). There is no data on the use of vaccines for *T. gondii* in Africa including SA. The non-use of vaccines against *T. gondii* in SA might be due to the lack of serological and molecular prevalence studies whose data will determine whether it is needed or not.

### 2.15.2 In humans

Human infections can be prevented and controlled by thoroughly washing hands with soap prior to handling meat (Klun *et al.*, 2006). Meat must be cooked thoroughly before consumption at temperatures above 67 ºC (Pal, Alem and Tuli, 2014; Ibrahim, 2017). Freezing meat at –12 ºC for at least 24 hours is effective in killing tissue cysts, however, sporulated oocysts are not killed by a temperature of -20 ºC for up to 28 days (Ibrahim, 2017). Milk should also be pasteurized before consumption (Robert-Gangneux, 2014).

A pregnant woman must avoid contact with cats and clean their litter boxes. Gloves should be worn when gardening and fruits and vegetables should be washed thoroughly before eating, as oocysts can contaminate them (Ross, Jones and Lynch, 2006). Serological testing for women who are pregnant can help prevent congenital infections (Tenter, 2000). Despite the global importance of toxoplasmosis as a zoonotic disease, no human vaccinations exist at this time (Innes *et al.*, 2019).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Study design

This was a prospective quantitative study constituting of four components. The first component entailed utilizing a questionnaire to determine the risk factors associated with animal exposure to toxoplasmosis in the NW province. The second component entailed determining the seroprevalence of T. gondii in the NW province using ELISA. The third component entailed using PCR to detect *T. gondii* in diagnostic tissue samples received at ARC-OVR from the FS province and collected tissue samples from the NW province. The fourth and final component entailed the use of bioinformatics to examine isolates of the *T. gondii* GRA6 and B1 housekeeping genes deposited in the GenBank from different species and countries to evaluate if they could be used as genetic markers.

## 3.2 Study area

The study was conducted on communal and commercial farms of both the NW (figure 3.1) and FS Provinces (figure 3.2). In the FS province, routine diagnostic samples were used to assess the occurrence. In the North West Province, villages were selected randomly from each municipality. In each village, efforts were made to consider all directions (East, West, North and South of the village using an abstract transect) to avoid bias. Farmers who did not give consent for their animals to be sampled were replaced with the next farms within the village.

### 3.2.1 North West province

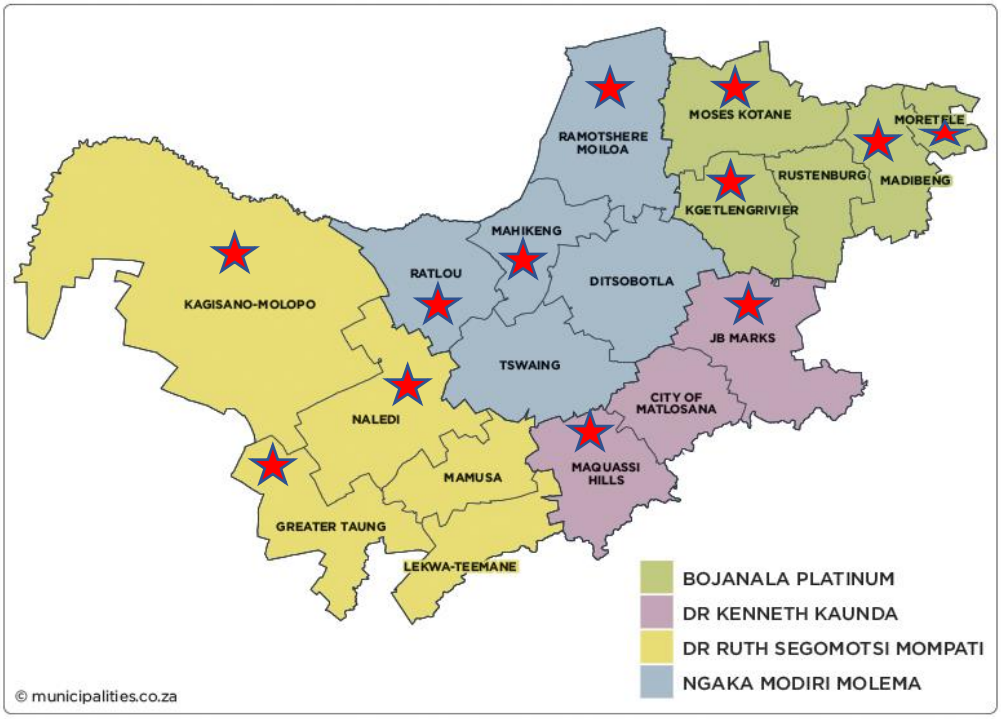


**Figure 3.1:** A map showing study sites in the NW province (sourced from https://municipalities.co.za/provinces/view/8/north-west)

NW province is in the north of SA on the Botswana border, fringed by the Kalahari Desert in the west, Gauteng province to the east and the Free State to the south. It covers an area of 104 882km² and has a population of 3 748 436. Most of the province comprises flat areas of scattered trees and grassland. Mahikeng is the capital city of the province that lies near the Botswana border and forms a single urban area with its neighboring town, Mmabatho.

Between Potchefstroom and Klerksdorp in the south, Rustenburg in the east, and Brits in the west, there is the largest amount of economic activity. The southern region is widely known for its cattle rearing.

The four district municipalities that make up North West are further divided into 18 local municipalities. Sampling was conducted in all districts (marked in red stars on figure 3.1). Bojanala district at the Kgetleng River, Madibeng, Moses Kotane and Moretele municipalities, in Dr Kenneth Kaunda district at the JB Marks and Maquassi Hills municipalities, in Dr Ruth Segomotsi Mompati at Greater Taung, Kagisano-Molopo and Naledi municipality, in Ngaka Modiri-Molema district at Mahikeng, Ramotshere Moiloa and Ratlou municipalities. The

samples were processed and analysed at the Agricultural Research Council, Onderstepoort Veterinary Research (ARC-OVR), Bacterial PCR Laboratory.

### 3.2.2 Free State province

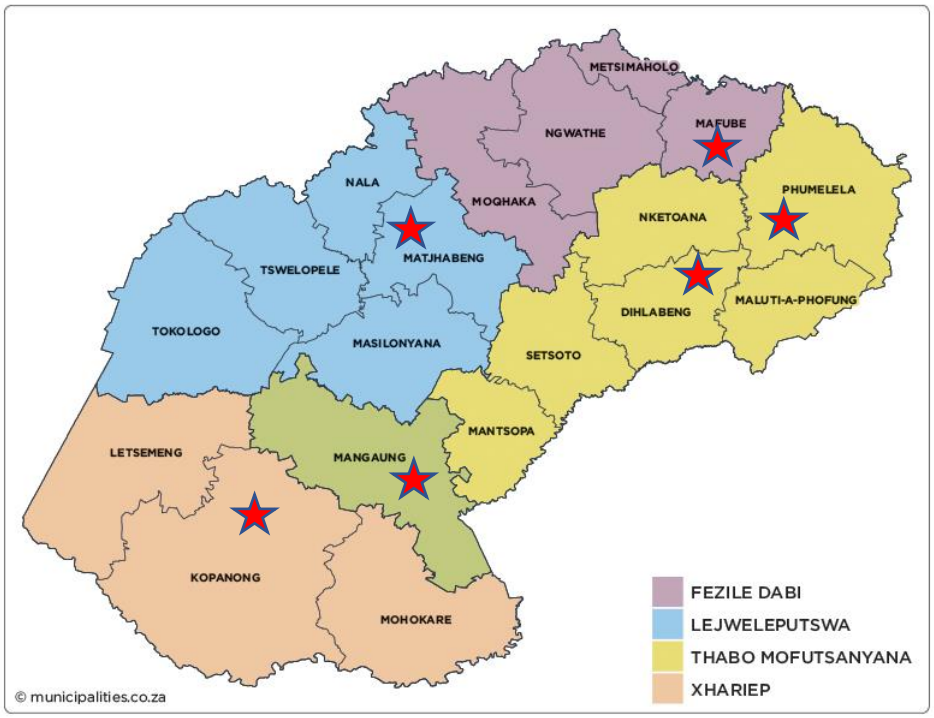


**Figure 3.2:** A map showing study sites in the FS province (sourced from https://municipalities.co.za/provinces/view/2/free-state)

The FS province is geographically located in the center of South Africa, bordered by the Northern Cape, Eastern Cape, North West, Mpumalanga, KwaZulu-Natal, and Gauteng provinces, as well as Lesotho. The FS is a rural province of farmland, mountains, goldfields, and widely dispersed towns.

It has the second-smallest population and the second-lowest population density in South Africa despite being the third-largest province. The province has a population of 2 834 714, making up 5.1% of the total country's population, and a land area of 129 825 km². Bloemfontein, its capital city, serves as the seat of South Africa's legal system. Other significant cities include Bethlehem, Welkom, Kroonstad, and Sasolburg.

The province consists of four district municipalities, which are further divided into 18 local municipalities, and one metropolitan municipality (Mangaung Metropolitan Municipality) with its economy dominated by agriculture, mining, and manufacturing. Diagnostic tissue sample

submissions came from the local municipalities marked in red (figure 3.2) in Fezile Dabi, Lejweleputswa, Thabo Mofutsanyana, and Xhariep district, as well as Mangaung metropolitan municipality (red-marked).

### 3.3 Sample size

The sample size for the study was calculated using Thrusfield's (Thrusfield, 2004): *n* = 1.962 p (1 p)/d2, with p being the estimated prevalence, d the estimated precision, and *n* the estimated sample size. Due to the lack of data on *T. gondii* prevalence in the study population, an expected prevalence (P exp) of 50% was used and 5% was set as the estimated precision (d).

$$n_0 = \frac{\{1.96^2 \times P_{exp} \times (1 - P_{exp})\}}{d^2}$$

$n_0 = \frac{3.84 \times 0.5 \times 0.5}{0.0025} = \frac{0.96}{0.0025} = 384 n_0 = 384$ heads of small ruminants in the North West.

However, for the study, a total of 439 (164 sheep and 275 goats) serum samples, 408 vaginal swabs, 94 milk and 31 sheath scrapes (Table 3.1).

**Table 3.1:** Number and type of samples collected from sheep and goats in NW and FS provinces

| Province | Species | Type of Sample | Number (n) |
|---|---|---|---|
| North West | Sheep | Serum | 164 |
| | | Sheath scrape | 13 |
| | | Milk | 29 |
| | | Vaginal swabs | 151 |
| | Goats | Serum | 275 |
| | | Sheath scrape | 18 |
| | | Milk | 65 |
| | | Vaginal swabs | 257 |
| Free State | Sheep | Tissue | 9 |
| | Goats | Tissue | 2 |

### 3.4 Sample collection

Four different tissue samples were collected as shown in table 3.1 (whole blood, sheath scrapping, vaginal swabs, and milk from lactating animals). A purposive sampling of animals with a history of abortions or reproductive diseases and a random sampling of animals without a history of abortions or reproductive diseases was also conducted.

#### 3.4.1 Blood

The animals were captured and restrained humanely to aseptically collect 5 ml of whole blood sample in the jugular vein, located on the neck of the animal with a 20-gauge vacutainer needle (Greiner Bio-One, Frickenhausen, Germany) and a vacutainer needle holder (Greiner bio-one, Frickenhausen, Germany) to collect the blood. The red stopper tube (Greiner bio-one, Frickenhausen, Germany) was labelled with animal identification and the sample collection site was used, placed into a cooler with ice packs and transported to the laboratory. The blood was then centrifuged at 3000 rpm for 10 minutes to obtain serum. The serum was harvested into sterile 2 ml micro-centrifuged tubes with sample identification and stored at -20 °C until required for analysis.

#### 3.4.2 Milk

Milk samples were collected from nursing ewes and does after disinfecting teats and using latex gloves, approximately 100 ml of milk was collected into a sterile container by manual milking. The initial stream of milk was discarded, and a sterile container was filled with the next stream. The container was labelled and placed into a cooler box with frozen icepacks, transported to ARC-OVR, Bacterial PCR Laboratory and stored at -20 °C until analysis.

#### 3.4.3 Sheath scraping

A Sheath scraping was conducted using a dry AI pipette attached to a 20 ml sterile reusable hypodermic syringe with a silicon rubber (Irons, Henton and Bertschinger, 2002). Rams and bucks were properly restrained on the neck. The collected aspirate was transferred into a container containing 4 ml of phosphate-buffered solution (PBS), and transported to ARC-OVR, Bacterial PCR Laboratory in a cooler box with ice packs and stored at -20 °C until analysis.

#### 3.4.4 Vaginal swabs

Vaginal swabs were collected using sterile swabs by gently swabbing the vaginal wall. The swab was moistened with sterile saline in the absence of vaginal discharge to avoid discomfort

and irritation. The swabs were transported to ARC-OVR, Bacterial PCR Laboratory, and stored at -20 °C until analysis.

### 3.4.5 Diagnostic submissions

Diagnostic tissue samples submitted at ARC-OVR from aborted cases in sheep and goats of the Free State province were also analysed (table 3.2).

**Table 3.2:** Breakdown of diagnostic submissions received at ARC-OVR from Free State province

| Species | District | Municipality | Town | Type of sample (s) | Number (n) |
|---|---|---|---|---|---|
| **Sheep** | Fezile Dabi | Mafube | Frankfort | Tissues | 2 |
| | Thabo Mofutsanyana | Dihlabeng | Bethlehem | | 4 |
| | Xhariep | Phumelela | Vrede | | 1 |
| | | Kopanong | Reddersburg | | 1 |
| | | Mangaung | Bloemfontein | | 1 |
| **Goat** | | Mangaung | Bloemdal | | 1 |
| | Lejweleputswa | Matjhabeng | Hennenman | | 1 |

## 3.5 Questionnaire

During sample collection, an investigative questionnaire survey (Appendix A) was done to determine risk variables related to toxoplasmosis by questioning animal herders and/or farm owners. The hypothesized risk factors for *T. gondii* included; age (< 1 year,> 1 year), breed, sex (male, female), location (municipality, district), animal management system (free-ranging), hygiene practices (frequency of cleaning the animal stables), feed storage (storage room, outside), knowledge of reproductive diseases, disposal of aborted material (burn, burry, hang on the tree/kraal), drinking water supply (dam, river, borehole, tap), the presence of cats (domestic and/or feral), as well as existence or absence of rodent control. Questions were derived from the literature (Abdallah *et al.*, 2019; Tagwireyi, Etter and Neves, 2019).

### 3.6 Ethical clearance

The University of South Africa College of Agriculture and Environmental, Animal Research Ethics, and Health Research Ethics committees gave their approval for this study, with the ethics clearance number 2020/CAES_AREC/146 and REC-170616-051 (Appendix C). Approval was also granted by the Onderstepoort Veterinary Research Animal Ethics Committee with approval number: AEC 19.18. Further approval was obtained for Section 20 from the Department of Agriculture, Forestry and Fisheries (DAFF) with reference number 12/11/1/1 (Appendix D).

### 3.7 Test Methods

#### 3.7.1 Serological assay for *T. gondii* IgG antibody

Sera from the animals (n=439) was assayed using a two-strip IDEXX Toxotest antibody ELISA test kit for the detection of IgG antibodies against *T. gondii* was used following manufactures guidelines (IDEXX Laboratories, Liebelfd-Bern, Switzerland). Frozen sera and the test kit's reagents were thawed simultaneously at 25℃, and the wash buffer was diluted 1:400 with distilled water. The sera, as well as the positive and negative controls supplied with the test kit, were pre-diluted 1:400 in the diluted buffer.

The diluted controls and sera were poured in a volume of 100 µl into a *T. gondii* antigen precoated microtiter plate, and they were gently mixed by tapping on the sides. After mixing, the plate was covered with a plate cover and incubator at 37 ˚C for 1 hour. With 300 µl of the wash buffer, each well was washed three times after incubation. Gently tapping the plate on absorbent paper removed the wash buffer residues. After adding 100 µl of the conjugate to each well, covering it with a lid, and incubating it at 37°C for an hour, the bound antigen-antibody complexes were conjugated. After incubation, the above-mentioned wash step was repeated to eliminate any unbound complexes, and 100 µl of 3, 30, 5, 50-Tetramethylbenzidine (TMB) was added. The plates were again washed after incubation as described wash step was repeated after incubation to remove any unbound complexes, after which 100 µl of 3, 30, 5, 50-Tetramethylbenzidine (TMB) substrate was added into each well and incubated in the dark at room temperate for 15 minutes. After incubation, a stop solution of 100 µl was added to each well and the absorbance was measured at 450 nanometers with a Thermo Labsystems MultiskanMS Original microplate reader (Thermo Fischer Scientific, Waltham, MA, USA).

The validity of the assay was assessed as follows: the two negative controls average optical density value of optical density of the two negative controls (NCx) at 450 nm (A450) should be less or equal to (≤) 0.500. The two positive controls average value (PCx) at 450 nm (A450). Should be ≤ 2.500 and then the PCx-NCx (A450) should be greater than or equal to (≥) 0.300. Sample to positive (S/P) was calculated as per the formula below:

$$\text{S/P \%} = 100\text{x}\frac{\text{Sample A(450)} - \text{NCx}}{\text{PCx} - \text{NC}}$$

For results interpretation, S/P % <20 signified a negative result, 20 ≤ S/P % <30 signified a suspect, 30 ≤ S/P% <100 signified a weak positive result, and S/P % ≥100 signified a positive result. All samples that gave a suspect result were retested.

### 3.7.2 Molecular detection

Molecular detection by PCR was conducted on sheath scrapes, milk, vaginal swabs, and diagnostic tissue samples received during the study period. As shown in table 3.3, a total of 198 samples consisting of 138 vaginal swabs, 26 milk, 23 sheath scrapes from North West, and 11 diagnostic tissue samples from the Free State province were analysed. The aim was to use all the tissue samples collected (408 vaginal swabs, 94 milk samples, and 31 sheath scrapes) to determine the molecular prevalence; however, due to power outages and the breakdown of -80 freezers, we lost some of the samples and previously extracted were denatured, hence only 198 of the collected samples could be analysed. To ensure the integrity of the used samples a nano drop was used to measure the concentration of the DNA to prevent false negative results.

**Table 3.3:** Number of samples used for PCR as per origin, species, and sample type

| Province | Species | Type of Sample | Number (n) |
|---|---|---|---|
| **North West** | Sheep | Sheath scrape | 8 |
| | | Milk | 6 |
| | | Vaginal swabs | 61 |
| | Goats | Sheath scrape | 15 |
| | | Milk | 20 |
| | | Vaginal swabs | 77 |

| | | | |
|---|---|---|---|
| | Sheep | Tissue | 9 |
| **Free State** | Goats | Tissue | 2 |
| **Total** | | | 198 |

### 3.7.2.1 Sample preparation

#### 3.7.2.1.1 Vaginal swabs and sheath scrapes

A volume of 2 ml of the distilled water covering the butt of the swab and 2 ml of sheath scrape were poured into 2 ml microcentrifuge tubes and centrifuged at 8 000 xg for 10 minutes. After centrifugation, the supernatant was discarded leaving approximately 200 µl to be re-suspended to the pellet and mixed with a vortex until it is dissolved.

#### 3.7.2.1.2 Milk

Two millimeters of milk were poured into a 2 ml microcentrifuge tube and centrifuged at 8 000 xg for 10 minutes. After centrifuging, the supernatant was discarded leaving the cream; this was achieved by inserting the pipette tip between the cream and the pellet to suck up the supernatant, leaving approximately 200 µl to be re-suspended to the pellet and the cream. It was then mixed with a vortex until it is dissolved.

### 3.7.2.2 DNA extraction

The DNA extraction from vaginal swabs, milk and sheath scrapes from the seropositive animals was performed using a high pure PCR template preparation kit (Roche, SA) following manufactures instruction. The extraction was done as follows:

#### 3.7.2.2.1 Sample lysis and DNA binding

A volume of 200 µl binding buffer and 40 µl of proteinase K was added to each of the tubes of the above-prepared samples mixed and incubated at 70 °C for 10 minutes. After incubation, 100 µl of isopropanol was added, mixed, and applied to a high pure filter tube attached to a collection tube, which was centrifuged at 8 000 xg for 1 minute. The filter was then removed from the collection tube after centrifuging, and the collection was discarded.

#### 3.7.2.2.2 Washing

Five hundred microliters of inhibition removal buffer were added to the filter tube and centrifuged at 8 000 xg for 1 minute. The collecting tube was thrown away, and the filter tube was inserted into a clean collection tube in which 500 µl wash buffer, was centrifuged again at 8 000 xg. The filter tube was removed and inserted into a clean collection tube, discarding the old collection tube. The previous step was repeated, and the filter tube was placed and inserted into another clean collection tube and spun at 13 000 xg for 10 seconds to remove residual wash buffer.

### 3.7.2.5 Elution of DNA

The filter tube that was washed in the above step was inserted into a clean sterile 2 ml microcentrifuge tube and 200 µl of pre-warmed (70 °C) elution buffer (10 mM Tris-HCl, pH 8.5) was added to the filter tube and centrifuged at 8 000 xg for 1 minute. The filter tube was discarded, and the eluted DNA was stored at -20 °C until analysis.

## 3.7.2 Gene Amplification and Visualization

### 3.7.2.1 Universal ribosomal RNA (18S) amplification

The success of the DNA extraction and the presence of parasitic DNA in the samples were checked using universal ribosomal 18S rRNA gene primers (Table 3.4). Each PCR was carried in a 25 µl reaction mixture containing 12.5 µl master mix, 0.5 µl of each primer in Table 3.4 (Inqaba Biotechnical Industries (Pty) Ltd, Tshwane, South Africa), 6.5 µl of nuclease-free water and 5 µl of *T. gondii* DNA. The amplification was carried out with the Bio-Rad T100 thermal cycler by cycling the reaction for 35 cycles, with initial denaturation at 95 °C for 10 minutes, followed by denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds, and lastly, extension at 74 °C for 1 minute. Nuclease-free water was used as the negative control for non-DNA.

**Table 3.4:** Universal 18S rRNA primers (Wang *et al.*, 2014)

| **Primers** | **Sequence** |
|---|---|
| **Forward (1A)** | 5 ' -AACCTGGTTGATCCTGCCAGT-3 ' |
| **Reverse (564R)** | 5 ' - GGCACCAGACTTGCCCTC -3' |

#### 3.7.2.1.1 Visualization and confirmation of PCR amplicons

Five microliters of amplicons were used to confirm DNA amplification on a 2% ethidium bromide-stained agarose gel with an expected size of 700 bp using a quick load 100 bp molecular weight ladder (New England Biolabs, Ipswich, MA, USA). Bio-Rad Laboratories, SA). The gel was run for three hours at 80 volts using 1X TBE buffer (Bio-Rad Laboratories, SA). The PCR products were visualized using a gel documentation system (Bio-Rad Laboratories, SA).

### 3.7.2.2 Amplification of B1 gene

Nested PCR was performed using the extracted DNA as a template and two set of primers targeting the B1 gene of *T. gondii* (Inqaba Biotechnical Industries (Pty) Ltd, Tshwane, South Africa) (Table 3.5), as described by Jones et at, 2000 (Jones *et al.*, 2000).

#### 3.7.2.2.1 First amplification

For the first round of amplification, PCR was carried out in a reaction of 25 µl containing 12.5 µl of Master Mix Red (Ampliqon, Denmark), 0.5 µl of each primer (Table 3.5), and 5µl of DNA. The amplification was carried out with the Bio-Rad T100 thermal cycles where the reaction mixture was denatured for 10 seconds at 93 °C, followed by 10 seconds of annealing at 57 °C, and finally, 30 seconds of extension for 40 cycles at 72 °C. Inhouse *T.gondii* positive control (assertion number: OP029036) that was sequenced from the B1 gene in another study was used, and nuclease-free water was used as the negative control for non-DNA.

**Table 3.5:** Primer sequences for B1 gene (Jones *et al.*, 2000)

| Primers | Sequence | Sequence Position |
|---|---|---|
| **External forward** | 5'-GGAACTGCATCCGTTCATGAG-3' | 694–714 |
| **External reverse** | 5´-TCTTTAAAGCGTTCGTGGTC-3' | 887–868 |
| **Internal forward** | 5'- TGCATAGGTTGCAGTCACTG-3' | 757–776 |
| **Internal reverse** | 5'- GGCGACCAATCTGCGAATACACC-3' | 853–831 |

#### 3.7.2.2.2 Visualization and confirmation of PCR amplicons

Five microliters of amplicons were used to confirm DNA amplification on a 2% agarose gel stained with ethidium bromide with an expected size of 193 bp on the first round of amplification and 96 bp on the nested amplification using a quick load molecular weight ladder of 100 bp (New England Biolabs, Ipswich, MA, USA). The gel was run for two hours at 80

volts using 1X TBE buffer (Bio-Rad Laboratories, SA). The PCR data were visualized using a gel documentation system (Bio-Rad Laboratories, SA).

#### 3.7.2.3 *Toxoplasma gondii* specific 18S rRNA gene amplification

Each PCR was carried in a 25 µl reaction mixture containing 12.5 µl master mix (Ampliqon, Denmark), 0.5 µl of each *T. gondii* specific 18S rRNA gene primer in Table 3.6 (Inqaba Biotechnical Industries (Pty) Ltd, Tshwane, South Africa), 6.5 µl of nuclease free water and 5 µl of DNA. The amplification was carried out with the Bio-Rad T100 thermal cycler by cycling the reaction for 35 cycles, with initial denaturation at 95 °C for 10 minutes, followed by denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds, and lastly, extension at 74 °C for 1 minute. Nuclease-free water served as the negative control for non-DNA and an inhouse *T. gondii* positive control (assertion number: OP029036) that was sequenced from the B1 gene in another study was used.

**Table 3.6:** *Toxoplasma gondii* rRNA 18S gene primer sequences (Jones *et al.*, 2000)

| Primers | Sequence | Sequence Position |
|---|---|---|
| **Forward** | 5'-CCTTGGCCGATAGGTCTAGG-3' | 170–189 |
| **Reverse** | 5´-TCTTTAAAGCGTTCGTGGTC-3' | 253–231 |

##### 3.7.2.3.1 Visualization and confirmation of PCR amplicons

Five microliters of amplicons were used to confirm DNA amplification on a 2% agarose gel stained with ethidium bromide with an expected size of 88 bp using a quick load molecular weight ladder of 50 bp (New England Biolabs, Ipswich, MA, USA). The gel was run for three hours at 80 volts using 1X TBE buffer (Bio-Rad Laboratories, SA). The PCR products were visualized using a gel documentation system (Bio-Rad Laboratories, SA).

#### 3.7.2.4 Sequencing of universal 18S rRNA fragment for *T. gondii* confirmation

Sequencing of the universal 18S rRNA PCR products was done at Inqaba Biotechnical industries (Pty) Ltd (Tshwane South Africa). Sequencing was performed from both ends using the forward and reverse primer sequences that were initially used for amplification. Following sequencing, the sequences from both strands were manually modified, and pairwise alignments

were carried out using the BioEdit Sequence alignment editor (version 7.2.5). Using the basic local alignment tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the generated consensus sequences were examined for gene sequence identity and similarity on the NBI platform.

### 3.7.3 Genetic and phylogenic analysis of the B1 and GRA6 gene sequences

The B1 gene is found in 35 copies of the *T. gondii* genome, making PCR directed at the B1 gene more sensitive than PCR directed at single copy locations like GRA6 (Parameswarana *et al.*, 2009). As a result, these two housekeeping genes are commonly used for detection and confirmation of the presence of genetic material of T. *gondii*. The B1 and GRA6 gene sequence were then retrieved from the GenBank (https://www.ncbi.nlm.nih.gov/) to evaluate if they could be used as phylogenetic markers. Since there are no *T. gondii* sequence isolates from the study areas and South Africa on GenBank, isolates from other countries were used. The retrieved sequence isolates were then manually trimmed, aligned, and analyzed for the presence of single nucleotide polymorphism (SNP) and ultimately used to construct phylogenetic trees toa analyze their phylogenetic relationship.

#### 3.7.3.1 Sequence analysis and phylogenetic tree construction

Using Molecular Evolutionary Genetics Analysis (MEGA) (version 11), sequences were manually edited, trimmed, and aligned from the 5' end to the 3' end so that all sequences start and end at the same sequence. Multiple sequence alignments of both individual genes were carried out using ClastalW in MEGA (version 11) to calculate the degree of similarity between each gene sequence (Thompson, Higgins and Gibson, 1994). After aligning the sequences, single nucleotide polymorphisms (SNPs) were manually identified and examined. Phylogenetic trees were constructed using MEGA version 11's neighbor-joining technique, and the maximum composite likelihood method was used to validate them (Saitou and Nei, 1987; Tamura, Stecher and Kumar, 2021). One thousand replicate were used in the bootstrapping method.

## 3.8 Data analysis

A 95% confidence interval was used to calculate different prevalence values. Association of risk factors (age, gender, species, breed, type of breeding, origin of animals, history of abortion

disposal of aborted material, district, municipality, type of farm, presence of cats, water source, feeding system, feed storage and disposal of manure) with seroprevalence of *T. gondii* was investigated. All the data was entered into a spreadsheet of Microsoft Excel and analysed in Stata 15 (StataCorp, College Station, TX, USA). The univariable logistic regression model was used to test variables at the individual level against disease exposure. For the initial analysis, the Chi-square test (P-value ≤0.05) was used to test all variables individually for their unconditional association with the result. The variables that produced the highest p-value of ≥ 0.05 during the analysis of univariable logistic regression were excluded.

The correlation and agreement between serological and molecular data were calculated using the proportion of agreement expected formula:($P_e$) = ((row total x column total)/ grand total) X 100 and Cohen's Kappa (κ) test using the formulas below (Cohen, 1960). The degree of agreement based on was assessed using the following criteria: 0-20 none, 0.21-0.39 fair agreement, 0.40-0.59 minimal agreement, 0.60-0.79 moderate agreement, 0.80-0.89 strong agreement, and > 0.90 almost perfect agreement (Petrie and Watson, 2013).

$$k\frac{\Pr(a)-\Pr(e)}{1-\Pr(e)}$$

Pr(e) indicates chance agreement, whereas Pr(a) indicates the actual observed agreement.

$$\text{Expected agreement } (\Pr(e)) = \frac{\left(\frac{\text{cm}^1\text{x rm}^1}{\text{n}}\right)+\left(\frac{\text{cm}^2\text{x rm}^2}{\text{n}}\right)}{\text{n}}$$

where:

- $cm^1$ stands for column 1 marginal,
- $cm^2$ stands for column 2 marginal,
- $rm^1$ stands for row 1 marginal,
- $rm^2$ stands for row 2 marginal, and
- n stands for the total number of tested samples.

# CHAPTER 4

# RESULTS

## 4.1 Sample distribution by sex and species

Table 4.1 displays the number of sampled of animals' species across all four districts and local municipalities in the NW province. A total of 439 animals were sampled with goats making up 62.6% of the total and sheep making up 37.3%, with females (92.3%) outnumbering males (7.7%). The sheep and goats sampled geographic distribution among the district and local municipalities is shown in figure 4.1.

**Table 4.1:** Demographic data on tested animals

| District | Municipality | Species | *n* |
|---|---|---|---|
| | Kgetleng River | | |
| | | Sheep | 3 |
| | | Goats | 7 |
| | Madibeng | | |
| | | Sheep | 2 |
| **Bojanala Platinum** | | Goats | 4 |
| | Moses Kotane | | |
| | | Sheep | 3 |
| | | Goats | 17 |
| | Moretele | | |
| | | Sheep | 9 |
| | | Goats | 30 |
| | JB Marks | | |
| | | Sheep | 6 |
| | | Goats | 6 |
| **Dr Kennet Kaunda** | Maquassi Hills | | |
| | | Sheep | 2 |
| | | Goats | 16 |
| | Greater Taung | | |
| | | Sheep | 11 |
| **Dr Ruth Segomotsi** | | Goats | 20 |
| **Mompati** | Kagisano-Molopo | | |
| | | Sheep | 10 |
| | | Goats | 21 |

| | | | |
|---|---|---|---|
| | Naledi | | |
| | | Sheep | 15 |
| | | Goat | 10 |
| | Mahikeng | | |
| | | Sheep | 71 |
| | | Goats | 94 |
| | Ramotshere Moiloa | | |
| **Ngaka Modiri** | | Sheep | 12 |
| **Molema** | | Goats | 10 |
| | Ratlou | | |
| | | Sheep | 19 |
| | | Goats | 40 |
| **Total** | 12 | | 439 |

*n:* number of animals sampled

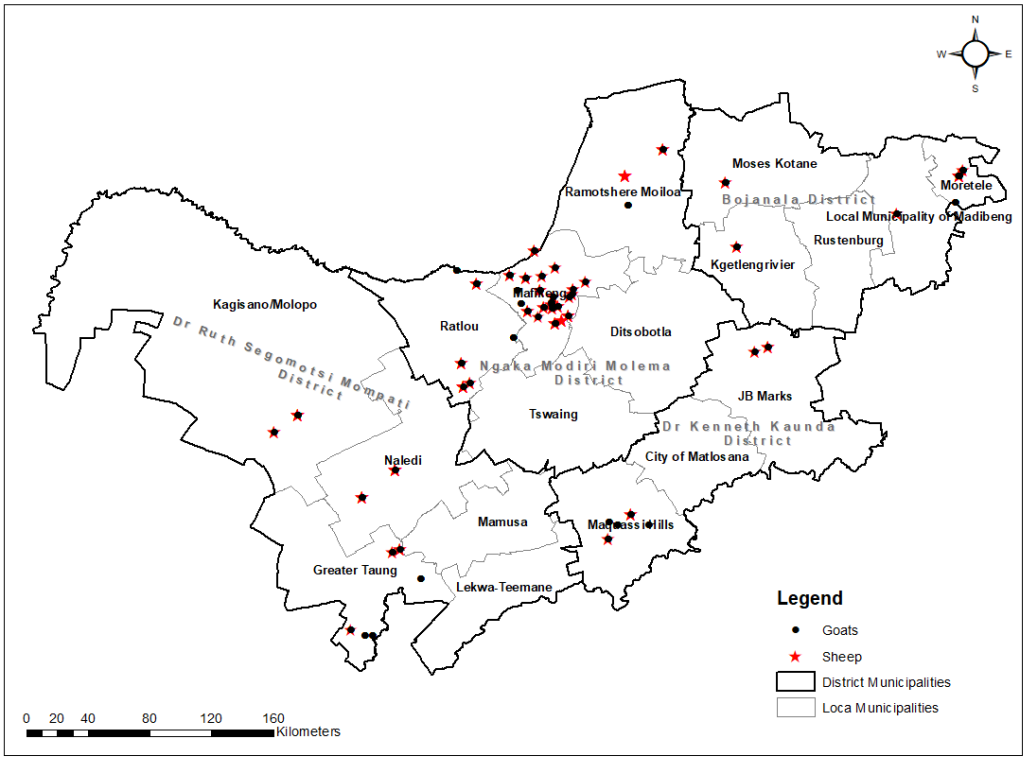


**Figure 4.1:** Geographic distribution of sampled sheep and goats in the districts and local municipalities

## 4.2 Overall seroprevalence

Of the 439 sera tested, 13.9% (95% CI: 0.69-4.07) were positive for antibodies against *T. gondii*. The seroprevalence for both sheep and goats were 19.5% (32/164) (95% CI: 0.24-1.92) and 10.5% (29/285) (95% CI: 0.42- 3.31) respectively (Table 4.2). *Toxoplasma gondii* infection among the species is presented in Table 4.2. In females, the variation in seroprevalence among the sexes was more pronounced than in males, with 61/405 (15%) in females and 2/34 (5.8%) in males (Table 4.3). The seroprevalence in Dr Ruth Segomotsi Mompati was the highest at 21.6% (19/87) followed by Ngaka Modiri Molema at 15.1% (37/245), Bojanala Platinum at 5.2% (4/77), and lastly, Dr Kennet Kaunda districts were at 3.3% (1/30) respectively (Table 4.3). Figure 4.1 displays the distribution of positive sheep and goats in the district and local municipalities.

**Table 4.2:** Seroprevalence of *T. gondii* infection among species

| Species | *n* | No. of positive samples | Percentage (%) | 95% CI |
|---|---|---|---|---|
| Sheep | 164 | 32 | 19.5 | 0.24-1.92 |
| Goats | 275 | 29 | 10.5 | 0.42- 3.31 |
| Total | 439 | 61 | 13.9 | 0.69-4.07 |

*n:* number of animals tested; No.: number; CI: confidence interval

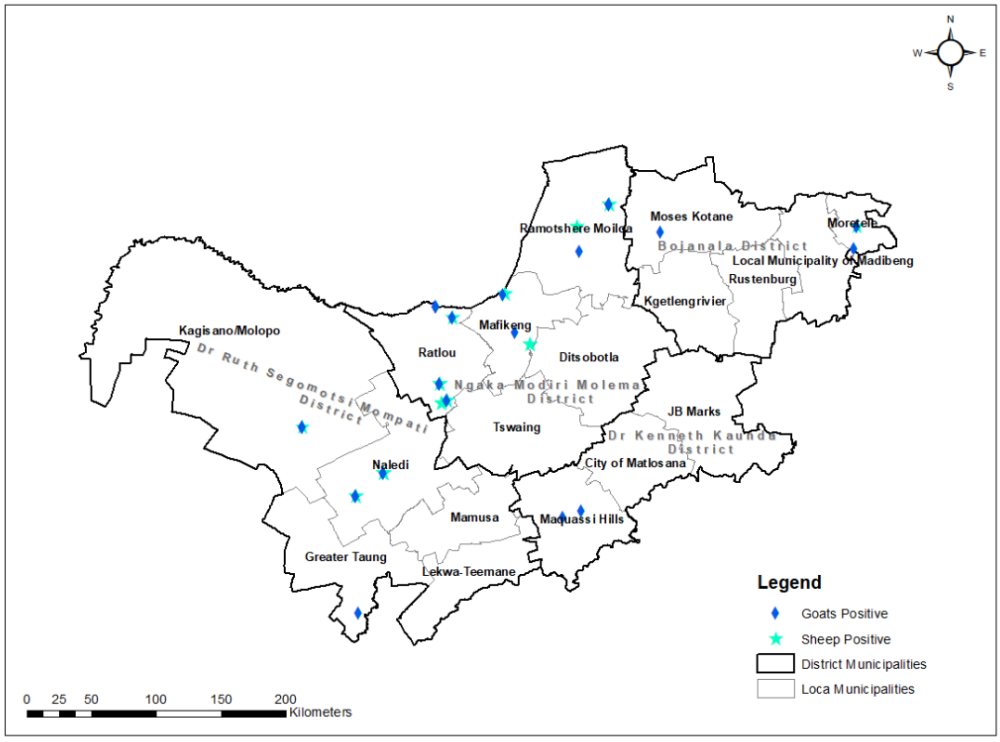

**Figure: 4.2:** Geographic distribution of positive sheep and goats in the districts and local municipalities

### 4.3 Risk Factors

Among the risk factors linked to higher *T. gondii* exposure among the animals, the following were statistically significant based on univariable analysis: breed, gender, species, the origin of animals, history of abortion, disposal of aborted material, district, municipality, feeding system, feed storage and presence of cats in the farms (Table 4.3, 4.4 and 4.5).

Among the factors associated with biological characteristics of the animals (Table 4.3), breed (OR= 4.34; 95%CI= 0.38-2.56; *p* =<0,01) was statistically significant with the mixed breed (17.9%) of both sheep and goats showing more susceptibility to exposure followed by Boerbok (5.6%) and White Dorper (5.9%). Both Polled Dorset and Saanen breeds showed 0% prevalence. Within the species (OR= 2.27; 95%CI= 1.37-5.39; *p*= <0.01), sheep (20.1%) showed more seropositivity than goats (10.9%). The gender (OR= 9.57; 95%CI= 2.19-41.76; p= <0.01) of the animals was also a risk factor with pronounced seropositivity in females (15%) than in males (5.8%). Type of breeding (OR= 7.34; 95%CI= 0.31-13.73; p= 0.04) was also a risk factor with the animals that breed naturally having the highest risk that the ones that breed naturally (13.9%) and artificially by insemination (5.9%).

**Table 4.3:** Univariate risk factors associated with biological characteristics of the animals

| **Risk factors** | | ***n*** | **No. of positive** | **Percentage (%)** | **OR** | **95% CI** | ***p*-value** |
|---|---|---|---|---|---|---|---|
| **Age (years)** | 1 | 7 | 2 | 28.5 | 1.71 | 0.6-3.3 | >0.05 |
| | 1.2 | 1 | 0 | 0 | | | |
| | 2 | 137 | 15 | 10.9 | | | |
| | 2.5 | 6 | 1 | 16.6 | | | |
| | 3 | 217 | 33 | 15.2 | | | |
| | 3.5 | 2 | 0 | 0 | | | |
| | 4 | 53 | 11 | 20.1 | | | |
| | 5 | 10 | 0 | 0 | | | |
| | 6 | 4 | 0 | 0 | | | |
| | 7 | 2 | 0 | 0 | | | |
| **Gender** | Male | 34 | 2 | 5.8 | 9.57 | 2.19-41.76 | <0.01*[a] |
| | Female | 405 | 61 | 15 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Species** | Sheep | 164 | 33 | 20.1 | 2.72 | 1.37-5.39 | <0.01*[a] |
| | Goats | 275 | 30 | 10.9 | | | |
| **Type of Breeding** | Natural | 422 | 59 | 13.9 | 7.34 | 0.31-13.73 | 0.04* |
| | Natural and AI | 17 | 1 | 5.9 | | | |
| **Breed** | Boerbok | 108 | 6 | 5.6 | | | |
| | Dorper | 18 | 0 | 0 | | | |
| | Kalahari red | 1 | 0 | 0 | | | |
| | Mixed | 291 | 52 | 17.9 | 4.34 | 0.38- 2.56 | >0.01* |
| | Polled Dorset | 2 | 0 | 0 | | | |
| | Saanen | 2 | 0 | 0 | | | |
| | White Dorper | 17 | 1 | 5.9 | | | |

CI: confidence interval; *n*: number of animals tested; No.: number of positive animals; OR: Odds Ratio; *: statistically significant; AI: artificial insemination; [a]based on chi-square

Origin of the animals (OR= 2.76; 95%CI= 0.15-6.61; p= <0.01) showed that it also plays a role in their exposure to *T. gondii* with animals bought from the local market and auction showing a higher seroprevalence (63.2%), followed by the ones bought on the local market and own breed (26.6%), local market (16.1%), then the ones bought on auctions (6.2%), and lastly, own breed with the showing the lowest seroprevalence (5.8%) Animals with a history of abortion (OR= 3.34; 95%CI= 1.55-7.20; *p*= <0.01) showed a higher prevalence (24.7%), than those with no history of abortion (11.9%) (Table 4.4). The disposal of aborted material (OR= 1.96; 95% CI= 0.43-1.16; *p*= <0.001) from the animals had no significance in the animals' exposure to *T. gondii* infection with burying, burring and feeding the pets, burning the material, burning or burring the aborted material, burning or hanging on the tree or kraal, feeding to pets, those that get sent state veterinary service and animals that never aborted having a seroprevalence of 16.7%, 32.3, 0%, 33.3%, 0%, 14.3%, 13.1%, 0%, respectively (Table 4.4).

Different districts (OR= 3.7; 95%CI= 3.27-14.79; *p*= <0.01) showed different in seroprevalence (Table 4.5), with Dr Ruth Segomotsi Mompati having the highest number of seropositive animals (21.6%), followed by the Ngaka Modiri Molema (15.1%), then Bojanala Platinum (5.2%), and Dr Kennet Kaunda (3.3%). Within municipalities (OR= 3.66; 95%CI= 1.84-7.27; *p*= >0.01), Naledi had the highest number of seropositive animals (52%), followed by Ramotshere Moiloa (40.9), Ratlou (36.2), Kagisano-Molopo (16.1%), Moretele (7.3%),

Maquassi Hills (5.6%), Moses Kotane (5%), Mahikeng (4.2%), Greater Taung (3.2%) and the lowest being JB Marks, Kgetlengriver, and Madibeng all with 0% seroprevalence. There was a higher prevalence (24%) from farms with the presence of cats (OR= 3.46; 95% CI= 1.79-6.69; p= <0.01) than the ones that did not have cats (10%). Feeding systems (OR= 7.87; 95% CI= 2.95 - 21.01, p= <0.01) showed varying seroprevalence with the free and home-fed animals (21.1%) having the highest seroprevalence, followed by the free grazing animals (17.9%), then free grazing and farm fed (2.3%) and lastly, home fed (0%). Animals from farms where feed is stored (OR=21.0; 95% CI=3.05-217.63; p= <0.01) in a car garage, designated room, designated shack, storeroom, in the house and those that are not fed feed showed a seroprevalence of 17.9%, 3.9%, 5.6%, 17.9%, 42.9%, and 18.7% respectively. The disposal of manure (OR= 3.18; 95%CI= 0.06-1.05; p= <0.01) was a risk factor with the animals from farms who use the manure as a fertilizer having the highest seroprevalence (16.7%), followed by the ones that dispose it in the bins (13.6%), the one that never clean their kraals (4.2%), and that burry it in the soil had 0% seroprevalence. Age (OR= 7.68; 95%CI= 0.25-1.00; p= 0.66), type of farm (OR= 1.51; 95%CI= 0.96, 2.39; p= 0.6), and water source (OR= 0.49; 95%CI= 0.24-1.00; p=0.06) were found to be insignificant to the animal's exposure to *T. gondii* infection (Table 4.3 and 4.5).

**Table 4.4:** Univariate risk factors associated with the origin and abortion history of the animals

| **Risk factors** | | ***n*** | **No. of positive** | **Percentage (%)** | **OR** | **95% CI** | ***p*-value** |
|---|---|---|---|---|---|---|---|
| **Origin of animals** | Auction | 112 | 7 | 6.2 | 2.76 | 1.15- 6.61 | >0.01*[a] |
| | Local market | 174 | 28 | 16.1 | | | |
| | Local market and auction | 19 | 12 | 63.2 | | | |
| | Own breed | 104 | 6 | 5.8 | | | |
| | Local market and own breed | 30 | 8 | 26.6 | | | |
| **History of abortion** | Yes | 81 | 20 | 24.7 | 3.34 | 1.55-7.20 | <0.01*[a] |
| | No | 339 | 40 | 11.9 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Burry | 90 | 15 | 16.7 | | | |
| | Burry or feed pets | 31 | 13 | 41.9 | | | |
| | Burn | 8 | 0 | 0 | | | |
| | Burn or burry | 9 | 3 | 33.3 | | | |
| **Disposal of aborted material** | Burn or hang on the tree or kraal | 26 | 0 | 0 | 1.96 | 0.43-1.16 | <0.001*[a] |
| | Feed to pets | 7 | 1 | 14.3 | | | |
| | No history | 251 | 33 | 13.1 | | | |
| | Send to the state vet | 17 | 0 | 0 | | | |

CI: confidence interval; *n*: number of animals tested; No.: number of positive animals; OR: Odds Ratio; *: statistically significant; AI: artificial insemination; [a]based on chi-square

**Table 4.5** Univariate risk factors associated with the rearing environment of the animals

| **Risk factors** | | ***n*** | **No. of positive** | **Percentage (%)** | **OR** | **95% CI** | ***p*-value** |
|---|---|---|---|---|---|---|---|
| **District** | Bojanala Platinum | 77 | 4 | 5.2 | | | |
| | Dr Kennet Kaunda | 30 | 1 | 3.3 | | | |
| | Dr Ruth Segomotsi Mompati | 88 | 19 | 21.6 | 3.7 | 3.27-14.79 | <0.01* |
| | Ngaka Modiri Molema | 245 | 37 | 15.1 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Greater Taung | 31 | 1 | 3.2 | | | |
| | JB Marks | 12 | 0 | 0 | | | |
| | Kagisano-Molopo | 31 | 5 | 16.1 | | | |
| | Kgetlengrivier | 10 | 0 | 0 | | | |
| | Madibeng | 6 | 0 | 0 | | | |
| **Municipality** | Mahikeng | 165 | 7 | 4.2 | | | |
| | Maquassi Hills | 18 | 1 | 5.6 | 3.66 | 1.84-7.27 | >0.01 |
| | Moretele | 41 | 3 | 7.3 | | | |
| | Moses Kotane | 20 | 1 | 5 | | | |
| | Naledi | 25 | 13 | 52 | | | |
| | Ramotshere Moiloa | 22 | 9 | 40.9 | | | |
| | Ratlou | 58 | 21 | 36.2 | | | |
| **Type of farm** | Commercial | 83 | 9 | 10.8 | 1.51 | 0.96, 2.39 | 0.06 |
| | Communal | 356 | 53 | 14.9 | | | |
| **Presence of cats** | Yes | 129 | 31 | 24.0 | 3.46 | 1.79-6.69 | <0.01* [a] |
| | No | 310 | 31 | 10 | | | |
| | Borehole | 261 | 42 | 16.1 | | | |
| | Borehole and dam | 76 | 9 | 11.8 | | | |
| **Water source** | Borehole and municipal | 26 | 0 | 0 | 0.49 | 0.24-1.00 | 0.06 |
| | Borehole and river | 16 | 1 | 6.3 | | | |
| | Dam | 37 | 4 | 10.8 | | | |
| | Municipal (tap) | 21 | 6 | 28.6 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Car garage | 28 | 5 | 17.9 | | | |
| | Designated room | 128 | 5 | 3.9 | | | |
| **Feed storage** | Designated shack | 18 | 1 | 5.6 | 21.0 | 3.05-217.63 | <0.01*[a] |
| | Storeroom | 160 | 27 | 16.9 | | | |
| | In the house | 14 | 6 | 42.9 | | | |
| | Not fed feed | 91 | 17 | 18.7 | | | |
| | Dispose in a bin | 59 | 8 | 13.6 | | | |
| **Disposal of manure** | Burry in the soil | 10 | 0 | 0 | | | |
| | Kraals are never cleaned | 71 | 3 | 4.2 | 3.18 | 0.06-1.05 | <0.01*[a] |
| | Use as fertilizer for plants | 299 | 50 | 16.7 | | | |
| | Free grazing | 101 | 17 | 16.8 | | | |
| **Feeding system** | Free grazing and farm-fed (CRF) | 130 | 3 | 2.3 | | | |
| | Free grazing and home-fed (CNF) | 199 | 42 | 21.1 | 7.87 | 2.95 - 21.01 | <0.01* |
| | Home-fed (CNF) | 9 | 0 | 0 | | | |

CI: confidence interval; *n*: number of animals tested; No.: number of animals tested; *: statistically significant; OR: odds ratio; [a]based on chi-square; CRF: commercial CNF: communal

### 4.4 Molecular detection

#### 4.4.1 Amplification of universal ribosomal 18S RNA

Out of the 198 samples tested using universal 18S ribosomal RNA primers, 190 were amplified. This proved that our extraction method worked and that the samples had parasite DNA. An example of a gel picture with some of the tested samples is shown in figure 4.3.

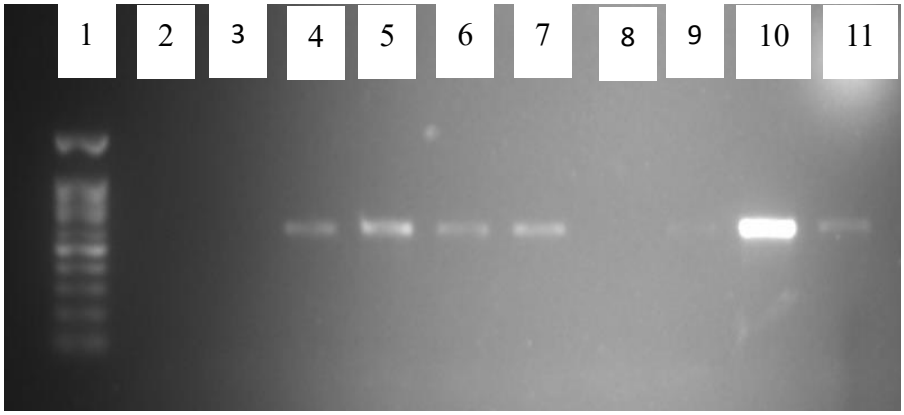


**Figure 4.3:** Amplification of universal ribosomal 18S PCR products. **Lane 1** is the DNA molecular weight ladder of 100 bp (New England Biolabs, Ipswich, MA, USA); **lane 2** is the nuclease free water, a negative control; **lane 3** to **lane 11** are some of the tested samples.

### 4.4.2 Amplification of B1 gene

Out of the 198 samples tested using B primers with an expected size of 194 bp, none of them amplified. Figure 4.4 shows an example of some of the tested samples.

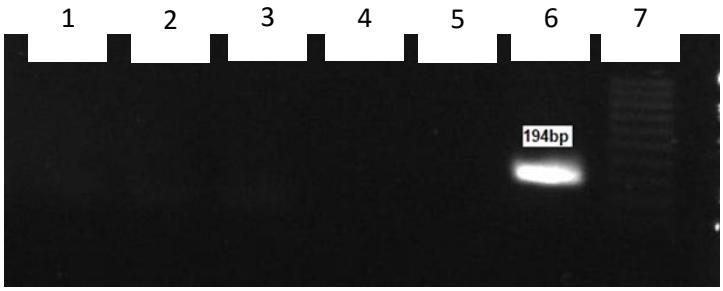


**Figure 4.4:** Amplification of 194 bp of B1 PCR products. **Lane 1** to **lane 4** are some of the tested samples; **lane 5** is nuclease free water, a negative control; **lane 6** is a *T. gondii* positive control; and **lane 7** is a DNA molecular weight ladder of 100 bp (New England Biolabs, Ipswich, MA, USA).

### 4.4.3 Amplification of ribosomal *T. gondii* RNA (18S) gene

All the 198 samples tested by *T. gondii* 18S rRNA amplified using 18S rRNA primers did not amplify. A 0% molecular detection was recorded. Figure 4.5 shows an example of some of the tested samples.

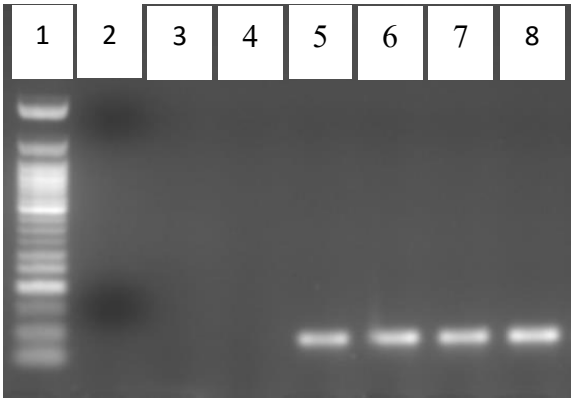


**Figure 4.5:** Amplification of 88 bp ribosomal RNA 18S PCR products. **Lane 1** is the DNA molecular weight ladder 100 bp of (New England Biolabs, Ipswich, MA, USA). **Lane 2** is nuclease free water, a negative control; **lane 3** and lane **4** are some of the diagnostic samples; **lane 5** to **lane 8** are *T. gondii* positive controls.

### 4.4.4 Sequencing of the universal 18S rRNA fragment for *T. gondii* confirmation

Four amplicons (#7, #45, #48, and B1) from the amplified samples were sequenced. After sequencing, the sequences were edited and Blasted to confirm if they are *T. gondii*. The results of the Blast showed that they are eukaryotic DNA, not *T. gondii* or any other organism that causes reproductive illnesses and abnormalities in sheep and goats.

### 4.4.5 Correlation and agreement between the serological and molecular detection

The Cohen's kappa (k) test showed that there is an agreement of 50% and a fair correlation between the seropositivity (ELISA) and molecular detection (PCR) with a kappa of 0.33 as shown in table 4.6.

**Table 4. 6:** Correlation and agreement between serological (ELISA) and molecular detection (PCR) data

| | | **PCR** | | Row marginals | | Agreement | Cohen's Kappa |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | | | | |
| **ELISA** | Positive | 61 | 126 | 187 | $rm^1$ | | |
| | Negative | 0 | 187 | 187 | $rm^2$ | 0.50 | 0.33 |
| Column Marginals | | 61 | 313 | 374 | n | | |
| | | $cm^1$ | $cm^2$ | | | | |

*cm: column marginals; rm: row marginals; CI: Confidence interval

## 4.5 Sequence and phylogenetic analysis of the *T. gondii* B1 and GRA6 gene sequences

### 4.5.1 *Toxoplasma gondii* B1 gene sequence analysis for 803 bp fragments

Single nucleotide polymorphism was found in 29% (7/24) of the analysed gene sequences from Mexican sheep isolates, as shown in Appendix E with SNPs highlighted in red. The SNPs were at position 506 in isolate Tecuanillo178, at positions 574, 616, and 667 in isolate ElReal11, at position 581 in isolate ElReal109a, at position 525 in isolate Camelalote106, at position 228 in isolate Estacion98 and at the same position (132) on isolate Camalote102b and Estacion101b. These isolates did not show pronounced differences in their SNPs position, which is not surprising given they are from the origin and species. Appendix H contains a list of the analysed isolates, their assertion numbers, as well as the host and country from which they were isolated.

### 4.5.2 *Toxoplasma gondii* B1 gene sequence analysis from fragment sizes of between 300 to 1000 bp that were trimmed and aligned

As shown in Appendix F with yellow (absent sequences) and red (SNPs) highlighted colors, SNP we identified in 46% (38/83) of the analysed isolate sequences. Isolate TgCatAu_7 from a cat in Australia had absent sequences from position 50 until position 110 and it was the only isolate with absent sequences. This isolate exhibited a distinction from the rest of the isolate, even the ones that were isolated from the same species in Australia as it was the only one with absent codons at these positions.

Isolate Estacion98 from sheep in Colima, Mexico had SNP at position 16, isolate Camolote102b from sheep in Michoacan, Mexico had it at position 222, isolate Estacion101b from sheep in Colima, Mexico had it at position 126, isolate 16A from sheep in Iran, at positions 396, 325, 331 and 396, sheep isolates 1A, 2A, 16A, and 26 from Camel in Iran all had it at position 325, isolate CR34 from California mussel in the USA had them at positions 187, and 354, isolate SR217 from California mussel in the USA at positions 199 and 325, isolate 2A from sheep in Iran at positions 287 and 237, isolate 25 from Camel in Iran had them at position 187. The Iranian sheep and camel isolates had SNPs at the same position which could be mean they are the same strain. This was the same observation from all the sheep although they are not of the same origin which could be an indication of them being affected by a strain that is most isolated in sheep.

Isolate SR231 from California muscle in California, isolate SR222 from California muscle in USA, TGK-KLK-365-IMNO from an *Ixodes ricinus* tick in Poland, and isolate SR222 from California muscle in the USA, all had them at position 187, 45, and 199, respectively. Isolate

TGK-KLR-IMNO from an *Ixodes ricinus* tick had them at positions 39 and 452, isolate 781-L-IMNO also from an Ixodes Ricinus tick in Poland had them at positions 4, 35, 452, and 472, isolate 782-L-IMNO from an *Ixodes ricinus* tick in Poland had them at positions 4, 35, 452, and 472, and at positions 118, 119, 199, and 243 in isolates of the *Ixodes ricinus* tick from Poland (TGK-KLR-625-IMNO, TGK-KLR-631-IMNO, TGK-KLR-583-IMNO, and TGK-KLR-610-IMNO). The *Ixodes ricinus* ricks and the California muscle isolates indicate a possible relation as they show SNPs at the same positions.

The SNP was also identified from black bears isolate 220 from the USA had it at position 325, while isolate 222 had them at positions 325, 396, and 459. Isolate TGK-KLR-983-IMNO from an *Ixodes ricinus* tick in Poland had them at positions 402, 325, and 234, isolate TGK-KLR-744-IMNO had them at positions 452, 456, and 471, and isolate TGK-KLR-836-IMNO had SNPs at positions 452, and 471. Clones from Iran: clone SY5 from sheep had SNP at position 402, clone CG21 from chicken had it at position 187, clone SY4 from sheep had it at positions 234, and 325, while clones SY12 from sheep and clone CQ7 from cattle both had it at the same position (325). It is interesting to note that there are SNP positions shared among these isolates although they were isolated from different species originating from different countries. The isolates from Iran also present with SNPs at different locations, except for cow for the cow and sheep isolate. Appendix I contains a list of the isolates and/or clones, their assertion numbers, as well as the hosts and countries from which they were isolated.

### 4.5.3 *Toxoplasma gondii* B1 gene sequence analysis from fragment sizes of between 300 to 1000 bp that were trimmed and aligned

As shown in the sequences marked in red (SNPs) and yellow (absent sequences) in Appendix G, SNPs and absent sequences were found in 83% (63/76) and 80% (61/76) of the isolates' analysed GRA6 gene sequences, respectively. Apart from isolates TgCoP02, TgCoP03, and TgCo04 from coyotes in the United States, isolate TgA18001 from a Jaguar in French Guiana, isolate TgSoUs14 from sea otter in the USA, isolate TgBobcatMS1 from a cat in Mississippi, and isolates (TgWolfMN11, TgWolfMN12, TgWolfMN13, TgWolfMN19, TgWolfMN25, TgWolfMN26, TgWolfMN27, TgWolfMN28 and TgWolfMN29) from grey wolves, all the sequences had absent sequences from position 272 to position 274. On isolate TgA105037 from chicken in Gabon, sequences were absent from positions 241 to 303. All Turkish cat isolates (TgCatTr_ Izmir02, TgCatTr_ Izmir03, TgCatTr_ Izmir06, TgCatTr_ Izmir09, TgCatTr_Izmir11, TgCatTr_Izmir12, TgCatTr_ Izmir18, TgCatTr_ Izmir29, TgCatTr_

Izmir20, and TgCatTr_Izmir22) showed SNPs at the same locations, at positions 21 and 89 and 151, respectively. The grey wolf isolates TgWolfMN11, TgWolfMN12, TgWolfMN13, TgWolfMN19, TgWolfMN25, TgWolfMN26, TgWolfMN27, TgWolfMN28 and TgWolfMN29 from the USA had SNPs at position 75, 126, 142, 279, 401, and 423, respectively, except for isolate TgWolfMN20, which had additional SNPs at locations 21, 86, 151, 319, 544, 559, and 597 in addition to the same SNPs at positions 126 and 146 shared with the other isolates.

Isolates TgCkPr01 (chicken), TgCkPr02 (chicken), TgCkPr04 (chicken), TgCkPr16 (chicken), TgPiPr02 (pig), TgCkPr14 (pig) from Portugal all had SNPs at the same positions (21, 319, 544, 597 and 599), except for isolates TgPiPr05 (pig) and TgCkPr03 (chicken) which only had them at position 21 and 151. Gabon isolates TgA105001 (chicken), TgA105002 (chicken), TgA10511 (goat), TgA105015 (chicken), TgA105016 (chicken), TgA105018 (chicken), and TgA105043 (chicken) shared SNPs at locations 21, 86, 319, 544, 559, and 597. (chicken). Additionally, position 150 was shared by isolates TgA18005, TgA05002, TgA105043, and TgA105001. Compared to the other isolates, TgA32129 (sheep) only had SNPs at positions 21 and 279. The SNPs for the USA coyotes isolate TgCoPa02, TgCoPa03, TgCoPa04, and TgCoPa07 were located at positions 75, 126, 273, 279, and 401, respectively. French Guiana isolate TgA18001 (jaguar) had SNPs at positions 75,126, and 279, while isolate TgA105002 (grison) had them at positions 21, 151, 151, 319, 544, 559, and 562. Again, we see isolatesfr

Iranian isolate 4A (goat) had SNPs at positions 21,151, 287, and 319, isolate 22 (camel) had them at positions 59, and 151, isolate 7B (sheep) at positions 21, 89, and 151, isolate 5A (sheep) at positions 21, 51, 89,287, and 319, isolate 5B (sheep) at positions 89, and 151, isolate 11 (sheep) at positions 51, 89, 287, 319, 559, and 579, 22R (sheep) at positions 21, 89, 151, and 319, lastly isolate 16A (sheep) at positions 21, 59, 151, 421, and 510. Appendix J contains a list of the isolates/clones, their assertion numbers, the host, and the countries from which they were isolated.

In this analysis, we saw similarities in SNPs position from isolates isolated from the same species of the same origin. This is interesting since it indicates that the strains of these isolates are not species specific.

### 4.5.4 Phylogenetic relationship of *T. gondii* B1 gene from 803 bp sequences

Figure 4.6 shows the phylogenetic relatedness of sheep from Mexican sheep isolates that resulted from trimming and alignment of gene sequences from various countries and species

that were retrieved from the GenBank based on B1 gene sequence (803 bp) (https://www.ncbi.nlm.nih.gov/). Despite being from different parts of Mexico, the majority of isolates clustered (cluster 2) together in the phylogenetic tree, suggesting high sequence similarity. Although isolate Camalote102b and Estacion101b are not from the same state, they formed their own cluster (cluster 1), suggesting they are more related than the rest of the isolates.

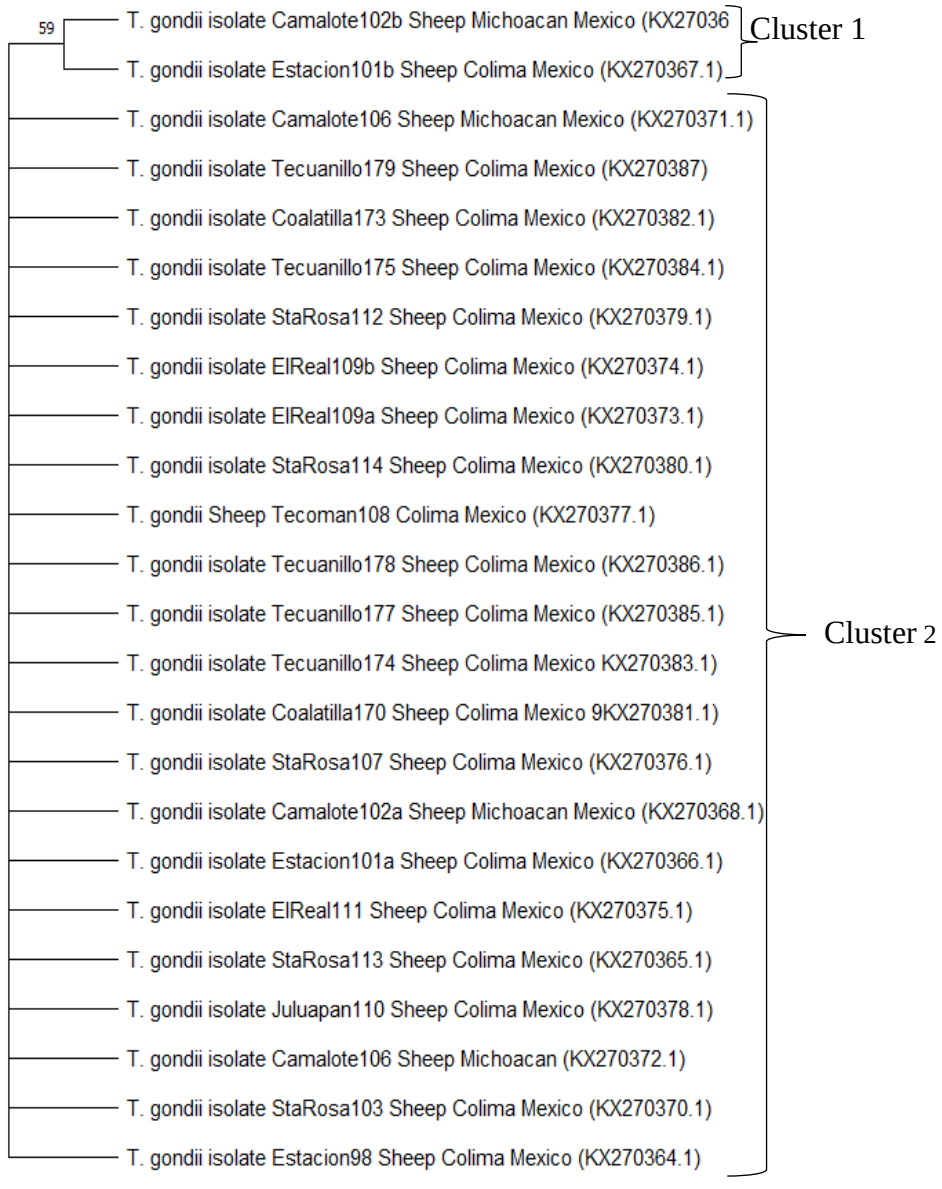


**Figure 4.6:** Phylogenetic tree of *T. gondii* B1 gene (803 bp fragments). The Neighbor-Joining approach was used to infer the evolutionary history (Saitou and Nei, 1987). The bootstrap consensus tree produced from 1000 repeats is supposed to represent the evolutionary history of the taxa under consideration (Felsenstein, 1985). Branch collapse occurs for partitions repeated in less than 50% of bootstrap repetitions. The percentage of duplicate trees in the bootstrap test (1000 iterations) where the associated taxa clustered together is shown next to the branches (Felsenstein, 1985). The evolutionary distances, which are measured in terms of the number of base substitutions

per site, were calculated using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004). There were 24 nucleotide sequences in this investigation.

### 4.5.5 Phylogenetic relationship of *T. gondii* B1 gene sequences ranging from 300 bp to 1000 bp that we trimmed and aligned

Figure 4.7 shows phylogenic relatedness from isolates/clones from various countries and species from trimmed and aligned *T. gondii* B1 sequences ranging from 300 to 1000 bp retrieved from GenBank (https://www.ncbi.nlm.nih.gov/). Following the construction of the phylogenic tree, the isolates were grouped into 7 clusters. The first cluster was composed of isolates 220 and 222 from American black bears, isolate SR217 from California muscle, isolate 26 from Iranian camel, isolates 1A, 16A, and 16B, clones SY12 and SY4 from Iranian sheep, and clone CQ7 from Iranian cattle. Within cluster 1, isolate 1A, 16A, 16B, 24A, 220, 222, SR217, and clones SY4 and SY12 all formed a sub-cluster. Cluster two formed from isolates. Iranian chicken clone CG19, Iranian goat clones GQ2, GQ3, GY3, and GY4, and isolates 4B and 15B, as well as Iranian camel isolate 22 all contributed to the formation of cluster 2. Cluster 2 also included the Iranian sheep isolates 28B, 22A, 5A, and clones SY3 AND SY5. ElReal109a, ElReal111, Tecuanillo174, Tecuanillo175, Tecuanillo177, Tecuanillo178, Tecuanillo179, Coalatilla 170, Coalatilla 173, StaRosa114, Estacion98, Juluapan110, and Estacion101a are sheep isolates from Colima, Mexico, making up the second cluster. Cluster 2 was similarly produced by an isolate of Camolote106 from sheep in Michoacan, Mexico. Iranian chicken clone CG19, Iranian goat clones GQ2, GQ3, GY3, and GY4, and isolates 4B and 15B, as well as Iranian camel isolate 22 all contributed to the formation of cluster 2. Cluster 2 also included the Iranian sheep isolates 28B, 22A, 5A, and clones SY3 AND SY5, as well as clone D from duck and CY2 cattle both from Iran. Isolate ElReal109a, ElReal111, Tecuanillo174, Tecuanillo175, Tecuanillo177, Tecuanillo178, Tecuanillo179, Coalatilla 170, Coalatilla 173, StaRosa114, Estacion98, Juluapan110, and Estacion101a are from sheep isolates in Colima, Mexico, making up the second cluster. Cluster 2 was similarly produced by an isolate of Camolote106 from sheep in Michoacan, Mexico. Ixodes ricinus tick isolated from Poland, isolate TG-KLR-583-IMNO, TG-KLK-1018-IMNO, TG-KLK983-IMNO and TF-KLK-720-IMNO, TG-KLK-720-IMNO, TG-KLK-830-IMNO, TG-KLK-555-IMNO, TG-KLK-365-IMNO, and TG-KLR-625-IMNO formed part of cluster 2. Australian cat isolates TgCatAu_6 and TgCatAu_8 were the final isolates making up cluster 2. Clone CG21 from Iranian chicken, isolate 25 from Iranian camels, isolate SR231 from California muscle, and

isolate CR34 from California muscle made up the third cluster. Both muscles isolates and subclustered under the third cluster. Sheep isolates Camalote102b from Michoacan and Estacion101b from Colima, both in Mexico, formed Cluster 4. Isolates from the Polish Ixodes ricinus 836-L-IMNO, 774-L-IMNO, 782-L-IMNO, and 781-L-IMNO collectively formed cluster 5, with isolates 836-L-IMNO and 774-L-IMNO forming subcluster 5.1 and isolates 782-L-IMNO and 781-L-IMNO forming subcluster 5.2. Isolates TG-KLR-610-L-IMNO and SR222 from California muscle, California, as well as the Polish isolates TG-KLR-631-L-IMNO and TG-KLR-610-L-IMNO of Ixodes ricinus formed Cluster 6. Three isolates, SR215 from California muscle, TgCatAu_7 from an Australian cat, and C-F-TG-56 from a South Korean cat formed the final cluster, cluster 7, which also formed subcluster 7.1 with TgCatAu_7 and C-F-TG-56.

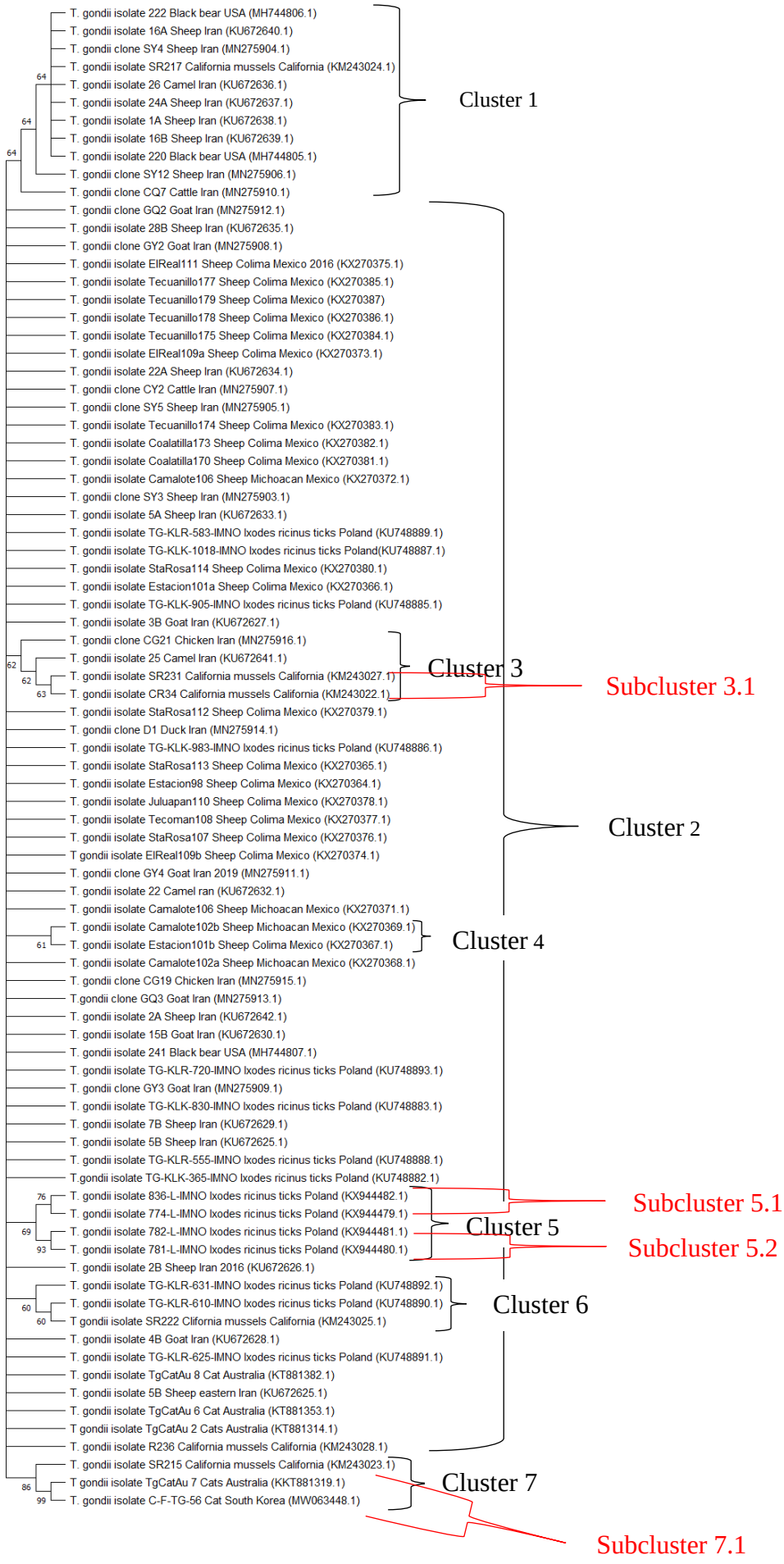
T. gondii isolate 222 Black bear USA (MH744806.1)
T. gondii isolate 16A Sheep Iran (KU672640.1)
T. gondii clone SY4 Sheep Iran (MN275904.1)
T. gondii isolate SR217 California mussels California (KM243024.1)
T. gondii isolate 26 Camel Iran (KU672636.1)
T. gondii isolate 24A Sheep Iran (KU672637.1)
T. gondii isolate 1A Sheep Iran (KU672638.1)
T. gondii isolate 16B Sheep Iran (KU672639.1)
T. gondii isolate 220 Black bear USA (MH744805.1)
T. gondii clone SY12 Sheep Iran (MN275906.1)
T. gondii clone CQ7 Cattle Iran (MN275910.1)
T. gondii clone GQ2 Goat Iran (MN275912.1)
T. gondii isolate 28B Sheep Iran (KU672635.1)
T. gondii clone GY2 Goat Iran (MN275908.1)
T. gondii isolate ElReal111 Sheep Colima Mexico 2016 (KX270375.1)
T. gondii isolate Tecuanillo177 Sheep Colima Mexico (KX270385.1)
T. gondii isolate Tecuanillo179 Sheep Colima Mexico (KX270387)
T. gondii isolate Tecuanillo178 Sheep Colima Mexico (KX270386.1)
T. gondii isolate Tecuanillo175 Sheep Colima Mexico (KX270384.1)
T. gondii isolate ElReal109a Sheep Colima Mexico (KX270373.1)
T. gondii isolate 22A Sheep Iran (KU672634.1)
T. gondii clone CY2 Cattle Iran (MN275907.1)
T. gondii clone SY5 Sheep Iran (MN275905.1)
T. gondii isolate Tecuanillo174 Sheep Colima Mexico (KX270383.1)
T. gondii isolate Coalatilla173 Sheep Colima Mexico (KX270382.1)
T. gondii isolate Coalatilla170 Sheep Colima Mexico (KX270381.1)
T. gondii isolate Camalote106 Sheep Michoacan Mexico (KX270372.1)
T. gondii clone SY3 Sheep Iran (MN275903.1)
T. gondii isolate 5A Sheep Iran (KU672633.1)
T. gondii isolate TG-KLR-583-IMNO Ixodes ricinus ticks Poland (KU748889.1)
T. gondii isolate TG-KLK-1018-IMNO Ixodes ricinus ticks Poland(KU748887.1)
T. gondii isolate StaRosa114 Sheep Colima Mexico (KX270380.1)
T. gondii isolate Estacion101a Sheep Colima Mexico (KX270366.1)
T. gondii isolate TG-KLK-905-IMNO Ixodes ricinus ticks Poland (KU748885.1)
T. gondii isolate 3B Goat Iran (KU672627.1)
T. gondii clone CG21 Chicken Iran (MN275916.1)
T. gondii isolate 25 Camel Iran (KU672641.1)
T. gondii isolate SR231 California mussels California (KM243027.1)
T. gondii isolate CR34 California mussels California (KM243022.1)
T. gondii isolate StaRosa112 Sheep Colima Mexico (KX270379.1)
T. gondii clone D1 Duck Iran (MN275914.1)
T. gondii isolate TG-KLK-983-IMNO Ixodes ricinus ticks Poland (KU748886.1)
T. gondii isolate StaRosa113 Sheep Colima Mexico (KX270365.1)
T. gondii isolate Estacion98 Sheep Colima Mexico (KX270364.1)
T. gondii isolate Juluapan110 Sheep Colima Mexico (KX270378.1)
T. gondii isolate Tecoman108 Sheep Colima Mexico (KX270377.1)
T. gondii isolate StaRosa107 Sheep Colima Mexico (KX270376.1)
T gondii isolate ElReal109b Sheep Colima Mexico (KX270374.1)
T. gondii clone GY4 Goat Iran 2019 (MN275911.1)
T. gondii isolate 22 Camel ran (KU672632.1)
T. gondii isolate Camalote106 Sheep Michoacan Mexico (KX270371.1)
T. gondii isolate Camalote102b Sheep Michoacan Mexico (KX270369.1)
T. gondii isolate Estacion101b Sheep Colima Mexico (KX270367.1)
T. gondii isolate Camalote102a Sheep Michoacan Mexico (KX270368.1)
T. gondii clone CG19 Chicken Iran (MN275915.1)
T.gondii clone GQ3 Goat Iran (MN275913.1)
T. gondii isolate 2A Sheep Iran (KU672642.1)
T. gondii isolate 15B Goat Iran (KU672630.1)
T. gondii isolate 241 Black bear USA (MH744807.1)
T. gondii isolate TG-KLR-720-IMNO Ixodes ricinus ticks Poland (KU748893.1)
T. gondii clone GY3 Goat Iran (MN275909.1)
T. gondii isolate TG-KLK-830-IMNO Ixodes ricinus ticks Poland (KU748883.1)
T. gondii isolate 7B Sheep Iran (KU672629.1)
T. gondii isolate 5B Sheep Iran (KU672625.1)
T. gondii isolate TG-KLR-555-IMNO Ixodes ricinus ticks Poland (KU748888.1)
T.gondii isolate TG-KLK-365-IMNO Ixodes ricinus ticks Poland (KU748882.1)
T. gondii isolate 836-L-IMNO Ixodes ricinus ticks Poland (KX944482.1)
T. gondii isolate 774-L-IMNO Ixodes ricinus ticks Poland (KX944479.1)
T. gondii isolate 782-L-IMNO Ixodes ricinus ticks Poland (KX944481.1)
T. gondii isolate 781-L-IMNO Ixodes ricinus ticks Poland (KX944480.1)
T. gondii isolate 2B Sheep Iran 2016 (KU672626.1)
T. gondii isolate TG-KLR-631-IMNO Ixodes ricinus ticks Poland (KU748892.1)
T. gondii isolate TG-KLR-610-IMNO Ixodes ricinus ticks Poland (KU748890.1)
T gondii isolate SR222 Clifornia mussels California (KM243025.1)
T. gondii isolate 4B Goat Iran (KU672628.1)
T. gondii isolate TG-KLR-625-IMNO Ixodes ricinus ticks Poland (KU748891.1)
T. gondii isolate TgCatAu 8 Cat Australia (KT881382.1)
T. gondii isolate 5B Sheep eastern Iran (KU672625.1)
T. gondii isolate TgCatAu 6 Cat Australia (KT881353.1)
T gondii isolate TgCatAu 2 Cats Australia (KT881314.1)
T. gondii isolate R236 California mussels California (KM243028.1)
T. gondii isolate SR215 California mussels California (KM243023.1)
T gondii isolate TgCatAu 7 Cats Australia (KKT881319.1)
T. gondii isolate C-F-TG-56 Cat South Korea (MW063448.1)
64
64
64
62
62
63
61
76
69
93
60
60
86
99
Cluster 1
Cluster 3
Subcluster 3.1
Cluster 2
Cluster 4
Subcluster 5.1
Cluster 5
Subcluster 5.2
Cluster 6
Cluster 7
Subcluster 7.1

**Figure 4.7:** Phylogenetic tree of *T. gondii* B1 gene from trimmed fragment sizes of 300 to 1000 bp sequences. The Neighbor-Joining approach was used to infer the evolutionary history (Saitou and Nei, 1987). The bootstrap consensus tree produced from 1000 repeats is supposed to represent the evolutionary history of the taxa under consideration (Felsenstein, 1985). Branch collapse occurs for partitions repeated in less than 50% of bootstrap repetitions. The percentage of duplicate trees in the bootstrap test (1000 iterations) where the associated taxa clustered together is shown next to the branches (Felsenstein, 1985). The evolutionary distances, which are measured in terms of the number of base substitutions per site, were calculated using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004). There were 83 nucleotide sequences in this analysis.

### 4.5.6 Phylogenetic relationship of *T. gondii* GRA 6 gene isolates

The phylogenic tree shown in figure 4.8 illustrates relatedness based on GRA6 gene sequences that vary in length from 400 base pairs to 1000 base pairs that were retrieved from GenBank from isolates from different countries and species. Six clusters were formed through the construction of the phylogenic tree. The chicken isolates TgA105015, TgA105016, and TgA105018 from Gabon formed cluster 1 together with isolate TgPiPr02 and TgPiPr14 from pigs in Portugal, isolate TgA105011 from a goat in Gabon, isolate TgFoxPa03 from a red fox in Portugal, TgCkPr04 from a chicken in Portugal, TgWolfMN20 from a grey wolf in the USA, and finally, pig isolates from Portugal TgCKPr01, TgCKPr02, TgCKPr04, and TgCKPr16. Isolates TgWtdUs10 from a white-tailed deer from the USA and TgA18005 from a Grison in French Guiana formed cluster 2. Cluster 3 formed from 3 isolates (TgA105002, TgA105043 and TgA105001) from chicken in Gabon, with isolate TgA105043 and TgA105001 forming subcluster 3.1.

Cluster 4 was made up of the Iranian sheep isolates 5A, 8A, and 11, as well as the red fox isolate TgFoxPa06 and the sheep isolate Tgshir2 from Mashhad, Iran. Isolate 5A and 8A formed a subcluster, while isolate 11 formed subcluster 4.1 with Tgshir2. Gray wolf isolates TgWolfMN11, TgWolfMN12, TgWolfMN13, TgWolfMN19 TgWolfMN25, TgWolfMN26, TgWolfMN27, TgWolfMN28, and TgWolfMN29 from the USA all clustered under cluster 5. Additionally, grouped under cluster 5 were the isolates TgA105037 from chickens in Gabon, TgA18001 from a jaguar in French Guiana, TgBobcatMS1 from a cat in Mississippi, USA, and TgSoUs14 from a coyote in the USA. Within cluster 5, isolates TgWolfMN11 and TgBobcatMS1 formed subcluster 5.1. Cluster 6 was the final cluster, and it included the chicken isolates TgA32129, TgA105051, TgA105053, TgA105003, and TgA105004 from Gabon, as well as the isolates TgCkPr03 TgCkPr11 from chicken in Portugal, TgFoxPa10 from red fox in the USA, TgPiPr09 and TgPiPr13 from pigs in Portugal, and TgCoPa01, TgCoPa07

and TgCoPa08 from a coyote in the USA. Cat isolates TgCatTR Izmir02, TgCatTR Izmir03, TgCatTR Izmir06, TgCatTR Izmir09, T TgCatTR Izmir02, TgCatTR Izmir11, TgCatTR Izmir12, TgCatTR Izmir18, TgCatTR Izmir19, and TgCatTR Izmir22 from Turkey, isolates KM from Chinese cat, TgWtdUs08 from white-tailed deer, isolate 5B from Iranian sheep, TgA32129 from Gabon sheep, and TgA32129 from France sheep also clustered under cluster 6. The Iranian camel isolates (isolates 22 and 24) were also in cluster 6.

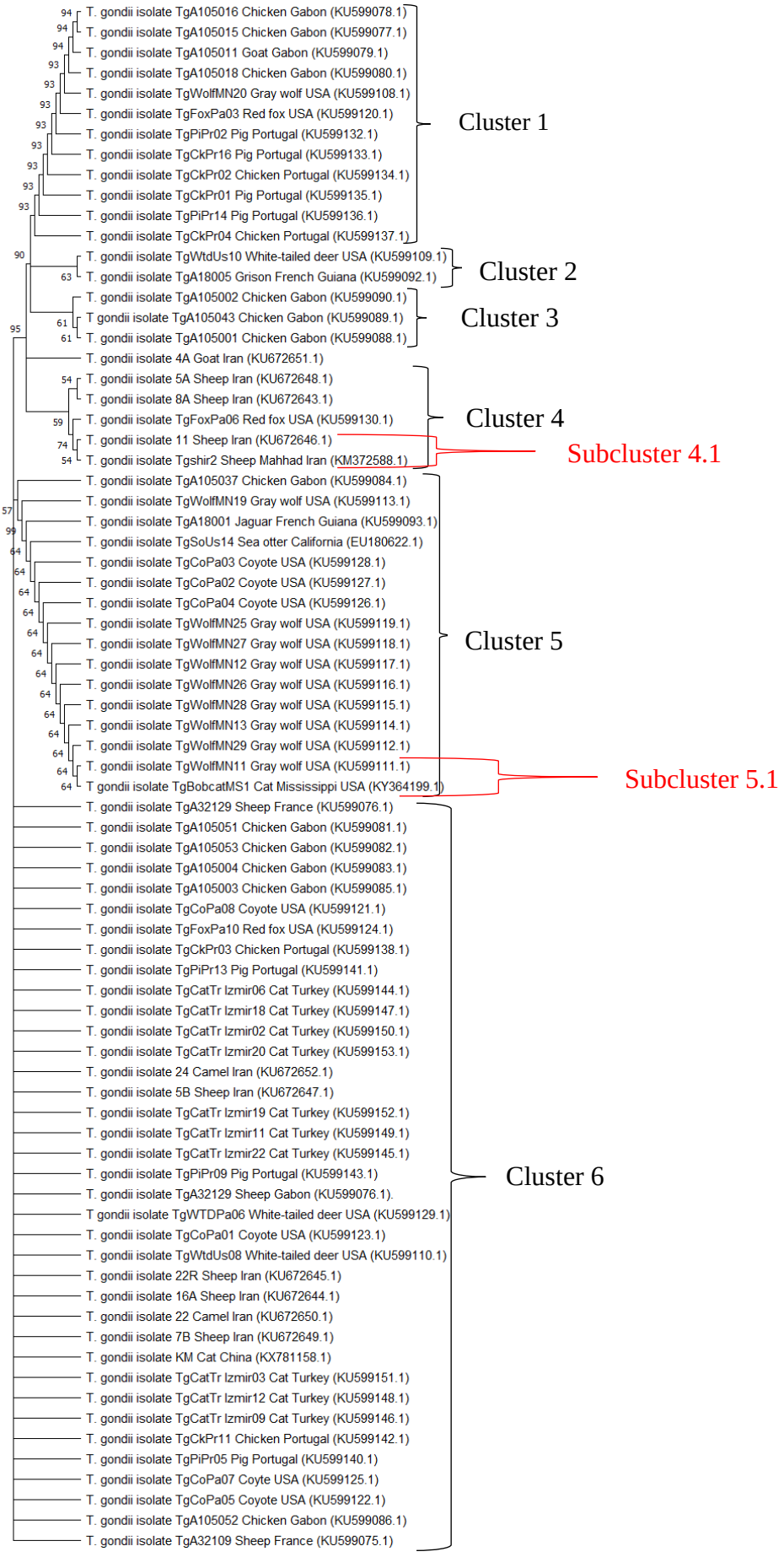
T. gondii isolate TgA105016 Chicken Gabon (KU599078.1)
T. gondii isolate TgA105015 Chicken Gabon (KU599077.1)
T. gondii isolate TgA105011 Goat Gabon (KU599079.1)
T. gondii isolate TgA105018 Chicken Gabon (KU599080.1)
T. gondii isolate TgWolfMN20 Gray wolf USA (KU599108.1)
T. gondii isolate TgFoxPa03 Red fox USA (KU599120.1)
T. gondii isolate TgPiPr02 Pig Portugal (KU599132.1)
T. gondii isolate TgCkPr16 Pig Portugal (KU599133.1)
T. gondii isolate TgCkPr02 Chicken Portugal (KU599134.1)
T. gondii isolate TgCkPr01 Pig Portugal (KU599135.1)
T. gondii isolate TgPiPr14 Pig Portugal (KU599136.1)
T. gondii isolate TgCkPr04 Chicken Portugal (KU599137.1)
Cluster 1
T. gondii isolate TgWtdUs10 White-tailed deer USA (KU599109.1)
T. gondii isolate TgA18005 Grison French Guiana (KU599092.1)
Cluster 2
T. gondii isolate TgA105002 Chicken Gabon (KU599090.1)
T gondii isolate TgA105043 Chicken Gabon (KU599089.1)
T. gondii isolate TgA105001 Chicken Gabon (KU599088.1)
Cluster 3
T. gondii isolate 4A Goat Iran (KU672651.1)
T. gondii isolate 5A Sheep Iran (KU672648.1)
T. gondii isolate 8A Sheep Iran (KU672643.1)
T. gondii isolate TgFoxPa06 Red fox USA (KU599130.1)
T. gondii isolate 11 Sheep Iran (KU672646.1)
T. gondii isolate Tgshir2 Sheep Mahhad Iran (KM372588.1)
Cluster 4
Subcluster 4.1
T. gondii isolate TgA105037 Chicken Gabon (KU599084.1)
T. gondii isolate TgWolfMN19 Gray wolf USA (KU599113.1)
T. gondii isolate TgA18001 Jaguar French Guiana (KU599093.1)
T. gondii isolate TgSoUs14 Sea otter California (EU180622.1)
T. gondii isolate TgCoPa03 Coyote USA (KU599128.1)
T. gondii isolate TgCoPa02 Coyote USA (KU599127.1)
T. gondii isolate TgCoPa04 Coyote USA (KU599126.1)
T. gondii isolate TgWolfMN25 Gray wolf USA (KU599119.1)
T. gondii isolate TgWolfMN27 Gray wolf USA (KU599118.1)
T. gondii isolate TgWolfMN12 Gray wolf USA (KU599117.1)
T. gondii isolate TgWolfMN26 Gray wolf USA (KU599116.1)
T. gondii isolate TgWolfMN28 Gray wolf USA (KU599115.1)
T. gondii isolate TgWolfMN13 Gray wolf USA (KU599114.1)
T. gondii isolate TgWolfMN29 Gray wolf USA (KU599112.1)
T. gondii isolate TgWolfMN11 Gray wolf USA (KU599111.1)
T gondii isolate TgBobcatMS1 Cat Mississippi USA (KY364199.1)
Cluster 5
Subcluster 5.1
T. gondii isolate TgA32129 Sheep France (KU599076.1)
T. gondii isolate TgA105051 Chicken Gabon (KU599081.1)
T. gondii isolate TgA105053 Chicken Gabon (KU599082.1)
T. gondii isolate TgA105004 Chicken Gabon (KU599083.1)
T. gondii isolate TgA105003 Chicken Gabon (KU599085.1)
T. gondii isolate TgCoPa08 Coyote USA (KU599121.1)
T. gondii isolate TgFoxPa10 Red fox USA (KU599124.1)
T. gondii isolate TgCkPr03 Chicken Portugal (KU599138.1)
T. gondii isolate TgPiPr13 Pig Portugal (KU599141.1)
T. gondii isolate TgCatTr Izmir06 Cat Turkey (KU599144.1)
T. gondii isolate TgCatTr Izmir18 Cat Turkey (KU599147.1)
T. gondii isolate TgCatTr Izmir02 Cat Turkey (KU599150.1)
T. gondii isolate TgCatTr Izmir20 Cat Turkey (KU599153.1)
T. gondii isolate 24 Camel Iran (KU672652.1)
T. gondii isolate 5B Sheep Iran (KU672647.1)
T. gondii isolate TgCatTr Izmir19 Cat Turkey (KU599152.1)
T. gondii isolate TgCatTr Izmir11 Cat Turkey (KU599149.1)
T. gondii isolate TgCatTr Izmir22 Cat Turkey (KU599145.1)
T. gondii isolate TgPiPr09 Pig Portugal (KU599143.1)
T. gondii isolate TgA32129 Sheep Gabon (KU599076.1).
T gondii isolate TgWTDPa06 White-tailed deer USA (KU599129.1)
T. gondii isolate TgCoPa01 Coyote USA (KU599123.1)
T. gondii isolate TgWtdUs08 White-tailed deer USA (KU599110.1)
T. gondii isolate 22R Sheep Iran (KU672645.1)
T. gondii isolate 16A Sheep Iran (KU672644.1)
T. gondii isolate 22 Camel Iran (KU672650.1)
T. gondii isolate 7B Sheep Iran (KU672649.1)
T. gondii isolate KM Cat China (KX781158.1)
T. gondii isolate TgCatTr Izmir03 Cat Turkey (KU599151.1)
T. gondii isolate TgCatTr Izmir12 Cat Turkey (KU599148.1)
T. gondii isolate TgCatTr Izmir09 Cat Turkey (KU599146.1)
T. gondii isolate TgCkPr11 Chicken Portugal (KU599142.1)
T. gondii isolate TgPiPr05 Pig Portugal (KU599140.1)
T. gondii isolate TgCoPa07 Coyte USA (KU599125.1)
T. gondii isolate TgCoPa05 Coyote USA (KU599122.1)
T. gondii isolate TgA105052 Chicken Gabon (KU599086.1)
T. gondii isolate TgA32109 Sheep France (KU599075.1)
Cluster 6

**Figure 4.8:** Phylogenic tree of *T. gondii* GRA6 gene sequences. The Neighbor-Joining approach was used to infer the evolutionary history (Saitou and Nei, 1987). The bootstrap consensus tree produced from 1000 repeats is supposed to represent the evolutionary history of the taxa under consideration (Felsenstein, 1985). Branch collapse occurs for partitions repeated in less than 50% of bootstrap repetitions. The percentage of duplicate trees in the bootstrap test (1000 iterations) where the associated taxa clustered together is shown next to the branches (Felsenstein, 1985). The evolutionary distances, which are measured in terms of the number of base substitutions per site, were calculated using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004). There were 76 nucleotide sequences in this analysis.

# CHAPTER 5

# DISCUSSION

In many farms across the world, reproductive diseases like toxoplasmosis have a significant impact on animal production in sheep and goats, and many instances go unnoticed within the herd, resulting in unforeseen and unexplained abortions, foetal and new-borns deaths (Dubey *et al.*, 2020). In other cases, these diseases cause recurrent illnesses in the herds, resulting in low reproductive output over time, which becomes a thorn in the herds' economic sustainability. Various diagnostic and screening methods can be used to evaluate sheep and goats' seroprevalence of toxoplasma-specific IgG antibodies. Furthermore, there is no universally recognized *T. gondii* reference material against which different diagnostic and screening tests can be compared (Ahmad *et al.*, 2015). In addition to determining the prevalence and risk assessment of toxoplasmosis in commercial and communal sheep and goats in the NW province and its occurrence in the FS province, the objective of this study was to also compare and evaluate the variation in the B1 and GRA gene sequences from isolates deposited in the GenBank as well as their phylogenetic relationships. This is, as far as we know, the first study of its kind on sheep and goats in the study areas.

## 5.1 Overall seroprevalence

The Seroprevalence of *T. gondii* in sheep and goats has been reported throughout the world, as well as in Africa, and in Gauteng, Free State, KwaZulu-Natal and in the Eastern and Western Cape provinces of South Africa (Samra *et al.*, 2007; Hammond-Aryee, Van Helden and Van Helden, 2015; Tagwireyi, Etter and Neves, 2019). In this study, the overall seroprevalence of *T. gondii* in sheep and goats of the North West province was found to be 13.9% using an ELISA test, with sheep (19.5%) having a higher seroprevalence than goats (10.5%). This could be attributed to the fact that sheep have more likelihood of contracting infection from the pasture and soil since they graze near the ground, whereas goats prefer browsing, reducing their chances of coming into contact with oocysts in the pasture and soil (Bentum *et al.*, 2019). The seroprevalence in sheep found in this study is higher than the 8% found in the Western Cape and the 4.3% (ELISA) and 5.6% (IFAT) (Samra *et al.*, 2007). Although both studies were conducted in South Africa, the difference in detected seroprevalence could be due to a variety of factors, including the type of farming systems (communal vs. commercial), the geo-climatic conditions as it is colder in the Western Cape compared to the North West which is warmer, and, most importantly, the serological tests used (ELISA vs. IFAT) due to different sensitivities

(Hove, Lind and Mukaratirwa, 2005; Ishaku *et al.*, 2018; Tagwireyi, Etter and Neves, 2019). A study conducted in the Eastern Cape found a seroprevalence of 64.46% and 53.9% for sheep and goats respectively, which is higher than the one found in this study (Tagwireyi, Etter and Neves, 2019). The higher seroprevalence in the Eastern Cape might be attributed to the fact that is much more humid in the coastal and the humidity promotes the viability of *T. gondii* oocysts compared to the dry semi-desert climate in the North West province (Fayer, 1981; Hammond-Aryee, Van Helden and Van Helden, 2015; Ibrahim, 2017).

An overall seroprevalence of 67.2% using IFAT was found in sheep and goats in Zimbabwe ,which is almost 5 times higher than the one found in this study (Hove, Lind and Mukaratirwa, 2005). The vast difference in the seroprevalence between the two studies might be influenced by factors such as location (Zimbabwe vs. North West province), farm management system (communal vs. commercial and communal), a period when the samples were collected (the year 1999-2000 vs. the year 2019-2021), and serological test that is used (IFAT vs. ELISA). These factors have an influence on the exposure of the animals to *T. gondii* infections (Kamani, Mani and Egwu, 2010; Andrade *et al.*, 2013; Hoda *et al.*, 2015; Areshkumar, Divya and Yasotha, 2018; Bentum *et al.*, 2019). The seroprevalence of goats in neighbouring Botswana was 10% using IHAT, which matches the seroprevalence of goats identified in this study (Sharma *et al.*, 2003). This could be because they are both dry semi-desert regions, which is not conducive to long-term oocyte survival. In studies conducted in other African countries, the seroprevalence of sheep and goats was found to be, 17.68% using DAT, 30.5% using ELISA, 5.7% using ELISA, 18.8% using ELISA, 37.4% using MAT in Ethiopia, Ghana, Nigeria (Borno), Algeria, and Tunisia, respectively (Van Der Puije *et al.*, 2000; Kamani, Mani and Egwu, 2010; Sharif *et al.*, 2017; Abdallah *et al.*, 2019; Al Hamada *et al.*, 2019; Lachkhem *et al.*, 2021a). Nigeria's seroprevalence is lower than the study's average and that of the rest of Africa. This could be explained by the fact that the study was only conducted in Borno state, which is just one of the 36 states in Nigeria. The variations in seroprevalence between African countries could be attributed to climatic differences, with arid areas having a lower rate of seroprevalence (Samra *et al.*, 2007; Julie *et al.*, 2019).

In Asia, a systematic review and meta-analysis conducted in China showed a sheep seroprevalence of 8.5%, where different serological techniques were used and are lower than the results of this study (Wang *et al.*, 2021). Iran had a seroprevalence of 9.7% for sheep, using MAT which is consistent with the findings of this study, although the investigations utilised different tests (Raeghi, Akaberi and Sedeghi, 2011). Thailand observed a seroprevalence of

27.9% using LAT in goats, which is 3 times greater than what was found in this study (Jittapalapong *et al.*, 2005). Thailand is a tropical country, and the humidity and moisture are known to help oocysts survive longer, increasing the likelihood of infection (Innes, 2010; Ibrahim, 2017).

In Europe, the seroprevalence of sheep and goats in Romania was reported to be 50.64% and 75% using ELISA, respectively (Hotea *et al.*, 2021). Because Romania is humid compared to the dry North West, climatic differences are known to play a role in variations between the two regions with increased seroprevalence in humid regions (Hotea *et al.*, 2021). The seroprevalence found in the sheep and goats of Greece was using ELISA was 48.6% and 30.7%, respectively, which is higher than what was found in this study (Tzanidakis *et al.*, 2012). Belgium reported a seroprevalence of 87.4% using ELISA, ten times more than what was found in this study (Verhelst *et al.*, 2014). A seroprevalence of 56.6% for sheep using ELISA was found in Scotland (Katzer *et al.*, 2011). This is two times the seroprevalence found in this study and the same range as the seroprevalence found in the Eastern Cape province, South Africa which used ELISA (Tagwireyi, Etter and Neves, 2019).

Seroprevalence in sheep in American countries like Argentina, Brazil, Colombia and Costa Rica was 17.3% using IFAT, 30.2% using IFAT, 23.5% using ELISA, and 41.1% using ELISA, respectively (Guimarães *et al.*, 2013b; Hecker *et al.*, 2013; Villagra-Blanco *et al.*, 2019a; Martínez-Rodriguez, Tafur-Gómez and Guzman-Barragan, 2020a). The seroprevalence found in Colombia and Argentina agrees with the findings of this study, with Costa Rica having the greatest seroprevalence. The discrepancy in seroprevalence between the results obtained in this study and those obtained in the American countries could be due to the different climatic conditions and serological assays used (IFAT vs ELISA) (Villagra-Blanco *et al.*, 2019b). Further studies using ELISA have found seroprevalence on the Caribbean islands to be 67%; 58%, in Dominica, 48%; 57% in Grenada, 89%; 80% in Montserrat, 57%; 42% in St. Kitts and Nevis in sheep and goats respectively (Hamilton *et al.*, 2014). In Norwegian dairy goats, a seroprevalence of 17% using DAT was discovered, which was higher than the results reported in this study for goats (Stormoen, Tharaldsen and Hopp, 2012). The results of the varying seroprevalence could have been influenced by the different assays (ELISA vs DAT) used in the studies (Martínez-Rodriguez, Tafur-Gómez and Guzman-Barragan, 2020b). The Caribbean islands had a higher seroprevalence for both sheep and goats compared to the one found in this study and agree with the one reported by Tagwireyi, Etter and Neves, 2019 in the Eastern Cape province. In comparison to the dry North West region, the islands and the Eastern Cape

province provide a warm and humid environment suitable for long-term survival of oocysts (Dubey *et al.*, 1990). Our findings provide the first evidence of *T. gondii* infections in communal and commercial sheep and goats of the North West province.

### 5.2 Risk factors

The association of toxoplasmosis with the biological characteristics of animals has shown that species and breed can influence the exposure of sheep and goats to the disease infection (Arwa Lachkhem and Wahiba Sakly, 2015). In the Univariate analysis, seropositivity was found to be higher in sheep than in goats and it was also higher in the mixed breeds of both sheep and goats. The difference in seroprevalence between the two species is attributed to the fact that sheep are grazers who eat short grasses and clovers near the soil, whereas goats are natural browsers who consume leaves and twigs from taller bushes and shrubs, making them more likely to encounter oocysts (Hamilton *et al.*, 2014; Stelzer *et al.*, 2019). Mixed-bred animals are more likely to be infected than pure breeds due to inbreeding, thus altering the genetic make-up of the animals resulting in the animals being easily susceptible to infections as a result of weaker altered genetic make-up from inbreeding (Webster, 2010; Chaklu *et al.*, 2020). The history of abortion was found to play a role in animal exposure to *T. gondii* with those who have a history of abortion having higher seropositivity (24.7%) than those who had not (11.9%). Primary infection with *T. gondii* during the first or second trimester of pregnancy is linked to abortions in sheep, whereas primary infection during the later stages of pregnancy results in the birth of lambs with congenital infection (Rodger *et al.*, 2006; Katzer *et al.*, 2011). Although farmers did not give information about the stage of pregnancy in which these abortions occur, this confirms that animals were exposed to *T. gondii* which might have played a role in these abortions. These findings provide further support for previous studies that reported *T. gondii* as a predisposing factor for abortions in sheep and goats (Sharma *et al.*, 2003; Buxton *et al.*, 2006; Rodger *et al.*, 2006; Franco *et al.*, 2011). Animals from farms where aborted material was buried and fed to pets had a higher seropositivity rate (41.9%) than those from farms where it was burned (0%) and sent to the state veterinarian (0%), implying that disposal of aborted material played a role in the exposure. The pets can acquire *T. gondii* infection if they dig up and eat the buried material, or if they are fed the aborted material, which then contaminates the animals' water, pastures, and soil, exposing the rest of the farm's animals to the oocysts that may be present. Those who are burned ensure that the cysts are burned alongside the aborted

material, while those who are sent to the state veterinarian limit exposure by not contaminating the farm environment. A similar study conducted in Botswana found the disposal of aborted material to be significant in the exposure of sheep and goats to toxoplasmosis (Sharma *et al.*, 2003).

Since small ruminants are herbivorous, their principal source of *T. gondii* contamination is felids and oocysts shed in the environment. Therefore, the presence of other wild felids that may be shedding oocysts into the environment may pose a risk of transmission to small ruminants (Dubey *et al.*, 2021). This renders the environmental or rearing factors associated with the exposure of the animals to the disease important. The main identified risk factors were the district municipality, the local municipality, the presence of cats on the farms, and the disposal of manure. Analysis of epidemiological data shows that on most farms in the study areas, cat activities are not monitored and are free to roam around the farms and have access to pastures, pens, and stables. As a result, oocyst shedding is widespread, increasing the risk of *T. gondii* infection. Since cats tend to bury their faeces, having access to feed storage facilities raises the potential for contamination; these sites are excellent for such unpleasant feline activities. This finding is comparable to that of other studies that found an increase in seropositivity with the presence of cats on farms (Dubey, 2009b; Tagwireyi, Etter and Neves, 2019; Adesiyun *et al.*, 2020).

Dr Ruth Segomotsi district municipality had the highest seropositivity among the district municipalitiess (21.6%), while Dr Kenneth Kaunda district municipality had the lowest (3.3%). Within local municipalities, Naledi had the highest seropositivity (52%) while JB Marks, Kgetlengrivier and Madibeng local municipalities had no seropositive animals (0%). Dr Ruth Segomotsi district municipality is the largest district municipality in the North West province with the poorest rural areas, and Naledi local municipality falls within this district municipality, meaning they have more communal farms than commercial, and the higher seropositivity could be attributed to the fact that most of these farmers tend to not know about reproductive diseases like toxoplasmosis which might lead to poor hygiene practices in their farms to which cats have easy access (Martínez-Rodriguez, Tafur-Gómez and Guzman-Barragan, 2020b). In comparison to Dr Ruth Segomotsi district municipality, Dr Kenneth Kaunda district municipality only has commercial farms with farmers who tend to be more knowledgeable about reproductive diseases like toxoplasmosis and who have good hygiene practices on the farms as they are farming for profit (Stelzer *et al.*, 2019). These good hygiene practices therefore, prevent the attraction of cats to their farms as they have rodent controls, which are

the main attraction of cats, reducing the shedding of oocysts by cats, and thus reducing the exposure of animals (Hamilton *et al.*, 2014; Ibrahim, 2017). These findings are in agreement with the ones reported in other studies where there was higher seropositivity in sheep and goats on communal than commercial farms (Hove, Lind and Mukaratirwa, 2005; Tagwireyi, Etter and Neves, 2019). The 0% seropositivity in JB Marks, Kgetlengrivier, and Madibeng local municipalities could be due to a lower number of sampled animals, resulting in less precision as the number of animals present in each municipality is misrepresented (Kasiulevičius, Šapoka and Filipavičiūtė, 2006). There was a high seroprevalence from farms that use manure as a fertilizer, a similar finding to that of Tagwireyi et el.,2019 (Tagwireyi, Etter and Neves, 2019). The use of manure as fertilizer could act as a vector for spreading oocyst, especially if there are cats in that farms that shed their faeces on the manure.

The age of the animals usually influences the exposure of animals to infections with older animals being more at risk due to their declining immunity than the young one who has a much stronger immunity (Schares *et al.*, 2017; Stelzer *et al.*, 2019). However, in this study, the age had no significant association with the exposure of the animals to *T. gondii* infection and this is in accordance with other studies in the East Hararghe zone of Oromia region, Ethiopia and Nigeria where they also had more positive young animals (<1 year) than older animals (>1 year) (Bártová *et al.*, 2017; Tilahun *et al.*, 2018). Gender of the animals was statistically significant in the exposure of the animals to the parasite with females having higher seroprevalence than males, a finding similar to the one found in the Eastern Cape (Tagwireyi, Etter and Neves, 2019). The increased seroprevalence in females may result from physiological changes, hormonal fluctuations, immunosuppression associated with pregnancy, and lactation stress (Khalife *et al.*, 2022). Animals that were allowed to only breed naturally had a seroprevalence than the ones they allowed them to breed naturally and artificially inseminate them. This finding is agreement with the findings of study by Lopez et al., 2013 which examined the viability of the sexual transmission of *T. gondii* in reproductive female sheep (Lopes *et al.*, 2013). Artificial insemination procedures are conducted aseptically to prevent contaminations and infection, while animals can mate without the same measures. factors in the rearing of the animal's environment including the type of farm, water supply, feed storage, and waste disposal were all found to have no statistically significant association with *T. gondii* seropositivity in this study. These findings corroborate the findings of the studies conducted in Brazil (Piaui), Central Ethiopia, China, Dutch, Northern Portugal and Northern Italy (Lopes

*et al.*, 2013; Gebremedhin *et al.*, 2013; Gazzonis *et al.*, 2015; Liu, Li and Pan, 2015; Deng *et al.*, 2016; Rêgo *et al.*, 2016)

### 5.3 Molecular detection

Infections with the *T. gondii* can occur in three forms as mentioned in the literature review: horizontally via oocyst, horizontally via tissue cysts, and vertically through tachyzoites (Ibrahim, 2017; Dubey *et al.*, 2020). As a result, the type of samples that are collected in accordance with the different routes and stages of infection will influence its detection with molecular methods (Liu *et al.*, 2015; Fernández-Escobar *et al.*, 2022). Vaginal swabs, sheath scrapes, milk and diagnostic tissue samples were tested to detect *T. gondii* using nested and conventional PCR.

Despite serological evidence of the animals' exposure to *T. gondii* infection from the ELISA results, the parasite was not detected from all the above-mentioned analysed samples. This non-detection is similar to the absence of detection that Clune et al. reported (Clune *et al.*, 2022). Studies by Lopes et al. and Santana et al. have shown that the parasite can be sexually transmitted by the tachyzoites in the semen of infected male sheep and goats to the females (Santana *et al.*, 2013; Lopes *et al.*, 2013). However, in these studies, detection and confirmation were done from the muscular tissues and organs of *T. gondii* seropositive animals that were sacrificed after the study instead of sheath scrapings or vaginal swabs like we did in this study. This may suggest that the tachyzoites were no longer being shed in the reproductive organs of the animals at the time of sampling or the infection occurred via a different route of infection. It is also important to note that tachyzoites are susceptible to harsh environmental conditions such as extreme heat and dryness and die off quickly outside the host (Tenter, 2000; Dubey *et al.*, 2021).

The absence of *T. gondii* from the milk samples differs from the findings by Gazzonis et al. in which they detected the parasitic DNA in 20.6% (13/63) of milk samples (Gazzonis *et al.*, 2019). Milk become contaminated with oocytes that are shed from the cats and the tachyzoite form of the parasite (Tenter, 2000; Dubey *et al.*, 2020). An experiment by Neto et al., 2018 in which they inoculated milk samples from naturally infected goats in Brazil detected *T. gondii* DNA from the brains of inoculated mice (Ferreira Neto *et al.*, 2018). These results would

therefore suggest that the milk was not contaminated with oocysts or tachyzoite form of *T. gondii* and that the animals had no current infections.

## 5.4 Correlation and agreement between serological (ELISA) and molecular detection (PCR)

The reliability of the results obtained during research studies depends on the consistency and agreement among the data collection and processing methods used (McHugh, 2012). Therefore, processes that gauge agreement among the various collected data need to be included in well-designed research studies. One aspect of overall confidence in the accuracy of a research study is the reliability of collected and processed data. Any research work has a variety of potential sources of errors, and the accuracy of the study's results and conclusions depends on how well the researcher manages these sources of error (McHugh, 2012; Petrie and Watson, 2013).

In this study, an agreement of 50% between the serological and molecular data was calculated, indicating that only 50% of the data is erroneous. This agreement is consistent with what was found in another similar study (Bachand *et al.*, 2019). Cohen's kappa was further calculated to determine the correlation between the data set, and kappa value was 0.33 indicating a fair correlation between the two tests. There are not many comparison studies that compare different techniques for diagnosing *T. gondii* infection. However, a comparison study by Schares et al., 2017 using similar methods resulted in moderate agreement ($k$= 0.60) (Schares *et al.*, 2017) . The sample sizes could have influenced the difference correlation as they had more samples which increased their change of molecular detection and thus better comparisons.

## 5.5 Sequence analysis of the *T. gondii* B1 and GRA6 gene isolates

In the past decades, numerous distinct loci have been studied through the sequencing of housekeeping genes, antigens, and neutral introns (Khan *et al.*, 2007; Chen *et al.*, 2012). This dawn of genomic research and technological advancements have enabled researchers to get in-depth knowledge of genetic structure, genome diversity, structures of different strains, polymorphisms, and their interactions (Beck *et al.*, 2009; Yucesan *et al.*, 2021). Similarly, estimating local rates of evolution based on numerous alignments allows for a quantitative evaluation of the strength of evolutionary constraints and the significance of functional features (Lau *et al.*, 2016).

One of the many ways of examining variation among sequences is the identification of single nucleotide polymorphism that is found in genomes at specific locations known as sequence-tagged sites (STS), and they can be utilized for gene mapping, identifying population structure, and conducting functional studies (Stuart Brown, 1998; Fazaeli and Ebrahimzadeh, 2007; Bawm *et al.*, 2020). Single nucleotide polymorphisms are regarded as the most helpful biomarkers for disease diagnosis because of their common frequency, ease of analysis, affordable genotyping, and ability to conduct relation studies using statistical and bioinformatics techniques (Biradar *et al.*, 2014; Cubas-Atienzar *et al.*, 2018; Vallejos-Vidal *et al.*, 2020). Single nucleotide polymorphisms were identified and used to establish a link between sequence variation and genetic traits during the analysis of the genome sequences in this study, which allowed us to identify a gene that could be used as a genetic marker between the B1 and GRA6 *T. gondii* housekeeping genes.

### 5.5.1 Sequence analysis of *T. gondii* B1 gene isolates

The B1 gene is one of the widely targeted genes when detecting *T. gondii* in clinical and environmental samples (Fernández-Escobar *et al.*, 2022). It is a multicopy gene and although multicopy genes are known to be more sensitive than single-copy genes, there are significant problems in targeting them (Costa and Bretagne, 2012). With multicopy genes, determining the number of repeats for each strain using multicopy genes and choosing primers and probes based on conserved sequences from among the numerous repeats of the three main *T. gondii* lineages are both difficult tasks (Saeij, Boyle and Boothroyd, 2005; Costa and Bretagne, 2012). Some studies have discovered that the B1 gene, although found in 35 copies of the gene, is less sensitive than other often targeted genes, leading to misdiagnosis of the parasite (Edvinsson *et al.*, 2006, 2007; Costa and Bretagne, 2012; Camilo *et al.*, 2017). Given that it is frequently used for detection, this suggests the need for its analysis to determine if it could be used as a phylogenetic genetic marker for studies.

Significant polymorphism was discovered in the B1 genomic sequences of the analysed isolates. With the B1 sequence analysis of the 803 bp fragments, seven of the 24 analysed isolates (Tecuanillo178, ElReal11, ElReal109a, Camalote102b, Camalote106, Estacion101b, and Estacion98) had SNPs. Although all these isolates where isolated from sheep in Mexico, they still showed slight variation through the identified SNPs as they were not identified at the same locations. This low variation was expected since the isolates are from the same country in neighboring states along the coast, which indicates they have might have similar

environmental adaptation and survival wich does not influence the altercation of their genomes as supported by other studies where gene isolates from neighboring areas did not show significant variation (Dubey, 2009b; Dubey *et al.*, 2020; Cong *et al.*, 2021). This was a limitation in the study since trimming and alignment of gene sequences from all the retrieved isolates only produced Mexican sheep isolates, which limited analysis of other sequences from other hosts and countries.

Of the total (*n*=83) analysed *T. gondii* B1 genes for the isolates with fragment sizes of 300 bp, we found 29% (24/83) of the isolates with SNPs. Isolate TgCatAu_7 from a cat in Australia showed a total variation from the rest of the isolates as it had absented sequences from position 50 until position 110. The relation was also observed through the absence of sequences on all the isolates at the same position (78), except for California muscle isolate SR222 from the USA. This data is in agreement with previous findings in which they found genetic variation from isolates originating from different hosts and geographic locations (Chen *et al.*, 2012; Wang *et al.*, 2013; Cubas-Atienzar *et al.*, 2018).

Isolate Estacion98 from sheep in Colima, Mexico, had an SNP at position 16, isolate Camolote102b from sheep in Michoacan, Mexico had it at position 222, isolate Estacion101b from sheep in Colima, Mexico, had it at position 126, isolate 16A from sheep in Iran, had them at positions 396, 325, 331 and 396, isolate from sheep 1A, 2A, 16A, and 26 from Camel in Iran, had them at position 325, isolate CR34 from California muscle in California, had them at positions 187 and 354, isolate SR217 from California muscle in California, had them at position 199 and 325, isolate 2A from sheep from Iran had them at position 287 and 237, isolate 25 from Camel in Iran had them at position 187. Isolates SR231 and SR222 from California muscle in the USA, TGK-KLK-365-IMNO from Ixodes Ricinus tick in Poland, and SR222 from California muscle in California all had SNPs at position 187, 45, and 199, respectively. Isolate TGK-KLR-IMNO from an Ixodes Ricinus tick had them at positions 39 and 452, isolate 781-L-IMNO from an Ixodes Ricinus tick in Poland had them at position 4, 35, 452, and 472, and isolate 782-L-IMNO from an Ixodes Ricinus tick in Poland had them at position 4, 35, 452, and 472. The single nucleotide polymorphisms were found at positions 118, 119, 199, and 243 in isolates of the Ixodes Ricinus tick from Poland (TGK-KLR-625-IMNO, TGK-KLR-631-IMNO, TGK-KLR-583-IMNO, and TGK-KLR-610-IMNO). The presence of SNPs at different locations among the sequences further supports studies that were able to show that variation among hosts exits irrespective of whether they are from the same geographic location or not (Maryam *et al.*, 2016; Arefkhah *et al.*, 2019; Fernández-Escobar *et al.*, 2022).

Black bears isolate 220 from the USA had it at position 325, while isolate 222 had them at positions 325, 396, and 459. Isolate TGK-KLR-983-IMNO from an Ixodes Ricinus tick in Poland had SNPs at positions 402, 325, and 234, isolate TGK-KLR-744-IMNO had them at positions 452, 456, and 471, and isolate TGK-KLR-836-IMNO had SNPs at positions 452, and 471. Clones from Iran: clone SY5 from sheep had SNP at position 402, clone CG21 from chicken had it at position 187, clone SY4 from sheep had it at positions 234, and 325, while clones SY12 from sheep and clone CQ7 from cattle both had it at position 325. Although some of these isolates had SNPs at different locations of the sequence, they are not far off to suggest that their sequences are different as supported by Galal *et al.*, (2019).

### 5.5.2 Sequence analysis of *T. gondii* GRA6 gene isolates

One of the well-known *T. gondii* markers is the parasitic molecules known as dense granule antigens (GRA), which are secreted into the parasitophorous vacuole and the dense granules of tachyzoites, both of which are connected to the network of the GRA (Lecordier *et al.*, 1995; Rome *et al.*, 2008; Beck *et al.*, 2009; Etheridge *et al.*, 2014; Maryam *et al.*, 2016). They are immunogenic and are in control of the parasites' ability to survive inside cells (Rome *et al.*, 2008). These antigens have a single copy gene and are polymorphic (Edvinsson *et al.*, 2007; Rome *et al.*, 2008; Beck *et al.*, 2009; Chen *et al.*, 2012; Maryam *et al.*, 2016; Arefkhah *et al.*, 2019). GRA6 is infrequently used as a marker for detection in studies, despite being referenced in the literature as a potential genetic marker and target GRA used for *T. gondii* detection like the rest of the dense granule antigens (Dubey *et al.*, 2011; Wang *et al.*, 2013; Fernández-Escobar *et al.*, 2022). This motivated us to study the GRA6 gene isolates and determine if they could indeed be used as a marker for the detection or if their limited usage is because of it being a poor marker.

Among the analysed GRA6 sequence isolates (n=76), 82.9% (63/76) of them had SNPs and some had absent sequences. 80,3% (61/76) of the isolates had absent sequences at the same positions (272 to position 274), 18% (14/76) did not have and 1% had them at a different position (241 to 303). This shows that isolates can still be different even when they were isolated from the same species and origin. Some studies suggest that these absence of sequences might be a result of gene mutations (Chaichan *et al.*, 2017; Vallejos-Vidal *et al.*, 2020). All Turkish cat isolates showed SNPs at the same locations which can be influenced by that they are all isolated from the host in the same location as was noted in other studies (Hassan *et al.*, 2019). The grey wolf isolates from the USA showed SNPs at the same locations, apart from

isolate TgWolfMN20, which had additional SNPs at different locations in addition to the same SNPs shared with the other isolates. According to a review by Chaichan et el., 2017, it is also common for isolates of the same origin and hosts to differ genetically (Chaichan *et al.*, 2017).

Chicken and pig isolates from Portugal had SNPs at the same positions, except for isolates TgPiPr05 (pig) and TgCkPr03 (chicken) which only had them at different positions. All the Gabon isolates shared SNPs at the same. Additionally, position 150 was shared by isolates TgA18005, TgA05002, TgA105043, and TgA105001 with isolate, TgA32129 (sheep) having them at different locations from the rest of the Gabon isolates. The SNPs for the USA coyotes isolates were located at positions. The French Guiana isolates had SNPs at different positions. The route of *T. gondii* infection is said to have an impact on the adaptation and genetic structure of the parasite on its host cells and the environment of the hosts, hence similarities that could be an indication of the same route of infections for the hots were seen (Guy, 2014; Saraf *et al.*, 2017; Innes *et al.*, 2019).

The Iranian isolates from all the different species had SNPs at completely different positions. Although these isolates contained SNPs in different locations, their phylogenetic tree still demonstrated that they had a close phylogenetic relationship, with just a slight difference in bootstrap difference values. This might indicates that the SNPs in some genes do greatly influence the sequence of the genome to a point of phylogenic variation (Vallejos-Vidal *et al.*, 2020).

Studies on sequence analysis in other targeted *T. gondii* genes during genotyping (B1, SAG1, SAG2, GRA3, GRA5, GRA7, and GRA14) have shown substantially lower levels of polymorphism than GRA6, which is more polymorphic in comparison to the others (Rome *et al.*, 2008; Chen *et al.*, 2012; Biradar *et al.*, 2014; Wang *et al.*, 2015; Maryam *et al.*, 2016; Bahadori *et al.*, 2018; Arefkhah *et al.*, 2019; Firouzeh and Foroughiborj, 2021). Climate is crucial for the preservation of *T. gondii* oocysts and tachyzoites with regions where the infections occur tend to have higher temperatures, less precipitation, and lower altitudes than those where it does not (Kantzoura *et al.*, 2013; Rouatbi *et al.*, 2020). These variations between the two gene sequences (B1 and GRA6) could also be a result of immune selection since GRA6 is highly immunogenic compared to the B1, therefore is probably extreme for targets of selection pressure by enabling their quick presentation as antigens inside the host cell (Saeij *et al.*, 2014). These findings demonstrate that GRA6 can be used as phylogenetic marker.

## 5.6 Phylogenetic analysis of the *T. gondii* B1 and GRA6 gene sequences

To describe and visually illustrate complicated interactions in population biology, a phylogenetic network is preferred to the conventional separating phylogenetic tree (Morrison, 2005). The maximization of comparability or the minimum evolution principle is frequently applied in the development of phylogenetic trees (Saitou and Nei, 1987; Rouatbi *et al.*, 2020). The standard algorithm of tree-making methods based on this theory is to look at all potential branching patterns or a predetermined number of topologies branching patterns that are likely to be close to the true tree, and then select the one that exhibits the least amount of overall evolutionary change as the final tree (Saitou and Nei, 1987). Through this method, we are then able to determine the genetic relationship between these isolates.

### 5.6.1. Phylogenetic analysis of the *T. gondii* B1 gene sequences

The highest bootstrap percentages (≥50%) validated the isolates clustering on the phylogenetic tree. The B1 phylogenetic tree from the 803 bp fragments resulted in the formation of two clusters from two Mexican states, Colima and Michoacan. Although the two clusters formed, the bootstrap values were the same for both, implying a low genetic variability among the isolates. This finding is similar to another study that found low genetic variation between isolates originating from the same hosts of the same geographic location (Wang 2015). Interestingly, cluster 1 is formed by isolates from one of each state. This relationship between the sequences of isolates Camalote102b and Estacion101b may be explained by the possibility that animals were transported between the neighbouring states. The observation that the two isolates shared SNPs at the same location (position 132) further supports their phylogenetic relation as it was expected.

With the phylogenetic tree construction of B1 gene isolates with fragment sizes of between 300 to 1000 bp that were trimmed and aligned, seven clusters were generated with some generating subclusters. Through the clustering of various isolates and/or clones from 5 distinct animal species (sheep, camel, California mussel, black bear, and cattle), we were able to see their phylogenetic relationship despite them being isolated from different species and countries. This demonstrates a close relationship between some of the Irian and the USA isolates which is interesting given the different climatic conditions in both countries. As surprising as this observation is, it is not an unusual occurrence as other studies were able to demonstrate a such relationship between different hosts of different origins (Tenter, 2000; Can *et al.*, 2014;

Fernández-Escobar *et al.*, 2022). The Iranian isolates and/or clones from dominated cluster 1 by making up 73% of the cluster (8/11).

Even more association between the isolates from various species and geographical regions could be seen in Cluster 2. The species diversity included both the most common *T. gondii* hosts (sheep, goat, cattle, cat, duck, and chicken) and less common hosts (mussel and Ixodes Ricinus ticks) (Dubey, 2009b). This is however not a new occurrence as more studies were able to show an association between isolates and/clones from different species originating from different locations (Galal *et al.*, 2018; Fernández-Escobar *et al.*, 2022). The association could be largely influenced by the ability of the *T. gondii* parasite to adapt to different hosts and environments (Stuen, Granquist and Silaghi, 2013). The Mexican isolates clustered again on cluster 4. In cluster 5 we saw the clustering of 4 Ixodes ricinus ticks which further subclustered into two groups. This suggests that, in contrast to other isolates from the same hosts that clustered separately, they have a strong ancestral association (Xia *et al.*, 2021). A study conducted in Asia also found similar results where isolates from the same type of host obtained from the showed a strong ancestral relation (Chaichan *et al.*, 2017). Once more, demonstrating that these isolates are not host specific, the tick isolates further clustered into cluster 6 with the American muscle strain.

The last cluster of the tree, cluster 7, seen cat isolates cluster together although one was isolated from Australia and the other in South Korea. It is also interesting to note that the Australian isolate (TgCatAu_7) had absent sequences from multiple locations in its sequence while the South Korean isolate (C-F_Tg-56) only had an absent sequence in one position. The discrepancy may be a result of the different living environments and geographic locations of the cats (Zheng *et al.*, 2016; Kakakhel *et al.*, 2021). The bootstrap values are the only difference between the cat isolate and the mussel isolate they clustered with.

### 5.6.2. Phylogenetic analysis of the GRA6 gene sequences

The highest bootstrap percentages (≥50%) validated the isolates clustering on the phylogenetic tree. Six distinct clusters were identified by phylogenetic analysis of the 76 *T. gondii* GRA6 isolates with fragment sizes of between 300 to 100 bp that were trimmed and aligned from the retrieved sequences. Cluster one and five comprised isolates from different hosts and geographical locations that showed similar genetic variation with only a difference in bootstrap values. Cluster 5 however clustered with more isolates from the USA (15/16) and it is also interesting to note that all the gray wolfs clustered in cluster five and they all had SNPs at the

same locations during the sequence analysis, proving a strong phylogenetic relationship. Grey wolf isolate and cayote originate from the same family, hence this relationship makes sense. The cat isolation TgBobcatMS1 from Mississippi, USA formed a subcluster with a grey wolf isolate from the same country, and while having SNPs in the same place as the cat isolate, the jaguar isolate did not subcluster with them. Studies have been able to demonstrate that despite having close genetic relationships among themselves, *T. gondii* isolates typically have limited genetic variation, even when originating from the same host (Khan *et al.*, 2007)

Cluster 2 formed from a white-tailed deer isolate from the USA and a grison isolate from Gabon. This relation was unexpected given that they are from two different continents and there were no similarities between them during the sequence analysis. However, studies have been able to show that this does happen amongst isolates as a result of mutations in their genes (Khan *et al.*, 2007). The third cluster of the tree was made up of 3 Gabon chicken isolates with two of them (TgA105043 and TgA105001) forming a subcluster. The have been other studies that observed this type of clustering from the isolates of the same host and geographic location, owing to common ancestral linage (Bridgett *et al.*, 2011).

# CHAPTER 6
# CONCLUSIONS AND RECOMMENDATIONS

## 6.1 Conclusions

Set study objectives were achieved as the seroprevalence ad risk factors that pose a risk for exposure to the animals to *T. gondii* were identified. Molecular detection and occurrence in the FS province were not achieved owing to the reasons discussed under discussion. We were able to demonstrate that the GRA6 gene is a good marker for *T. gondii* genotyping than the B1 gene, and their phylogenetic relationship, which allowed us to identify genetic diversity among the *T. gondii* B1 and GRA6 gene sequences isolates. As far as we are aware, this is the first study to identify *T. gondii* seroprevalence and risk factors that contribute to the animals' exposure to the parasite, and we were able to show that the disease exists on both communal and commercial farms in the NW province and determine occurrence in the FS province.

### 6.1.1 Seroprevalence

The seroprevalence of both the sheep and goats was determined from serum using ELISA with sheep having the highest seroprevalence compared to goats, proving that sheep are more susceptible to exposure to the parasite compared to goats. Additionally, the seroprevalence varied between districts and municipalities, with the highest seroprevalence seen in areas closest to Botswana's and the Northern Cape province's borders. During the study, it was also discovered that several of the villages in the Dr Ruth Segomotsi Mompati District (the district with the highest prevalence), which are at the border of the Northern Cape and the North West Province, import some of their sheep and goats from the Northern Cape.

### 6.1.2 Risk factors

Risk factors for the exposure of sheep and goats to *T. gondii* included breed, species, animal origin, district, municipality, history of abortion, handling of aborted material, presence of cats on the farms, feeding system, and feed storage. Age, gender, type of farm, water supply, feed storage, and waste disposal were all found to have no significance in the bearing of seropositivity.

### 6.1.3 Molecular detection

There was no detection of *T. gondii* on PCR from the samples that were tested. This indicates that the animals were only exposed to *T. gondii* and there was no current infection from them.

In addition, the samples that were analysed were likely not good type of samples to detect the *T. gondii* pathogen and were also not enough to afford greater chance of detection of the genetic material of the parasite.

### 6.1.4 B1 and GRA6 genes sequence analysis and phytogenic tree construction

There was no vast variation between the B1 genes for the 803pb fragments when compared to the B1 gene sequences and phylogenetic analysis of the fragment sizes of between 400 to 1000 pb although they are isolates from the same gene. This suggests that the fragment size might also have an impact on the variation amongst isolates of the same genes and using the same fragment sizes could not be ideal in studying gene variation amongst the isolates or intraspecific phylogenetic analysis.

When comparing the GRA6 gene to the B1 gene's sequence and phylogenetic results, GRA6 gene results presented more sequence variation and phylogenetic relationship among *T. gondii* isolates from various hosts and geographical locations, allowing for the genotype differentiation of the isolates under study. These results suggest that the GRA6 gene can indeed be used in population genetic studies of *T. gondii* isolates as a potential genetic marker and should be considered for use more frequent than it is currently used. A limitation of the study was the lack of isolates from the study area and South Africa, our 0% molecular prevalence, and non-detection from the Free State tissue samples. As a result, only the isolates from the rest of the worldwide isolates deposited in the GenBank.

## 6. 2 Recommendations

Farmers should take into consideration the risk factors that are associated with the seropositivity found in this study. This will allow them to have preventative measures that will limit the exposure of the animals within the farms as this study was able to show that the animals are indeed exposed to *T. gondii* infections.

According to the Animal Diseases Act of 1984 (ACT 35 1984) and the Animal Diseases Regulations (R.2026 of 1986) No. 10469 of September 26, 1986, toxoplasmosis is not regarded as a regulated and notifiable disease and it is not currently monitored regularly by farmers and state veterinary services, and the data obtained in this study demonstrates the need for routine monitoring of the disease to prevent misdiagnosis of abortion and stillbirth cases. In addition, toxoplasmosis should also be considered when investigating abortion or stillbirth cases.

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# APPENDICES

## APPENDIX A: RISK ASSESSMENT QUESTIONNAIRE

**Risk assessment questionnaire for communal and commercial farm in the North West and Free State Provinces**

Date:_____

### Section A: General Information

**1.1. Farm details:**

Province_____ District_____ Municipality_____

Farm/Village:_____ GPS coordinates_____

**1.2. Interviewee**

| | | | | | |
|---|---|---|---|---|---|
| Owner | Worker | Herd man | Family | Neighbour | Other: |

**1.3. Gender**

| | |
|---|---|
| Male | Female |

**1.4. Age group**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| <5 | 5-18 | 18-30 | 31-39 | 40-49 | 50-59 | 60-69 | 70-79 | >80 |

**1.5. Literacy status**

| | | | |
|---|---|---|---|
| Never went to school | Primary School | Secondary School | Tertiary School |

### Section B: Animal Details

**2.1. Species**

| | |
|---|---|
| Sheep | Goats |

**2.2. Breed**

| Breed | Number |
|---|---|
| | |
| | |
| | |
| | |
| | |

**2.3 Sex and number of animals**

1

**2.14. What do you do with the manure from the stables/kraals?**

---

## Section C: Knowledge of Animal Reproductive Diseases Responsible for Abortions

**3.1. Do you know that there are animal diseases that can lead to abortions in animals?**

| Yes | No |
|---|---|

**3.2. Do you have any animals with the history of aborting?**

| Yes | No |
|---|---|

**If yes, continue with question 3.3-3.5.**

**3.3. At what stage of the pregnancy does abortion occur?**

| Early | Mid | Late |
|---|---|---|

**3.4. What do you do when it occurs?**

---

**3.5. What do you do to the aborted foetus?**

| Burry | Burn | Leave it the stable/kraal | Feed pets | Dispose in the bin | Submit to state vet office |
|---|---|---|---|---|---|

**3.6. Where do you keep aborted animals?**

| Isolated from the herd | With the herd |
|---|---|

# APPENDIX B: CONCERNT FORM

## CONSENT TO PARTICIPATE IN THIS STUDY

I, _____ (participant name), confirm that the person asking my consent to take part in this research has told me about the nature, procedure, potential benefits and anticipated inconvenience of participation.

I have read (or had explained to me) and understood the study as explained in the information sheet.

I have had sufficient opportunity to ask questions and am prepared to participate in the study.

I understand that my participation is voluntary and that I am free to withdraw at any time without penalty (if applicable).

I am aware that the findings of this study will be processed into a research report, journal publications and/or conference proceedings, but that my participation will be kept confidential unless otherwise specified.

I agree to the recording of the <insert specific data collection method>.

I have received a signed copy of the informed consent agreement.

Participant Name & Surname..... (please print)

Participant Signature.....Date.....

Researcher's Name & Surname.....(please print)

Researcher's signature.....Date.....

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# APPENDIX C: UNISA CAES HEALTH AND ANIMAL ETHICS CLEARANCE

UNISA university of south africa

**UNISA-CAES HEALTH RESEARCH ETHICS COMMITTEE**
**UNISA-CAES ANIMAL RESEARCH ETHICS COMMITTEE**

Date: 09/11/2020

NHREC Registration # : REC-170616-051
REC Reference # : 2020/CAES_AREC/146
Name : Mr ME Masombuka
Student #: 67134238

Dear Mr Masombuka

**Decision: Ethics Approval from 05/11/2020 to 31/10/2021**

---

**Researcher(s):** Mr ME Masombuka
mthokozo@icloud.com

**Supervisor (s):** Dr G Mokolopi
kgobeg@unisa.ac.za; 011-471-3909

Dr N Gcebe
gceben@arc.agric.za; 012-529-9138

**Working title of research:**

Prevalence and risk assessment of toxoplasmosis in commercial and communal sheep and goats in the Free State and North West provinces

**Qualification:** MSc Agriculture

---

Thank you for the application for research ethics clearance by the Unisa-CAES Health and Animal Research Ethics Committees for the above mentioned research. Ethics approval is granted for one year, renewable until the completion of the project, **subject to further clarification, and submission of yearly progress reports. Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

**Due date for progress report: 31 October 2021**

*Please note the points below for further action:*

*Feedback from the Animal Research Ethics Committee:*

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1. How many farms/households will be sampled, and how many animals per farm/household?
2. Who will collect all the different samples – will the veterinarian (Dr Mphuthi) do it, or will the researcher collect some of the samples?
3. Will Dr Mphuthi collect the samples in both the Free State and North West, as he/she is situated in North West? Or will someone else collect the Free State samples?
4. The SAVC number of the veterinarian must be provided, as well as a commitment letter confirming her availability for the duration of the study.
5. There must be permission from the state veterinarian and/or the community leaders in each research area. The researcher is cautioned that sample collection may not commence until the permission has been obtained and submitted to the committee.
6. The ethics application form is incomplete and needs to be completed in detail.
7. How will the does and ewes be restrained during the procedures? Who will do it? What is their recent experience with such procedures?
8. Where will the sampling take place – will it be at communal (e.g. diptank) areas, or at farmsteads?
9. The researcher must specify the method of euthanasia that will be applied in the unlikely event that an animal is seriously injured during the research, e.g. when being restrained, for instance. The procedure must be on record, no matter how unlikely serious injury to the animals may be. Furthermore, who will perform the procedure?
10. The sample size formula needs to be corrected ($Z^2 \times P^{1-P}$)

*Feedback from the Health Research Ethics Committee:*

1. The supervisor has not signed the health ethics application form.
2. Is two minutes realistic for the completion of the interview? Will it not take longer than that?
3. With regard to the multivariate analysis, the committee recommends that the variables should be selected based on the objectives and the purpose for fitting the specific statistical model, rather than stepwise. The stepwise approach is numerically based and sometimes rejects a key variable needed for the success of the research.

*The **low risk application** was **reviewed** by the UNISA-CAES Health and Animal Research Ethics Committees on 05 and 06 November 2020 respectively in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:



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1. The researcher will ensure that the research project adheres to the relevant guidelines set out in the Unisa Covid-19 position statement on research ethics attached.
2. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
3. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
4. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
5. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
6. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
7. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
8. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

*Note:*
*The reference number **2020/CAES_HREC/146** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committees.*

Yours sincerely,

W.M. Strauss.

**Prof MA Antwi** **Dr WM Strauss**



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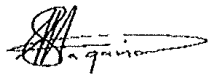

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## APPENDIX D: DAFF APPROVAL LETTER

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
**REPUBLIC OF SOUTH AFRICA**

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

**Enquiries:** Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
**Reference:** 12/11/1/1

Dr Nomakorinte Gcebe
Onderstepoort Veterinary Institute
100 Old Soutpan Road
Onderstepoort
0110
Email: GcebeN@arc.agric.za

Dear Dr Gcebe,

**RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)**

Your application sent per email on 17 May 2019, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

**Conditions:**

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 17/05/2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;

4. Samples may only be collected from animals where the state veterinary official has confirmed that the area is not under any restriction due to disease which the species is susceptible to;
5. Samples from abattoirs may only be removed subject to obtaining permission from the owner and the provincial veterinary official providing oversight for that specific abattoir;
6. All samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and the National Road Traffic Act, 1996 (Act No. 93 of 1996);
7. Isolates of *Coxiella burnetii* and *Toxoplasma gondi* as well as extracted DNA and purified protein derivatives from this study may be stored at the OVI Bacteriology laboratories and any further use or distribution is subject to obtaining a separate Section 20 approval;
8. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

**Title of research/study:** Prevalence of Q-fever and Toxoplasmosis in slaughtered and farmed animals in Free State, North West and Limpopo Provinces of South Africa and development of cell mediated immunity biomarkers.

**Researcher:** Dr Nomakorinte Gcebe

**Institution:** Onderstepoort Veterinary Institute

**Our ref Number**: 12/11/1/1

**Your ref**: n/a

**Expiry date:** 2022-04

Kind regards,

**DR. MPHO MAJA**
**DIRECTOR OF ANIMAL HEALTH**
**Date:** 2019 -07- 30



SUBJECT: S20 PERMISSION FOR: PREVALENCE OF Q-FEVER AND TOXOPLASMOSIS IN SLAUGHTERED AND FARMED ANIMALS IN FREE STATE, NORTH WEST AND LIMPOPO PROVINCES OF SOUTH AFRICA AND DEVELOPMENT OF CELL MEDIATED IMMUNITY BIOMARKERS.- LJVR

## APPENDIX E: ANALYSED *T. GODII* B1 SEQUENCES FOR 803 bp FRAGMENTS

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       10         20         30         40         50
Tecuanillo178   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Tecuanillo177   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Tecuanillo175   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Tecuanillo174   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Coalatilla173   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Coalatilla170   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
StaRosa114      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
StaRosa112      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Juluapan11      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Tecuanillo179   TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Tecoman108      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
StaRosa107      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
ElReal111       TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
ElReal109b      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
ElReal109a      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Camalote106     TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Camalote104     TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Camalote102b    TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
StaRosa103      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Camalote102a    TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Estacion101b    TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Estacion101a    TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
StaRosa113      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC
Estacion98      TTGGTTCCGC CTCCTTCGTC CGTCGTAATA TCAGGCCTTC TGTTCTGTTC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       60         70         80         90        100
Tecuanillo178   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Tecuanillo177   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Tecuanillo175   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Tecuanillo174   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Coalatilla173   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Coalatilla170   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
StaRosa114      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
StaRosa112      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Juluapan11      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Tecuanillo179   GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Tecoman108      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
StaRosa107      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
ElReal111       GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
ElReal109b      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
ElReal109a      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Camalote106     GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Camalote104     GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Camalote102b    GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
StaRosa103      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Camalote102a    GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Estacion101b    GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Estacion101a    GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
StaRosa113      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
Estacion98      GCTGTCTGTC TAGGGCACCC TTACTGCAAG AGAAGTATTT GAGGTCATAT
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                      110        120        130        140        150
Tecuanillo178 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Tecuanillo177 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Tecuanillo175 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Tecuanillo174 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Coalatilla173 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Coalatilla170 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
StaRosa114    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
StaRosa112    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Juluapan11    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Tecuanillo179 CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Tecoman108    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
StaRosa107    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
ElReal111     CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
ElReal109b    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
ElReal109a    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Camalote106   CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Camalote104   CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Camalote102b  CGTCCCATGA AGTCGACCAC CTGTTTCCTC TTTTCACTGT CACGTACGAC
StaRosa103    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Camalote102a  CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Estacion101b  CGTCCCATGA AGTCGACCAC CTGTTTCCTC TTTTCACTGT CACGTACGAC
Estacion101a  CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
StaRosa113    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC
Estacion98    CGTCCCATGA AGTCGACCAC CTGTTTCCTC TCTTCACTGT CACGTACGAC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                      160        170        180        190        200
Tecuanillo178  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Tecuanillo177  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Tecuanillo175  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Tecuanillo174  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Coalatilla173  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Coalatilla170  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
StaRosa114     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
StaRosa112     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Juluapan11     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Tecuanillo179  ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Tecoman108     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
StaRosa107     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
ElReal111      ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
ElReal109b     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
ElReal109a     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Camalote106    ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Camalote104    ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Camalote102b   ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
StaRosa103     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Camalote102a   ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Estacion101b   ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Estacion101a   ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
StaRosa113     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
Estacion98     ATCGCATTCA AGGGAAGAGA TCCAGCAGAT CTCGTTCGTG TATTCGAGAC
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Tecuanillo178    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Tecuanillo177    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Tecuanillo175    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Tecuanillo174    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Coalatilla175    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Coalatilla170    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
StaRosa114       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
StaRosa112       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Juluapan11       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Tecuanillo179    AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Tecoman108       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
StaRosa107       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
ElReal111        AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
ElReal109b       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
ElReal109a       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Camalote106      AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Camalote104      AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Camalote102b     AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
StaRosa103       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Camalote102a     AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Estacion101b     AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Estacion10       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
StaRosa113       AAGAGAGGTC CGCCCCCACA AGACGGCTGA AGAATGCAAC ATTCTTGTGC
Estacion98       AAGAGAGGTC CGCCCCCACA AGACGGCCGA AGAATGCAAC ATTCTTGTGC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
Tecuanillo178    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Tecuanillo177    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Tecuanillo175    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Tecuanillo174    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Coalatilla175    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Coalatilla170    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
StaRosa114       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
StaRosa112       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Juluapan11       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Tecuanillo179    TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Tecoman108       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
StaRosa107       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
ElReal111        TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
ElReal109b       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
ElReal109a       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Camalote106      TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Camalote104      TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Camalote102b     TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
StaRosa103       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Camalote102a     TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Estacion101b     TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Estacion101a     TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
StaRosa113       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
Estacion98       TGCCTCCTCT CATGGCAAAT GCCAGAAGAA GGGTACGTGT TGCATCATAA
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
Tecuanillo178    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Tecuanillo177    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Tecuanillo175    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Tecuanillo174    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Coalatilla175    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Coalatilla170    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
StaRosa114       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
StaRosa112       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Juluapan11       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Tecuanillo179    CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Tecoman108       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
StaRosa107       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
ElReal111        CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
ElReal109b       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
ElReal109a       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Camalote106      CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Camalote104      CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Camalote102b     CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
StaRosa103       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Camalote102a     CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Estacion101b     CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Estacion101a     CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
StaRosa113       CAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA
Estacion98       CAAGAGCTGT ATTTCCCGCT GGCAAATACA GGTGAAATGT ACCTCCAGAA


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
Tecuanillo178    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Tecuanillo177    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Tecuanillo175    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Tecuanillo174    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Coalatilla175    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Coalatilla170    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
StaRosa114       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
StaRosa112       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Juluapan11       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Tecuanillo179    AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Tecoman108       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
StaRosa107       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
ElReal111        AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
ElReal109b       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
ElReal109a       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Camalote106      AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Camalote104      AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Camalote102b     AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
StaRosa103       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Camalote102a     AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Estacion101b     AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Estacion101a     AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
StaRosa113       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
Estacion98       AAGCCACCTA GTATCGTGCG GCAATGTGCC ACCTCGCCTC TTGGGAGAAA
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                      410        420        430        440        450
Tecuanillo178   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Tecuanillo177   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Tecuanillo175   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Tecuanillo174   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Coalatilla173   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Coalatilla170   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
StaRosa114      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
StaRosa112      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Juluapan11      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Tecuanillo179   AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Tecoman108      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
StaRosa107      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
ElReal111       AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
ElReal109b      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
ElReal109a      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Camalote106      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Camalote104     AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Camalote102a    AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
StaRosa103      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Camalote102b    AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Estacion101b    AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Estacion101a    AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
StaRosa113      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC
Estacion98      AAGAGGAAGA GACGCTGCCG CTGTTTTGCA AATGAAAAGG ATTCATTTTC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                      460        470        480        490        500
Tecuanillo178   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Tecuanillo177   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Tecuanillo175   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Tecuanillo174   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Coalatilla173   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Coalatilla170   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
StaRosa114      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
StaRosa112      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Juluapan11      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Tecuanillo179   GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Tecoman108      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
StaRosa107      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
ElReal111       GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGGGT
ElReal109b      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
ElReal109a      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Camalote106     GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Camalote104     GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Camalote102a    GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
StaRosa103      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Camalote102b    GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Estacion101b    GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Estacion101a    GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
StaRosa113      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
Estacion98      GCAGTACACC AGGAGTTGGA TTTTGTAGAG CGTCTCTCTT CAAGCAGCGT
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         510        520        530        540        550
Tecuanillo178    ATTGTTGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Tecuanillo177    ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Tecuanillo175    ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Tecuanillo174    ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Coalatilla173    ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Coalatilla170    ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
StaRosa114       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
StaRosa112       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Juluapan11       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Tecuanillo179    ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Tecoman108       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
StaRosa107       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
ElReal111        ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
ElReal109b       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
ElReal109a       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Camalote106      ATTGTCGAGT AGATCAGAAA GGAAATGCAT CCGTTCATGA GTATAAGAAA
Camalote104      ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Camalote102b     ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
StaRosa103       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Camalote102b     ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Estacion101b     ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Estacion101a     ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
StaRosa113       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA
Estacion98       ATTGTCGAGT AGATCAGAAA GGAACTGCAT CCGTTCATGA GTATAAGAAA


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
Tecuanillo178    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Tecuanillo177    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Tecuanillo175    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Tecuanillo174    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Coalatilla173    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Coalatilla170    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
StaRosa114       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
StaRosa112       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Juluapan11       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Tecuanillo179    AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Tecoman108       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
StaRosa107       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
ElReal111        AAAATGTGGG AATGAAAGAG ACGGTAATGT GTTTGCATAG GTTGCAGTCA
ElReal109b       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
ElReal109a       AAAATGTGGG AATGAAAGAG ACGCTAATGT ATTTGCATAG GTTGCAGTCA
Camalote106      AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Camalote104      AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Camalote102b     AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
StaRosa103       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Camalote102b     AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Estacion101b     AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Estacion101a     AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
StaRosa113       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
Estacion98       AAAATGTGGG AATGAAAGAG ACGCTAATGT GTTTGCATAG GTTGCAGTCA
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
Tecuanillo178 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Tecuanillo177 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Tecuanillo175 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Tecuanillo174 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Coalatilla173 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Coalatilla170 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
StaRosa114    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
StaRosa112    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Juluapan11    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Tecuanillo179 CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Tecoman108    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
StaRosa107    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
ElReal111     CTGACGAGCT CCCCTTTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
ElReal109b    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
ElReal109a    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Camalote106   CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Camalote104   CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Camalote102b  CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
StaRosa103    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Camalote102a  CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Estacion101b  CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Estacion101a  CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
StaRosa113    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG
Estacion98    CTGACGAGCT CCCCTCTGCT GGCGAAAAGT GAAATTCATG AGTATCTGTG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
Tecuanillo178 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Tecuanillo177 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Tecuanillo175 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Tecuanillo174 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Coalatilla173 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Coalatilla170 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
StaRosa114    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
StaRosa112    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Juluapan11    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Tecuanillo179 CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Tecoman108    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
StaRosa107    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
ElReal111     CAACTTTGGT GTATTCACAA ATTGGTCGCC TGCAATCGAT AGTTGACCAC
ElReal109b    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
ElReal109a    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Camalote106   CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Camalote104   CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Camalote102b  CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
StaRosa103    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Camalote102a  CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Estacion101b  CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Estacion101a  CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
StaRosa113    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
Estacion98    CAACTTTGGT GTATTCGCAG ATTGGTCGCC TGCAATCGAT AGTTGACCAC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
Tecuanillo178   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Tecuanillo177   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Tecuanillo175   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Tecuanillo174   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Coalatilla173   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Coalatilla170   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
StaRosa114      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
StaRosa112      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Juluapan11      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Tecuanillo179   GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Tecoman108      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
StaRosa107      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
ElReal111       GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
ElReal109b      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
ElReal109a      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Camalote106     GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Camalote104     GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Camalote102a    GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
StaRosa103      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Camalote102a    GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Estacion101b    GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Estacion101a    GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
StaRosa113      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG
Estacion98      GAACGCTTTA AAGAACAGGA GAAGAAGATC GTGAAAGAAT ACGAGAAGAG


                ....|....| ....|....| ....|....| ....|....| ....|....|.
                       760        770        780        790        800
Tecuanillo178   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Tecuanillo177   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Tecuanillo175   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Tecuanillo174   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Coalatilla173   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Coalatilla170   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
StaRosa114      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
StaRosa112      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Juluapan11      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Tecuanillo179   GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Tecoman108      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
StaRosa107      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
ElReal111       GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
ElReal109b      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
ElReal109a      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Camalote106     GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Camalote104     GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Camalote102b    GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
StaRosa103      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Camalote102a    GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Estacion101b    GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Estacion101a    GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
StaRosa113      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
Estacion98      GTACACAGAG ATAGAAGTCG CTGCGGAGAC AGCGAAGACT GCGGATGACTT
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## APPENDIX F: ANALYSED *T. gondii* B1 GENE SEQUENCES WITH FRAGMENT SIZES BETWEEN 400 AND 1000 bp

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Tecuanillo179   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Tecuanillo178   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Tecuanillo177   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Tecuanillo175   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Tecuanillo174   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Coalatilla173   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Coalatilla170   CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
StaRosa114      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
StaRosa112      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Juluapan11      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Tecoman108      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
StaRosa107      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
ElReal111       CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
ElReal109b      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
ElReal109a      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Camalote106     CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Camalote104     CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Camalote102b    CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Camalote102a    CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Estacion101b    CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Estacion101a    CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
StaRosa113      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
Estacion98      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
CG21            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
CG19            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
D1              CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
GQ3             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
GQ2             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
CQ7             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
GY3             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
GY2             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
CY2             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SY12            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SY5             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SY4             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
GY4             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SY3             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
241             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
222             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
220             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
836-L-IMNO     CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
782-L-IMNO     CGCTTCCTTC GTCCGTCGTA ATATCAGGCC TTCTATTCTG TTCGCTGTCT
781-L-IMNO     CGCTTCCTTC GTCCGTCGTA ATATCAGGCC TTCTATTCTG TTCGCTGTCT
774-L-IMNO     CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLR-720      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCCG TTCGCTGTCT
TG-KLR-631      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLR-625      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLR-610      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLR-583      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLR-555      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLK-101      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLK-983      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLK-905      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
2A              CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TG-KLK-830      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
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TG-KLK-365     CGCTTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
25             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
16A            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
16B            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
1A             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
24A            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
26             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
28B            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
22A            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
5A             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
22             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
15B            CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
7B             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
4B             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
3B             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
2B             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
5B             CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TgCatAu_8      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TgCatAu_6      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
TgCatAu_7      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTC-
TgCatAu_2      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
R236           CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SR231          CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SR222          CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGTTGTCT
SR217          CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
SR215          CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
CR34           CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT
C-F-TG-56      CGCCTCCTTC GTCCGTCGTA ATATCAGGCC TTCTGTTCTG TTCGCTGTCT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Tecuanillo179  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Tecuanillo178  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Tecuanillo177  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Tecuanillo175  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Tecuanillo174  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Coalatilla173  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Coalatilla170  GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
StaRosa114     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
StaRosa112     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Juluapan11     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Tecoman108     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
StaRosa107     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
ElReal111      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
ElReal109b     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
ElReal109a     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Camalote106    GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Camalote104    GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Camalote102b   GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Camalote102a   GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Estacion101b   GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Estacion101a   GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
StaRosa113     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
Estacion98     GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
CG21           GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
CG19           GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
D1             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
GQ3            GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
GQ2            GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
CQ7            GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
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GY3             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
GY2             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
CY2             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SY12            GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SY5             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SY4             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
GY4             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SY3             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
241             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
222             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
220             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
836-L-IMNO      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
782-L-IMNO      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
781-L-IMNO      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
774-L-IMNO      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLR-720      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLR-631      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLR-625      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLR-610      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLR-583      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLR-555      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLK-101      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLK-983      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLK-905      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
2A              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLK-830      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TG-KLK-365      GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
25              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
16A             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
16B             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
1A              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
24A             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
26              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
28B             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
22A             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
5A              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
22              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
15B             GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
7B              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
4B              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
3B              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
2B              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
5B              GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TgCatAu_8       GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TgCatAu_6       GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
TgCatAu_7       ---------- ---------- ---------- ---------- ----------
TgCatAu_2       GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
R236            GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SR231           GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SR222           GTCTAGGGCA CCCTTACTGC AAGAGAAAGT ATTTGAGGTC ATATCGTCCC
SR217           GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
SR215           GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
CR34            GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
C-F-TG-56       GTCTAGGGCA CCCTTACTGC AAGAGAA-GT ATTTGAGGTC ATATCGTCCC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Tecuanillo179   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Tecuanillo178   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Tecuanillo177   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Tecuanillo175   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Tecuanillo174   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Coalatilla173   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Coalatilla170   ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
StaRosa114      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
StaRosa112      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Juluapan11      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Tecoman108      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
StaRosa107      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
ElReal111       ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
ElReal109b      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
ElReal109a      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Camalote106     ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Camalote104     ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Camalote102b    ATGAAGTCGA CCACCTGTTT CCTCTTTTCA CTGTCACGTA CGACATCGCA
Camalote102a    ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Estacion101b    ATGAAGTCGA CCACCTGTTT CCTCTTTTCA CTGTCACGTA CGACATCGCA
Estacion101a    ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
StaRosa113      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
Estacion98      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
CG21            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
CG19            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
D1              ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
GQ3             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
GQ2             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
CQ7             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
GY3             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
GY2             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
CY2             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SY12            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SY5             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SY4             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
GY4             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
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836-L-IMNO      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
782-L-IMNO      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
781-L-IMNO      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
774-L-IMNO      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLR-720      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLR-631      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLR-625      ATGAAGTCGA CCACCTGCTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
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TG-KLR-583      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLR-555      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLK-101      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLK-983      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLK-905      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
2A              ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLK-830      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TG-KLK-365      ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
25              ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
16A             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
16B             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
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1A              ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
24A             ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
26            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
28B           ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
22A           ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
5A            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
22            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
15B           ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
7B            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
4B            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
3B            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
2B            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
5B            ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TgCatAu_8     ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TgCatAu_6     ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TgCatAu_7     ---------- CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
TgCatAu_2     ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
R236          ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SR231         ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SR222         ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SR217         ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
SR215         ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
CR34          ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA
C-F-TG-56     ATGAAGTCGA CCACCTGTTT CCTCTCTTCA CTGTCACGTA CGACATCGCA


                  ....|....| ....|....| ....|....| ....|....| ....|....|
                          160        170        180        190        200
Tecuanillo179  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Tecuanillo178  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Tecuanillo177  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Tecuanillo175  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Tecuanillo174  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Coalatilla173  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Coalatilla170  TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
StaRosa114     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
StaRosa112     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Juluapan11     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Tecoman108     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
StaRosa107     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
ElReal111      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
ElReal109b     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
ElReal109a     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Camalote106    TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Camalote104    TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Camalote102b   TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Camalote102a   TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Estacion101b   TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Estacion101a   TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
StaRosa113     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
Estacion98     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
CG21           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTACTCG AGACAAGAGA
CG19           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
D1             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
GQ3            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
GQ2            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
CQ7            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
GY3            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
GY2            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
CY2            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
SY12           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
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SY5             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
SY4             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
GY4             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
SY3             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
241             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
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836-L-IMNO      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
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781-L-IMNO      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
774-L-IMNO      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLR-720      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLR-631      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAAA
TG-KLR-625      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLR-610      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAAA
TG-KLR-583      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLR-555      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLK-1018     TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLK-983      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLK-905      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
2A              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLK-830      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TG-KLK-365      TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
25              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTACTCG AGACAAGAGA
16A             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
16B             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
1A              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
24A             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
26              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
28B             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
22A             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
5A              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
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15B             TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
7B              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
4B              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
3B              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
2B              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
5B              TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TgCatAu_8       TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TgCatAu_6       TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TgCatAu_7       TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
TgCatAu_2       TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
R236            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
SR231           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTACTCG AGACAAGAGA
SR222           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAAA
SR217           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
SR215           TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA
CR34            TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTACTCG AGACAAGAGA
C-F-TG-56       TTCAAGGGAA GAGATCCAGC AGATCTCGTT CGTGTATTCG AGACAAGAGA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Tecuanillo179   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Tecuanillo178   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Tecuanillo177   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Tecuanillo175   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Tecuanillo174   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Coalatilla173   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Coalatilla170   GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
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StaRosa114      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
StaRosa112      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Juluapan11      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Tecoman108      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
StaRosa107      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
ElReal111       GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
ElReal109b      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
ElReal109a      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Camalote106     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Camalote104     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Camalote102b    GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Camalote102a    GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Estacion101b    GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Estacion101a    GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
StaRosa113      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
Estacion98      GGTCCGCCCC CACAAGACGG CCGAAGAATG CAACATTCTT GTGCTGCCTC
CG21            GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
CG19            GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
D1              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
GQ3             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
GQ2             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
CQ7             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
GY3             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
GY2             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
CY2             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SY12            GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SY5             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SY4             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAATATTCTT GTGCTGCCTC
GY4             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SY3             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
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220             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
836-L-IMNO     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
782-L-IMNO     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
781-L-IMNO     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
774-L-IMNO     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLR-720      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLR-631      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLR-625      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLR-610      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLR-583      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GCGCTGCCTC
TG-KLR-555      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLK-1018     GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLK-983      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLK-905      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
2A              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLK-830      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TG-KLK-365      GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
25              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
16A             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
16B             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
1A              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
24A             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
26              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
28B             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
22A             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
5A              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
22              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
15B             GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
7B              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
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4B              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
3B              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
2B              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
5B              GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TgCatAu_8       GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TgCatAu_6       GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TgCatAu_7       GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
TgCatAu_2       GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
R236            GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SR231           GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SR222           GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SR217           GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
SR215           GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
CR34            GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC
C-F-TG-56       GGTCCGCCCC CACAAGACGG CTGAAGAATG CAACATTCTT GTGCTGCCTC


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                        260        270        280        290        300
Tecuanillo179   CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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Tecuanillo177   CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Tecuanillo175   CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Tecuanillo174   CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Coalatilla173   CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Coalatilla170   CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
StaRosa114      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
StaRosa112      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Juluapan11      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Tecoman108      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
StaRosa107      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
ElReal111       CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
ElReal109b      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
ElReal109a      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Camalote106     CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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Camalote102b    CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Camalote102a    CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Estacion101b    CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Estacion101a    CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
StaRosa113      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
Estacion98      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
CG21            CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
CG19            CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
D1              CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
GQ3             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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CQ7             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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CY2             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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836-L-IMNO      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
782-L-IMNO      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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TG-KLK-365      CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
25              CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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16B             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
1A              CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
24A             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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28B             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
22A             CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
5A              CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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4B              CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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5B              CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
TgCatAu_8       CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
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TgCatAu_7       CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
TgCatAu_2       CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
R236            CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
SR231           CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
SR222           CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
SR217           CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
SR215           CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
CR34            CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG
C-F-TG-56       CTCTCATGGC AAATGCCAGA AGAAGGGTAC GTGTTGCATC ATAACAAGAG


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                       310        320        330        340        350
Tecuanillo179   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Tecuanillo178   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Tecuanillo177   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Tecuanillo175   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Tecuanillo174   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Coalatilla173   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Coalatilla173   CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
StaRosa114      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
StaRosa112      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Juluapan11      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Tecoman108      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
StaRosa107      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
ElReal111       CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
ElReal109b      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
ElReal109a      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Camalote106     CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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Camalote104     CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Camalote102b    CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Camalote102a    CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Estacion101b    CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Estacion101a    CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
StaRosa113      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
Estacion98      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
CG21            CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
CG19            CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
D1              CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
GQ3             CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
GQ2             CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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GY2             CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
CY2             CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
SY12            CTGTATTTCC CGCTGGCAAA TACACGTGAA ATGTACCTCC AGAAAAGCCA
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836-L-IMNO     CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
782-L-IMNO     CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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774-L-IMNO     CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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TG-KLR-631      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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TG-KLR-610      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
TG-KLR-583      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
TG-KLR-555      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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TG-KLK-905      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
2A              CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
TG-KLK-830      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
TG-KLK-365      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
25              CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
16A             CTGTATTTCC CGCTGGCAAA TACACGTGAA ATGTACCTCC AGAAAAGCCA
16B             CTGTATTTCC CGCTGGCAAA TACACGTGAA ATGTACCTCC AGAAAAGCCA
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R236            CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
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SR217          CTGTATTTCC CGCTGGCAAA TACACGTGAA ATGTACCTCC AGAAAAGCCA
SR215          CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
CR34           CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA
C-F-TG-56      CTGTATTTCC CGCTGGCAAA TACAGGTGAA ATGTACCTCC AGAAAAGCCA


                  ....|....| ....|....| ....|....| ....|....| ....|....|
                          360        370        380        390        400
Tecuanillo179  CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
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Tecoman108     CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
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TG-KLR-555     CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TG-KLK-1018    CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
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2A              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TG-KLK-830      CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TG-KLK-365      CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
25              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
16A             CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAGGAGG
16B             CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
1A              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
24A             CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
26              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
28B             CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
22A             CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
5A              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
22              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
15B             CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
7B              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
4B              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
3B              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
2B              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
5B              CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TgCatAu_8       CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TgCatAu_6       CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TgCatAu_7       CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
TgCatAu_2       CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
R236            CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
SR231           CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
SR222           CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
SR217           CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
SR215           CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
CR34            CCTCGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG
C-F-TG-56       CCTAGTATCG TGCGGCAATG TGCCACCTCG CCTCTTGGGA GAAAAAGAGG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
Tecuanillo179   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Tecuanillo178   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Tecuanillo177   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Tecuanillo175   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Tecuanillo174   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Coalatilla173   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Coalatilla170   AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
StaRosa114      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
StaRosa112      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Juluapan11      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Tecoman108      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
StaRosa107      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
ElReal111       AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
ElReal109b      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
ElReal109a      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Camalote106     AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Camalote105     AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Camalote102b    AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Camalote102a    AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Estacion101b    AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Estacion101a    AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
StaRosa113      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
Estacion98      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
CG21            AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
CG19            AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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CQ7             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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GY2             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
CY2             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SY12            AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SY5             AGGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SY4             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
GY4             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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781-L-IMNO     AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
774-L-IMNO     AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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TG-KLR-631      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLR-625      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLR-610      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLR-583      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLR-555      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLK-1018     AAGAGACACT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLK-983      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLK-905      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
2A              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLK-830      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
TG-KLK-365      AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
25              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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16B             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
1A              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
24A             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
26              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
28B             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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15B             AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
7B              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
4B              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
3B              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
2B              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
5B              AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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TgCatAu_7       AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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R236            AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SR231           AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SR222           AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SR217           AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
SR215           AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
CR34            AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
C-F-TG-56       AAGAGACGCT GCCGCTGTTT TGCAAATGAA AAGGATTCAT TTTCGCAGTA
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Tecuanillo178   CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Tecuanillo177   CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Tecuanillo175   CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Tecuanillo174   CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Coalatilla173   CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Coalatilla170   CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
StaRosa114      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
StaRosa112      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Juluapan11      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Tecoman108      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
StaRosa107      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
ElReal111       CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
ElReal109b      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
ElReal109a      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Camalote106     CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Camalote104     CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Camalote102b    CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Camalote102a    CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Estacion101b    CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Estacion101a    CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
StaRosa113      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
Estacion98      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
CG21            CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
CG19            CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
D1              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
GQ3             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
GQ2             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
CQ7             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
GY3             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
GY2             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
CY2             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
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220             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
836-L-IMNO      CGCCATGAGT TGGATTTTGT GAAGCGTCTC TCT
782-L-IMNO      CGCCATGAGT TGGATTTTGT AAAGCGTCTC TCT
781-L-IMNO      CGCCATGAGT TGGATTTTGT AAAGCGTCTC TCT
774-L-IMNO      CGCCATGAGT TGGATTTTGT GAAGCGTCTC TCT
TG-KLR-720      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
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TG-KLR-625      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TG-KLR-610      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TG-KLR-583      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TG-KLR-555      CACCAGGAGT TGGATTTTGT GGAGCGTCTC TCT
TG-KLK-1018     CACCAGGAGT TGGATTTTGT AGAGCGTCTC CCT
TG-KLK-983      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TG-KLK-905      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
2A              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TG-KLK-830      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TG-KLK-365      CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
25              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
16A             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
16B             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
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1A              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
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28B             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
22A             CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
5A              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
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7B              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
4B              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
3B              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
2B              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
5B              CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TgCatAu_8       CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TgCatAu_6       CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TgCatAu_7       CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
TgCatAu_2       CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
R236            CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
SR231           CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
SR222           CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
SR217           CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
SR215           CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
CR34            CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
C-F-TG-56       CACCAGGAGT TGGATTTTGT AGAGCGTCTC TCT
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## APPENDIX G: ANALYSED *T. GONDII* GRA6 GENE SEQUENCES WITH TRIMMED AND ALIGNED FRAGMENT SIZES OF BETWEEN 400 AND 1000 bp

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
24              AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
4A              AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
22              AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
7B              AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
5A              AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
5B              AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
11              AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
22R             AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
16A             AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
8A              AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
KM              AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir20         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir19         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir03         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir02         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir11         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir12         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir18         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir22         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir09         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
Izmir06         AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgPiPr09        AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
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TgPiPr13        AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgA32129        AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgPiPr05        AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgCkPr03        AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgCkPr04        AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgPiPr14        AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgCkPr01        AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
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TgPiPr02        AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
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TgWolfMN11      AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgWtdUs08       AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT
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Tgshir2       AAATGGCACA CGGTGGCATC CATCTGAGGC AGAAGCGTAA CTTCTGTCCT
TgBobcatMS    AAATGGCACA CGGTGGCATC TATCTGAGGC AGAAGCGTAA CTTCTGTCCT


              ....|....| ....|....| ....|....| ....|....| ....|....|
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7B            CGTCAATTCG TTGGGTGGAG TCGCTGTCGC AGCAGACAGC GGTGGTGTTA
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22            CGACTCGTTA GCTGAAGATG A---TACAAC CTCCGATGCG GCGGAGGGCG
7B            CGACTCGTTA GCTGAAGATG A---TACAAC CTCCGATGCG GCGGAGGGCG
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22R           CGACTCGTTA GCTGAAGATG A---TACAAC CTCCGATGCG GCGGAGGGCG
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8A            CGACTCGTTA GCTGAAGATG A---TACAAC CTCCGAAGCG GCGGAGGGCG
KM            CGACTCGTTA GCTGAAGATG A---TACAAC CTCCGATGCG GCGGAGGGCG
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7B              ACGTTGACCC TTTTCCCGCG CTGGCGAATG AGGGGAAGTC GGAGGCGCGT
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TgBobcatMS    GGCCCGTCGC TCGAGGAAAG AATCGAAGAA CAGGGCACAA GACGACGTTA


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7B            CTCCTCTGTT CAAGAACCAC AAGCGAAGGT GCCTAGCAAA CGAACACAGA
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5B            CTCCTCTGTT CAAGAACCAC AAGCGAAGGT GCCTAGCAAA CGAACACAGA
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22R           CTCCTCTGTT CAAGAACCAC AAGCGAAGGT GCCTAGCAAA CGAACACAGA
16A           CTCCTCTGTT CAAGAACCAC GAGCGAAGGT GCCTAGCAAA CGAACACAGA
8A            CTCCTCTGTT CAAGAACCAC AAGCGAAGGT GCCTAGCAAA CGAACACAGA
KM            CTCCTCTGTT CAAGAACCAC AAGCGAAGGT GCCTAGCAAA CGAACACAGA
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TgBobcatMS    TTCCTCTGTT CAAGAACCAC AAACGAAGGT GCCTAGCAAA CGAACACAGA
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7B              AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
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5B              AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
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22R             AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
16A             AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
8A              AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
KM              AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
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TgSoUs14     AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
Tgshir2      AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG
TgBobcatMS   AACGCCACAG ACTCATTGGT GCTGTGGTGT TGGCAGTATC TGTGGCAATG


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4A           CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
22           CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
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TgWolfMN11    CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgWtdUs08     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgWtdUs10     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgWolfMN20    CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA18001      CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA18005      CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105002     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105043     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105001     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105052     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA105003     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA105037     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA105004     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA105053     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA105051     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA105018     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105011     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105016     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA105015     CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCCAGAACC
TgA32129      CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgA32109      CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgSoUs14      CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
Tgshir2       CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC
TgBobcatMS    CTTACCGCTT TCTTTCTTCG AAGGACTGGA CGACGCTCTC CCCAAGAACC


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                            560        570        580        590        600
24            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
4A            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
22            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
7B            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
5A            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
5B            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
11            ATCTGGGGAT GGTGGTGGAA ATGATGCACG CAATAATGCT GGGAACGGTG
22R           ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
16A           ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
8A            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
KM            ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir20       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir19       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir03       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir02       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir11       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir12       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir18       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir22       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir09       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Izmir06       ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
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TgPiPr09      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCkPr11      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgPiPr13      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA32129      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgPiPr05      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCkPr03      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCkPr04      ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgPiPr14      ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgCkPr01      ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgCkPr02      ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgCkPr16      ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgPiPr02      ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgFoxPa06     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWTDPa06     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa03      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa02      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa04      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa07      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgFoxPa10     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa01      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa05      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgCoPa08      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgFoxPa03     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgWolfMN25    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN27    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN12    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN26    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN28    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN13    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN19    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN29    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN11    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWtdUs08     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWtdUs10     ATCTGGGGAT GATGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgWolfMN20    ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgA18001      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA18005      ATCTGGGGAT GATGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105002     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105043     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105001     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105052     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105003     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105037     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105004     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105053     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105051     ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA105018     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgA105011     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgA105016     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgA105015     ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACCGTG
TgA32129      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgA32109      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgSoUs14      ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
Tgshir2       ATCTGGGGAT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
TgBobcatMS    ATCTGGGGGT GGTGGTGGAA ATGATGCAGG CAATAATGCT GGGAACGGTG
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24            GGAATG
4A            GGAATG
22            GGAATG
7B            GGAATG
5A            GGAATG
5B            GGAATG
11            GGAATG
22R           GGAATG
16A           GGAATG
8A            GGAATG
KM            GGAATG
Izmir20       GGAATG
Izmir19       GGAATG
Izmir03       GGAATG
Izmir02       GGAATG
Izmir11       GGAATG
Izmir12       GGAATG
Izmir18       GGAATG
Izmir22       GGAATG
Izmir09       GGAATG
Izmir06       GGAATG
TgPiPr09      GGAATG
TgCkPr11      GGAATG
TgPiPr13      GGAATG
TgA32129      GGAATG
TgPiPr05      GGAATG
TgCkPr03      GGAATG
TgCkPr04      GGAATG
TgPiPr14      GGAATG
TgCkPr01      GGAATG
TgCkPr02      GGAATG
TgCkPr16      GGAATG
TgPiPr02      GGAATG
TgFoxPa06     GGAATG
TgWTDPa06     GGAATG
TgCoPa03      GGAATG
TgCoPa02      GGAATG
TgCoPa04      GGAATG
TgCoPa07      GGAATG
TgFoxPa10     GGAATG
TgCoPa01      GGAATG
TgCoPa05      GGAATG
TgCoPa08      GGAATG
TgFoxPa03     GGAATG
TgWolfMN25    GGAATG
TgWolfMN27    GGAATG
TgWolfMN12    GGAATG
TgWolfMN26    GGAATG
TgWolfMN28    GGAATG
TgWolfMN13    GGAATG
TgWolfMN19    GGAATG
TgWolfMN29    GGAATG
TgWolfMN11    GGAATG
TgWtdUs08     GGAATG
TgWtdUs10     GGAATG
TgWolfMN20    GGAATG
TgA18001      GGAATG
TgA18005      GGAATG
TgA105002     GGAATG
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**TgA105043** GGAATG
**TgA105001** GGAATG
**TgA105052** GGAATG
**TgA105003** GGAATG
**TgA105037** GGAATG
**TgA105004** GGAATG
**TgA105053** GGAATG
**TgA105051** GGAATG
**TgA105018** GGAATG
**TgA105011** GGAATG
**TgA105016** GGAATG
**TgA105015** GGAATG
**TgA32129** GGAATG
**TgA32109** GGAATG
**TgSoUs14** GGAATG
**Tgshir2** GGAATG
**TgBobcatMS** GGAATG

**APPENDIX H: INFORMATION ON THE *T. GONDII* B1 ISOLATES FOR THE 803 bp USED FOR SEQUENCE ANALYSIS AND PHYLOGENETIC TREE CONSTRUCTION**

| Isolate | Species | Country of origin | Accession number |
|---|---|---|---|
| Tecuanillo178 | Sheep | Colima, Mexico | KX270386.1 |
| Tecuanillo177 | Sheep | Colima, Mexico | KX270385.1 |
| Tecuanillo175 | Sheep | Colima, Mexico | KX270384.1 |
| Tecuanillo174 | Sheep | Colima, Mexico | KX270383.1 |
| Coalatilla173 | Sheep | Colima, Mexico | KX270382.1 |
| Coalatilla170 | Sheep | Colima, Mexico | KX270381.1 |
| StaRosa114 | Sheep | Colima, Mexico | KX270380.1 |
| StaRosa112 | Sheep | Colima, Mexico | KX270379.1 |
| Juluapan110 | Sheep | Colima, Mexico | KX270378.1 |
| Tecuanillo179 | Sheep | Colima, Mexico | KX270387.1 |
| Tecoman108 | Sheep | Colima, Mexico | KX270377.1 |
| StaRosa107 | Sheep | Colima, Mexico | KX270378.1 |
| ElReal111 | Sheep | Colima, Mexico | KX270375.1 |
| ElReal109b | Sheep | Colima, Mexico | KX270367.1 |
| ElReal109a | Sheep | Colima, Mexico | KX270373.1 |
| Camalote106 | Sheep | Michoacan, Mexico | KX270372.1 |
| Camalote105 | Sheep | Michoacan, Mexico | KX270371.1 |
| Camelote102b | Sheep | Michoacan, Mexico | KX270369.1 |
| StaRosa103 | Sheep | Colima, Mexico | KX270370.1) |
| Camelote102a | Sheep | Michoacan, Mexico | KX270368.1 |

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|---|---|---|---|
| Estacion101b | Sheep | Colima, Mexico | KX270367.1 |
| Estacion101a | Sheep | Colima, Mexico | KX270366.1 |
| StaRosa113 | Sheep | Colima, Mexico | KX270365.1 |
| Estacion98 | Sheep | Colima, Mexico | KX270364.1 |

**APPENDIX I: INFORMATION ON THE *T. GONDII* B1 ISOLATES AND/CLONES OF TRIMMED FRAGMENT SIZES OF BETWEEN 300-100 bp USED FOR SEQUENCE ANALYSIS AND PHYLOGENETIC TREE CONSTRUCTION**

| Isolate/clone | Host | Country of origin | Accession number |
|---|---|---|---|
| Isolate Tecuanillo178 | Sheep | Colima, Mexico | KX270386.1 |
| Isolate Tecuanillo177 | Sheep | Colima, Mexico | KX270385.1 |
| Isolate Tecuanillo175 | Sheep | Colima, Mexico | KX270384.1 |
| Isolate Tecuanillo174 | Sheep | Colima, Mexico | KX270383.1 |
| Isolate Coalatilla173 | Sheep | Colima, Mexico | KX270382.1 |
| Isolate Coalatilla170 | Sheep | Colima, Mexico | KX270381.1 |
| Isolate StaRosa114 | Sheep | Colima, Mexico | KX270380.1 |
| Isolate StaRosa112 | Sheep | Colima, Mexico | KX270379.1 |
| Isolate Juluapan110 | Sheep | Colima, Mexico | KX270378.1 |
| Isolate Tecuanillo179 | Sheep | Colima, Mexico | KX270387.1 |
| Isolate Tecoman108 | Sheep | Colima, Mexico | KX270377.1 |
| Isolate StaRosa107 | Sheep | Colima, Mexico | KX270378.1 |
| Isolate ElReal111 | Sheep | Colima, Mexico | KX270375.1 |
| Isolate ElReal109b | Sheep | Colima, Mexico | KX270367.1 |
| Isolate ElReal109a | Sheep | Colima, Mexico | KX270373.1 |
| Isolate Camalote106 | Sheep | Michoacan, Mexico | KX270372.1 |
| Isolate Camalote105 | Sheep | Michoacan, Mexico | KX270371.1 |
| Isolate Camelote102b | Sheep | Michoacan, Mexico | KX270369.1 |
| Isolate StaRosa103 | Sheep | Colima, Mexico | KX270370.1) |
| Isolate Camelote102a | Sheep | Michoacan, Mexico | KX270368.1 |
| Isolate Estacion101b | Sheep | Colima, Mexico | KX270367.1 |

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|---|---|---|---|
| Isolate Estacion101a | Sheep | Colima, Mexico | KX270366.1 |
| Isolate StaRosa113 | Sheep | Colima, Mexico | KX270365.1 |
| Isolate Estacion98 | Sheep | Colima, Mexico | KX270364.1 |
| Isolate 2A | Sheep | Iran | KU672642.1 |
| Clone CG21 | Chicken | Iran | MN275916.1 |
| Clone CG19 | Chicken | Iran | MN275915.1 |
| Clone D1 | Duck | Iran | MN275914.1 |
| Clone GQ2 | Goat | Iran | MN275912.1 |
| Clone CQ7 | Cattle | Iran | MN275910.1 |
| Clone GY3 | Goat | Iran | MN275909.1 |
| Clone GY2 | Goat | Iran | MN275908.1 |
| Clone CY2 | Cattle | Iran | MN275907.1 |
| Clone SY12 | Sheep | Iran | MN275906.1 |
| Clone SY5 | Sheep | Iran | MN275905.1 |
| Clone SY4 | Sheep | Iran | MN275904.1 |
| Clone GY4 | Goat | Iran | MN275911.1 |
| Clone SY3 | Sheep | Iran | MN275903.1 |
| Isolate 25 | Camel | Iran | KU672641.1 |
| Isolate 16A | Sheep | Iran | KU672640.1 |
| Isolate 16B | Sheep | Iran | KU672639.1 |
| Isolate 1A | Sheep | Iran | KU672638.1 |
| Isolate 24A | Sheep | Iran | KU672637.1 |
| Isolate 26 | Camel | Iran | KU672636.1 |
| Isolate 28B | Sheep | Iran | KU672635.1 |
| Isolate 22A | Sheep | Iran | KU672634.1 |

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|---|---|---|---|
| Isolate 5A | Sheep | Iran | KU672633.1 |
| Isolate 22 | Camel | Iran | KU672632.1 |
| Isolate 15B | Goat | Iran | KU672630.1 |
| Isolate 7B | Sheep | Iran | KU672629.1 |
| Isolate 4B | Goat | Iran | KU672628.1 |
| Isolate 3B | Goat | Iran | KU672627.1 |
| Isolate 2B | Sheep | Iran | KU672626.1 |
| Isolate 5B | Sheep | Iran | KU672625.1 |
| Isolate 241 | Black bear | USA | MH744807.1 |
| Isolate 222 | Black bear | USA | MH744806.1 |
| Isolate 220 | Black bear | USA | MH744805.1 |
| Isolate 836-L-IMNO | Ixodes ricinus ticks | Poland | KX944482.1 |
| Isolate 782-L-IMNO | Ixodes ricinus ticks | Poland | KX944481.1 |
| Isolate 781-L-IMNO | Ixodes ricinus ticks | Poland | KX944480.1 |
| Isolate 744-L-IMNO | Ixodes ricinus ticks | Poland | KX944479.1 |
| Isolate TG-KLR-720-IMNO | Ixodes ricinus ticks | Poland | KU748893.1 |
| Isolate TG-KLR-631-IMNO | Ixodes ricinus ticks | Poland | KU748892.1 |
| Isolate TG-KLR-625-IMNO | Ixodes ricinus ticks | Poland | KU748891.1 |
| Isolate TG-KLR-610-IMNO | Ixodes ricinus ticks | Poland | KU748890.1 |
| Isolate TG-KLR-583-IMNO | Ixodes ricinus ticks | Poland | KU748889.1 |
| Isolate TG-KLR-555-IMNO | Ixodes ricinus ticks | Poland | KU748888.1 |
| Isolate TG-KLK-1018-IMNO | Ixodes ricinus ticks | Poland | KU748887.1 |
| Isolate TG-KLK-983-IMNO | Ixodes ricinus ticks | Poland | KU748886.1 |
| Isolate TG-KLK-905-IMNO | Ixodes ricinus ticks | Poland | KU748885.1 |
| Isolate TG-KLK-830-IMNO | Ixodes ricinus ticks | Poland | KU748883.1 |

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|---|---|---|---|
| Isolate TG-KLK-365-IMNO | Ixodes ricinus ticks | Poland | KU748882.1 |
| Isolate TgCatAu_8 | Cat | Australia | KT881382.1 |
| Isolate TgCatAu_6 | Cat | Australia | KT881353.1 |
| Isolate TgCatAu_7 | Cat | Australia | KT881319.1 |
| Isolate TgCatAu_2 | Cat | Australia | KT881314.1 |
| Isolate R236 | California mussel | USA | KM243028.1 |
| Isolate SR231 | California mussel | USA | KM243027.1 |
| Isolate SR222 | California mussel | USA | KM243025.1 |
| Isolate SR217 | California mussel | USA | KM243024.1 |
| Isolate SR215 | California mussel | USA | KM243023.1 |
| Isolate CR34 | California mussel | USA | KM243022.1 |
| Isolate C-F-TG-56 | Cat | South Korea | MW063448.1 |

**APPENDIX J: INFORMATION ON THE *T. GONDII* GRA6 ISOLATES AND/CLONES OF TRIMMED FRAGMENT SIZES OF BETWEEN 300-100 bp USED FOR SEQUENCE ANALYSIS AND PHYLOGENETIC TREE CONSTRUCTION**

| Isolate | Host | Country of origin | Accession number |
|---|---|---|---|
| 24 | Camel | Iran | KU672652.1 |
| 4A | Goat | Iran | KU672651.1 |
| 22 | Camel | Iran | KU672650.1 |
| 7B | Sheep | Iran | KU672649.1 |
| 5A | Sheep | Iran | KU672648.1 |
| 5B | Sheep | Iran | KU672647.1 |
| 11 | Sheep | Iran | KU672646.1 |
| 2R | Sheep | Iran | KU672645.1 |
| 16A | Sheep | Iran | KU672644.1 |
| 8A | Sheep | Iran | KU672643.1 |
| KM | Cat | China | KX781158.1 |
| TgCatTr_Izmir20 | Cat | Turkey | KU599153.1 |
| TgCatTr_Izmir19 | Cat | Turkey | KU599152.1 |
| TgCatTr_Izmir03 | Cat | Turkey | KU599151.1 |
| TgCatTr_Izmir02 | Cat | Turkey | KU599150.1 |
| TgCatTr_Izmir11 | Cat | Turkey | KU599149.1 |
| TgCatTr_Izmir12 | Cat | Turkey | KU599148.1 |
| TgCatTr_Izmir22 | Cat | Turkey | KU599147.1 |
| TgCatTr_Izmir18 | Cat | Turkey | KU599145.1 |
| TgCatTr_Izmir09 | Cat | Turkey | KU599146.1 |

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|---|---|---|---|
| TgCatTr_Izmir06 | Cat | Turkey | KU599144.1 |
| TgPiPr09 | Pig | Portugal | KU599143.1 |
| TgCkPr11 | Chicken | Portugal | KU599142.1 |
| TgPiPr13 | Pig | Portugal | KU599141.1 |
| TgPiPr05 | Pig | Portugal | KU599140.1 |
| TgCkPr03 | Chicken | Portugal | KU599138.1 |
| TgCkPr04 | Chicken | Portugal | KU599137.1 |
| TgPiPr14 | Pig | Portugal | KU599136.1 |
| TgCkPr01 | Chicken | Portugal | KU599135.1 |
| TgCkPr02 | Chicken | Portugal | KU599134.1 |
| TgCkPr16 | Chicken | Portugal | KU599133.1 |
| TgPiPr02 | Pig | Portugal | KU599132.1 |
| TgFoxPa06 | Red fox | USA | KU599130.1 |
| TgFoxPa03 | Red fox | USA | KU599120.1 |
| TgWolfMN25 | Gray wolf | USA | KU599119.1 |
| TgWolfMN27 | Gray wolf | USA | KU599118.1 |
| TgWolfMN26 | Gray wolf | USA | KU599116.1 |
| TgWolfMN28 | Gray wolf | USA | KU599116.1 |
| TgWolfMN13 | Gray wolf | USA | KU599115.1 |
| TgWolfMN28 | Gray wolf | USA | KU599115.1 |
| TgWolfMN13 | Gray wolf | USA | KU599114.1 |
| TgWolfMN19 | Gray wolf | USA | KU599113.1 |
| TgWolfMN29 | Gray wolf | USA | KU599112.1 |
| TgWolfMN11 | Gray wolf | USA | KU599111.1 |

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|---|---|---|---|
| TgWolfMN20 | Gray wolf | USA | KU599108.1 |
| TgA18001 | Jaguar | French Guiana | KU599092.1 |
| TgSoUs14 | Sea otter | USA | EU180622.1 |
| TgWTDPa06 | White-tailed deer | USA | KU599129.1 |
| TgWtdUs08 | White-tailed deer | USA | KU599110.1 |
| TgWtdUs10 | White-tailed deer | USA | KU599109.1 |
| TgCoPa03 | Coyote | USA | KU599128.1 |
| TgCoPa02 | Coyote | USA | KU599127.1 |
| TgCoPa04 | Coyote | USA | KU599126.1 |
| TgCoPa07 | Coyote | USA | KU599125.1 |
| TgCoPa10 | Coyote | USA | KU599124.1 |
| TgCoPa01 | Coyote | USA | KU599123.1 |
| TgCoPa05 | Coyote | USA | KU599122.1 |
| TgCoPa08 | Coyote | USA | KU599121.1 |
| GAB5-GAL-DOM01-(TgA105002) | Chicken | Gabon | KU599090.1 |
| GAB3-GAL-DOM11-(TgA105043) | Chicken | Gabon | KU599089.1 |
| GAB3-GAL-DOM02-(TgA105001) | Chicken | Gabon | KU599088.1 |
| GAB3-GAL-DOM08-(TgA105052) | Chicken | Gabon | KU599086.1 |
| GAB1-GAL-DOM10-(TgA105003) | Chicken | Gabon | KU599085.1 |
| GAB2-GAL-DOM01-(TgA105037) | Chicken | Gabon | KU599084.1 |
| GAB2-GAL-DOM02-(TgA105004) | Chicken | Gabon | KU599083.1 |
| GAB4-GAL-DOM01-(TgA105053) | Chicken | Gabon | KU599082.1 |

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| GAB2-GAL-DOM06-(TgA105040) | Chicken | Gabon | KU599081.1 |
| GAB1-GAL-DOM13-(TgA105018) | Chicken | Gabon | KU599080.1 |
| GAB1-CAP-AEG06-(TgA105011) | Goat | Gabon | KU599079.1 |
| GAB1-GAL-DOM11-(TgA105016) | Chicken | Gabon | KU599078.1 |
| GAB1-GAL-DOM06-(TgA105015) | Chicken | Gabon | KU599077.1 |
| FR-OVI-ARI022-(TgA32129) | Sheep | France | KU599076.1 |
| FR-OVI-ARI043-(TgA32109) | Sheep | France | KU599075.1 |
| Tgshir2 | Sheep | Mashhad, Iran | KM372588.1 |
| TgBobcatMS1 | Cat | Mississippi, USA | KY364199 |