



**Knowledge, attitudes and practices analysis, prevalence and molecular detection
of *Neospora caninum* and *Toxoplasma gondii* in livestock in the
Khomas region of Namibia**

by

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Declaration

I, Alaster Samkange, declare that the thesis which I hereby submit for the degree PhD (Veterinary Tropical Diseases) in the Faculty of Veterinary Science at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



.....

13 December 2023

Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required ethics approval/exemption for the research described in this work.

The author declares that they have observed the ethical standards required in terms of the University's Code of Ethics for scholarly activities.

Abstract

This study chiefly focussed on the Khomas region of Namibia, and the research areas were: (1) the knowledge, attitudes and practices regarding neosporosis and toxoplasmosis among livestock farmers in the Khomas region and animal health practitioners in the whole country; (2) the seroprevalence and associated risk factors of *Neospora caninum* in cattle; (3) the seroprevalence and associated risk factors of *Toxoplasma gondii* in sheep and goats; (4) molecular investigation of *N. caninum* DNA in cattle and, (5) the molecular detection of *T. gondii* in sheep and goats in abattoir samples.

Only 15.9% (10/63) of the livestock farmers had heard about neosporosis or toxoplasmosis or knew how animals get infected ($p < 0.0001$). Five per cent (3/63) of the farmers knew the risks associated with keeping dogs and cats concerning neosporosis and toxoplasmosis, respectively ($p < 0.0001$). None of the animal health practitioners ($n = 51$) routinely requested *N. caninum* or *T. gondii* laboratory tests in cases of cattle, sheep or goat abortions. Although all animal health practitioners indicated they routinely interacted with livestock farmers, none regularly discussed neosporosis or toxoplasmosis.

Five point seven per cent (42/736) of the bovine sera were seropositive to *N. caninum*. Eight of the 32 establishments had at least one positive animal, giving a herd-level seroprevalence of 25%. There was no significant association between *N. caninum* seropositivity in cattle and the presence of dogs, jackals, history of abortions, farm size, and the number of cattle or average annual rainfall. The establishments with moderate to high numbers of Feliformia were 9.8 times more likely to be seropositive to *N. caninum* than those with none to low levels of the former ($p = 0.0245$).

Overall, 3.68% (11/299) of the sheep sera were seropositive to *T. gondii*, and 61.54% (8/13) of the sheep flocks tested had at least one positive animal. Only 0.29% (1/345) of the goat sera were

seropositive to *T. gondii*, and one of the 19 goat flocks had at least one positive animal, giving a herd-level prevalence of 5.26%. Sheep flocks had significantly greater animal-level and flock-level prevalences than goats ($p < 0.05$) and were 13.14 times more likely to be seropositive (OR = 13.14; CI 95%: 1.686-102.382) than goat flocks. Seropositivity to *T. gondii* was positively associated with the total number of sheep at the farming establishment, history of abortions and farm size ($p < 0.05$), but not goats. The study concluded that sheep were probably more exposed to *T. gondii* infection than goats and that the *T. gondii* seroprevalence level in the Khomas region was very low compared to other countries.

One hundred and ninety-nine bovine abattoir samples were collected from different animals, comprising 110 brain samples and 75 heart muscle samples. In addition, there were 14 whole blood samples from *N. caninum* seropositive cattle. The collected samples were tested using a conventional PCR targeting the pNc5 gene. All the samples tested were negative. The authors concluded that the negative results could be due to the low prevalence of *N. caninum* infection caused by adverse weather conditions and that a future study targeting aborted fetuses over a more extended period could yield positive results.

The *T. gondii* molecular study analysed 174 brain and heart tissue samples from sheep and goats for the presence of *T. gondii* DNA using nested PCR targeting the B1 gene. The tissue samples were obtained from animals at abattoirs designated for human consumption. The study found that 16.7% of the samples tested positive for *T. gondii* DNA, with a higher prevalence in sheep (17.4%) than in goats (7.7%). Eight of the 29 positive samples were successfully sequenced using the Sanger method. All isolates identified were closely related to *T. gondii* type III genotype, exhibiting alignment scores ranging from 96.44% to 100%. This study emphasizes the public health hazards of consuming undercooked sheep and goat meat and highlights the pressing need to introduce control measures to mitigate human exposure.

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Dedication

I dedicate this doctorate to my beloved wife, Annatolia, my two sons, Donald and Anesu, and my mom and dad.

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List of Abbreviations

AHP	Animal health practitioners
BCG	Bacille Calmette-Guerin
BLAST	Basic Local Alignment Search Tool
BVDV	Bovine Viral Diarrhoea Virus
CDC	Centres for Disease Control and Prevention
CI	Confidence interval
CSF	Cerebrospinal fluid
DAT	Direct agglutination test
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
cELISA	Competitive-inhibition enzyme-linked immunoassay assay
EU	European Union
FAO	Food Agricultural Organisation
GDP	Gross domestic product
GIT	Gastro-intestinal tract
H & E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HRM	High-resolution melting
IFAT	Indirect fluorescent antibody assay
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IHA	Indirect hemagglutination assay
ITS	Internal transcribed spacer
KAP	Knowledge, attitudes and practices
LAMP	Loop-mediated isothermal amplification
LAT	Latex agglutination test
MAT	Modified agglutination test
MEGA	Molecular evolutionary genetics analysis
MGA	Metagenomics analysis
MLG	Multilocus genotyping
MLMG	Multilocus-microsatellite genotyping
MLST	Multilocus sequence typing
MSA	Microsatellite analysis
MS	Microsatellite markers
NAT	Nucleic acid testing
NCBI	National Centre for Biotechnology Information
OR	Odds ratio
PAS	Periodic acid Schiff
PCR	Polymerase chain reaction
PCR-RFLPs	PCR-restriction fragment length polymorphisms
POP7	Polymer One Populational Seven
RAPD-PCR	Random amplified polymorphic DNA-PCR

rDNA	Ribosomal DNA
RH	Relative humidity
ROP	Polymorphic rhostry proteins
ToxoDB	Genomic database for <i>Toxoplasma</i> genus
UNAM	University of Namibia
UP	University of Pretoria
USA	United States of America
WB	Western blotting
WGS	Whole-genome sequencing
WHO	World Health Organisation

Chapter 1: General Introduction

1.1. Introduction

Approximately 70% of Namibia's population depends directly or indirectly on agriculture, of which livestock production accounts for 80% (Humavindu & Stage, 2013; Meat Board of Namibia, 2015; Simasiku & Sheefeni, 2017). Valued at approximately N\$4.6 billion and equating to 4.3% of the gross domestic product (GDP) (Meat Board of Namibia, 2019), the livestock sector employs roughly 30% of the nation's total labour force (Humavindu & Stage, 2013; Simasiku & Sheefeni, 2017). In 2020, the agriculture sector in Namibia contributed 6.6% of the country's GDP, with livestock production accounting for more than 55% of it (Coleman, 2021). Furthermore, animal products, livestock and crop exports constitute roughly 10.7% of Namibian exports (BDO Namibia, 2019). In addition, the country is one of only two African countries currently exporting beef to the European Union (EU), which shows the integral part livestock plays in the national economy. Thus, any animal disease resulting in reduced livestock productivity has severe economic knock-on effects.

Between 1991 and 1992, the central parts of the country, including the Khomas region, experienced an outbreak of abortions, stillbirths and neonatal deaths in cattle, sheep and goats on 119 farms (PA Basson, unpublished data). Preliminary studies were conducted to determine the cause of the outbreak, and *Enterococcus casseliflavus* was suspected to be the causative agent. However, experimental infections with this pathogen did not result in the observed syndrome (PA Basson, unpublished data). It thus left the outbreak's actual cause undetermined, and tests for other potential causes of abortion did not yield consistent results. However, *Neospora caninum* and *Toxoplasma gondii* were not among the pathogens investigated.

From 2016 to 2018, many farms in Namibia's central areas experienced another outbreak of abortions and orchitis in cattle, sheep, and goats (Samkange et al., 2022). Several infectious diseases were suspected, but none was confirmed as the primary cause. Amongst those tested for were chlamydiosis, bovine herpes virus, bovine viral diarrhoea, bluetongue, Rift Valley fever, leptospirosis, brucellosis, trichomoniasis, vibriosis and Q-fever (Samkange et al., 2022).

However, other possible causes of abortions, including *Neospora caninum* or *Toxoplasma gondii*, were not tested for.

Neospora caninum is an intracellular coccidian parasite that is the cause of bovine neosporosis, which is a significant cause of abortions worldwide (Reichel et al., 2020). The disease is characterised by sporadic or epidemic abortions (Parkinson et al., 2019), neonatal deaths, stillbirths and neuromuscular disease in calves (Dubey et al., 1992; Regidor-Cerrillo et al., 2020). The resulting production losses, including abortions, are responsible for global losses worth hundreds of millions of US dollars annually (Reichel et al., 2013).

Toxoplasmosis is a zoonosis caused by the protozoan parasite *Toxoplasma gondii*. The disease occurs worldwide, and in small ruminants (sheep and goats), it commonly causes abortions, foetal mummification, stillbirths, or births of weak offspring, which can have profound economic implications (Lopes et al., 2014; Khan & Noordin, 2020).

Despite their global recognition as important causes of abortion and associated production losses in the livestock sector, there is a paucity of information on the epidemiology of these pathogens in Namibia. While evidence from human serosurveys indicates the circulation of *Toxoplasma gondii* in Namibia (Joubert and Evans, 1997; Colf et al., 2020) and serosurveys in wildlife suggest the presence of *Neospora caninum* (Seltmann et al., 2020), studies examining these parasites in livestock are notably absent.

1.2. Problem statement

Neospora caninum is regarded as one of the most important causes of abortions in cattle worldwide, while *T. gondii* is significant in sheep and goats for the same reason. Therefore, these parasites are significant economically, especially in a major beef-producing country like Namibia. Despite the importance of *N. caninum* and *T. gondii* globally as major causes of abortions, there is no data on Namibian livestock.

Despite experiencing major abortion outbreaks resulting in substantial economic losses, with no confirmed primary cause identified (Samkange et al., 2022), there has been a glaring lack of investigation into the epidemiology of these two pathogens. Neosporosis and toxoplasmosis are wholly neglected or overlooked in Namibia to the extent that they have never been diagnosed in livestock in Namibia. As a result, some authors have labelled toxoplasmosis as the most neglected disease in sub-Saharan Africa (Tegegne et al., 2016). Consequently, no one knows whether or not *N. caninum* and *T. gondii* are present in livestock in Namibia. This scenario has resulted in a situation where the country's veterinary fraternity seems oblivious to the importance of *N. caninum* and *T. gondii*, resulting in a farming community generally ignorant of these important diseases. The upshot of this knowledge gap is that control efforts of abortions in Namibia are hindered due to a lack of scientific information on *N. caninum* and *T. gondii*.

1.3. Research questions

This study aimed to answer the following research questions:

- 1) What are the knowledge, attitudes and practices (KAP) of livestock farmers in Namibia's Khomas region and the animal health practitioners (AHP) in the country concerning *N. caninum* and *T. gondii*?
- 2) What is the seroprevalence and associated risk factors of *N. caninum* in cattle in Namibia's Khomas region, if it is present?
- 3) What is the seroprevalence and associated risk factors of *T. gondii* in sheep and goats in Namibia's Khomas region, if it is present?
- 4) Is the *N. caninum* deoxyribonucleic acid (DNA) detectable in cattle, and is *T. gondii* DNA detectable in sheep and goats, in abattoir samples in the Khomas region of Namibia?

1.4. Hypotheses

In light of the above-mentioned research questions, the following list of hypotheses was formulated to answer those questions:

- 1) H_0 : There is no significant knowledge, attitudes, or practices among livestock owners and animal health practitioners concerning *N. caninum* and *T. gondii* in Namibia.

- H₁: There is significant knowledge, attitudes and practices among livestock owners and animal health practitioners concerning *N. caninum* and *T. gondii* in Namibia.
- 2) Null (H₀) hypothesis: *N. caninum* does not occur in cattle in the Khomas region of Namibia.
- H₁ hypothesis: *N. caninum* occurs in cattle in the Khomas region of Namibia.
- 3) Null (H₀) hypothesis: *T. gondii* does not occur in sheep or goats in the Khomas region of Namibia
- H₁ hypothesis: *T. gondii* occurs in sheep and goats in the Khomas region of Namibia
- 4) H₀: There are no significant risk factors associated with *N. caninum* and *T. gondii* occurrence in the Khomas region
- H₁: There are significant risk factors associated with *N. caninum* and *T. gondii* occurrence in the Khomas region
- 5) H₀: There is no significant presence of *N. caninum* DNA in cattle abattoir samples in the Khomas region
- H₁: There is a significant presence of *N. caninum* DNA in cattle abattoir samples in the Khomas region
- 6) H₀: There is no significant presence of *T. gondii* DNA in sheep and goat abattoir samples in the Khomas region
- H₁: There is no significant presence of *T. gondii* DNA in sheep and goat abattoir samples in the Khomas region

1.5. Objectives

The objectives of this study were as follows:

- 1) To assess the knowledge, attitudes and practices (KAP) of livestock farmers in the Khomas region and the animal health practitioners (AHP) in Namibia concerning *N. caninum* and *T. gondii*.
- 2) To determine the seroprevalence of *N. caninum* and the associated risk factors in cattle in the Khomas region of Namibia.
- 3) To determine the seroprevalence of *T. gondii* and the associated risk factors in sheep and goats in the Khomas region of Namibia.

- 4) To determine the presence of *N. caninum* DNA in cattle and *T. gondii* DNA in sheep and goat abattoir samples.

1.6. Application of the results

Unravelling the KAP of farmers and AHPs and determining the serological and molecular presence of these two pathogens in animals will contribute towards a better understanding of their epidemiology in Namibia. Such knowledge will provide much-needed scientific information in coming up with the most cost-effective options to control *N. caninum* and *T. gondii*, including the necessary change of the farmers' attitudes and perceptions. Better control measures, attitudes and perceptions employed by livestock producers would reduce reproductive losses attributable to *N. caninum* and *T. gondii* and the related economic losses. This scenario would increase Namibia's livestock productivity, thereby reducing poverty. Furthermore, if the study detects the presence of *T. gondii* in sheep and goat meat, appropriate recommendations will be made regarding measures to mitigate human infections.

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Chapter 2: Review of Literature

2.1. Introduction

Neospora caninum and *Toxoplasma gondii* are closely related coccidian parasites (Duarte et al., 2020; Dubey et al., 2007b), which occur worldwide and are important causes of livestock production losses through abortions. *Neospora caninum* is one of the major causes of abortions in cattle, causing losses worth billions of dollars a year (Reichel et al., 2020), whilst losses due to *T. gondii* infections are estimated to cost the small ruminant industry millions of dollars annually (Stelzer et al., 2019).

Toxoplasma gondii and *N. caninum*, both intracellular protozoan parasites, share similarities but also exhibit distinct differences in host range, pathogenesis, and epidemiology (Della Rosa et al., 2021; Gos et al., 2017; Howe & Sibley, 1999; Sokol-Borrelli et al., 2020). *Toxoplasma gondii* has a broader host range, including humans, while *N. caninum* primarily causes bovine neosporosis, leading to abortion (Howe & Sibley, 1999). Studies highlight *N. caninum* as a more frequent cause of ovine reproductive failure than *T. gondii* (Della Rosa et al., 2021) and suggest *T. gondii*'s temperature sensitivity allows it to infect avian hosts, unlike *N. caninum* (Rezende-Gondim et al., 2017). Antigenic diversity and species-specific responses during life cycle transitions further distinguish them (Howe & Sibley, 1999; Sokol-Borrelli et al., 2020).

Genetic tools for *N. caninum* research have advanced, enabling functional assessment comparable to *T. gondii* (Mineo et al., 2022). Seroprevalence data show varying impacts on local livestock populations (Amdouni et al., 2021; Gos et al., 2017). Immune response studies indicate divergent host manipulation and survival strategies between the two parasites (Coombs et al., 2020). Understanding each parasite within its ecological niche is crucial, whether through molecular identification techniques or serologic assays, with practical implications for animal husbandry practices (De Freitas et al., 2023; Novoa et al., 2023).

2.2. *Neospora caninum*

Neospora caninum is an obligate intracellular, tissue cyst-forming, coccidian protozoan parasite of the phylum Apicomplexa and family Sarcocystidae, which occurs worldwide (Al-Qassab et al., 2010; Dubey et al., 1988; Goodswen et al., 2013). Due to some morphological similarities and immunological and histochemical cross-reactions, *N. caninum* was previously misdiagnosed as *T. gondii* until 1988 (Dubey et al., 2002, 1988; Ellis et al., 1994). Neosporosis is recognised as a major cause of reproductive disease in livestock, particularly cattle. However, *N. caninum* also infects many other domestic animals like dogs, sheep, goats, camelids, water buffalo, horses, and chickens; wildlife and captive animals like deer, rhinoceros, rodents, rabbits, coyotes, wolves, and foxes are also affected (McAllister, 2022).

2.2.1. Life cycle and transmission

Neospora caninum has a heteroxenous life cycle involving definitive (final) and intermediate hosts (Figure 2.1). Canids are the final hosts, and these include coyotes (*Canis latrans*) (Luis F P Gondim et al., 2004), dogs (*Canis lupus familiaris*), the grey wolf (*Canis lupus*), dingoes (*Canis lupus dingo*) (Goodswen et al., 2013; McAllister et al., 1998), but not foxes (Nishikawa, 2017). Dogs are both intermediate and definitive hosts of *N. caninum* (Barimani et al., 2023; Dubey et al., 2019; Nishikawa, 2017), which might also be true for other canids, and cattle are the main intermediate hosts (Almería, 2013; Dubey et al., 2007b). *Neospora caninum* has three infectious stages: tachyzoites and tissue cysts (containing bradyzoites) that are chiefly found in tissues of intermediate hosts, and sporozoites in sporulated oocysts that are only shed by canids, which are the final hosts (Dubey et al., 2007b). Tissue cysts are mainly found in neural tissue cells but can also be found in other cells, including muscles, macrophages, fibroblasts, vascular endothelial cells, hepatocytes and renal tubular cells (Al-Qassab et al., 2010; Almería, 2013; Dubey et al., 2007b).

Neospora caninum is acquired by canids, the final hosts, when they consume infected tissues of intermediate hosts. Moreover, an infected bitch can transmit the parasite to its puppies through vertical transmission (Dubey et al., 2019). The prevailing belief is that sexual reproduction of the parasites occurs in the gastrointestinal tract (GIT) of canids, but some authors dispute this claim,

stating that it has not been definitively proven (Donahoe et al., 2015; McAllister, 2022). In the canid's intestine, oocysts release sporozoites that invade the epithelial cells, differentiate into tachyzoites, and rapidly multiply to cause enteritis, diarrhoea, and even death (Kul et al., 2015). In addition, gametogony occurs in canids, the definitive hosts, producing unsporulated oocysts. These oocysts are subsequently excreted into the environment through faeces. However, the precise mechanisms of sexual reproduction of *N. caninum* in the definitive host are not fully understood (Al-Qassab et al., 2010).

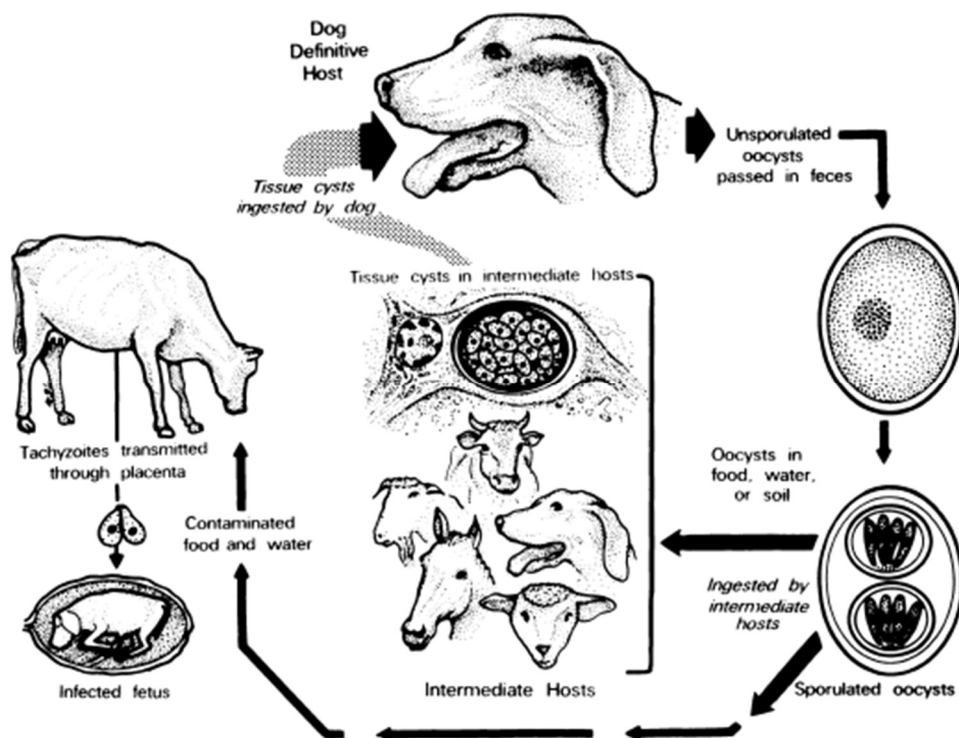


Figure 2.1: Life cycle of *Neospora caninum* (Dubey, 1999); used with permission

Oocysts can withstand adverse environmental factors and, given favourable conditions, undergo sporulation within one to three days (Almería, 2013; Donahoe et al., 2015). The intermediate hosts (domestic and wild herbivores) are infected by ingesting food and water contaminated by sporulated oocysts from the faeces of the final hosts (Dubey et al., 2007b; McAllister, 2022). In addition, neonatal calves are at risk of infection if they consume milk containing tachyzoites (Almería, 2013). However, the most important mode of transmission of *N. caninum* in cattle is

transplacental, to the foetus (Almería, 2013). Besides being the most important transmission route, transplacental transmission is very efficient (as high as 75-100%) (Goodswen et al., 2013; Reichel et al., 2013). *Neospora caninum* has also been demonstrated in frozen bull semen (Doosti et al., 2015), and experimental transmission using infected semen has been demonstrated in heifers (Serrano et al., 2006). This, therefore, makes venereal transmission of *N. caninum* a genuine possibility. However, other authors concur with this observation but still maintain that it is unlikely (Dubey et al., 2007b).

Within the GIT of an intermediate host, the sporozoites are released by the excystation of oocysts, and they invade the intestinal epithelium, where they transform into tachyzoites (Donahoe et al., 2015). The tachyzoites are then disseminated to virtually all host tissues, including the central nervous system (CNS), muscles, and placenta. They undergo repetitive cycles of intracellular multiplication, leading to the lysis of the infected host cell. Tachyzoites are released and infect surrounding cells, repeating this process for up to 20 cycles (Donahoe et al., 2015). Finally, the tachyzoites transform into bradyzoites due to the dominance of a robust, cell-mediated immune response by the host, which is primarily mediated by gamma interferon (Al-Qassab et al., 2010). These bradyzoites proliferate more slowly (by endodyogeny) and develop within tissue cysts, primarily in nervous tissue and skeletal muscle (Fereig & Nishikawa, 2020; Marugan-Hernandez, 2017).

Studies have shown that protective immunity in chronically infected cows does not prevent reactivation and vertical transmission during pregnancy (Williams et al., 2003) and that pregnancy-induced changes might trigger recrudescence without overt immunosuppression, leading to transplacental infection (Guy et al., 2001). Recrudescence involves the reactivation of bradyzoites, which then convert into tachyzoites and spread to the foetus via transplacental transmission (Donahoe et al., 2015; McAllister, 2022). After a cow becomes infected, it will remain persistently infected for life, and in 95% of cases, the infection will be transmitted to subsequent generations of calves (Donahoe et al., 2015; McAllister, 2022). The cycle is completed when the final hosts, typically canids, consume infected tissues of intermediate hosts.

2.2.2. Genetic diversity of isolates

Neospora caninum isolates from different geographical regions have shown marked differences in virulence and growth rates, which could impact their pathogenesis (Al-Qassab et al., 2009). Several studies have investigated the genetic diversity and pathogenicity of *N. caninum* isolates from various hosts and locations. Genetic studies have identified distinct lineages or subpopulations within *N. caninum* based on sequence analysis (Calarco & Ellis, 2020; Marková et al., 2019; Miller et al., 2002; Villa et al., 2021). These lineages are often associated with specific geographical regions or host species (Miller et al., 2002; Villa et al., 2021), suggesting a potential link between genotype and virulence. For example, a study in Italy found that *N. caninum* isolates from different regions clustered into two major clonal subpopulations (Villa et al., 2021). Another study in Sweden identified genetic variation among *N. caninum* isolates from moose and roe deer, with some lineages showing higher prevalence and potential virulence (Malmsten et al., 2011). In addition to genetic studies, experimental infection studies have provided further evidence of differences in virulence among *N. caninum* isolates. One study which compared the pathogenicity of different *N. caninum* strains in experimentally infected cows and buffaloes showed that certain strains caused more severe clinical signs and histological lesions than others (Chrysafidis et al., 2014).

Researchers have used a variety of genomic targets and multiple investigative approaches to study the differences among *N. caninum* isolates. However, the Random Amplified Polymorphic DNA–PCR (RAPD-PCR) technique is no longer used due to its limitations in determining *N. caninum* genetic diversity (Al-Qassab et al., 2010; Regidor-Cerrillo et al., 2006). Minor nucleotide sequence differences were found in the internal transcribed spacer sequences (*ITS1*) and PCR amplification of the Nc5 repeat sequence, but they were insufficiently polymorphic to differentiate between *N. caninum* isolates (Al-Qassab et al., 2010; Regidor-Cerrillo et al., 2006). The limited amount of nucleotide changes during *N. caninum*'s evolution from its recent ancestor could be the reason for the absence of significant nucleotide differences in the 18S-like ribosomal DNA (small subunit-rDNA) sequence among tested isolates (Al-Qassab et al., 2010; Regidor-Cerrillo et al., 2006).

Microsatellite markers (MS) have become prevalent in studying genetic diversity in *N. caninum* due to the absence of identified single nucleotide and insertion-deletion polymorphisms that are suitable for genotyping (Regidor-Cerrillo et al., 2020). They are characterised by short, tandem repeats consisting of 2 to 6 bases in length, whereas minisatellites are defined by longer repeat units ranging from 8 to 100 bases (Al-Qassab et al., 2009). The microsatellite repeat length variation within loci shows high levels of polymorphism in protozoan parasites, making these markers extremely informative for genetic studies within species (Regidor-Cerrillo et al., 2006). Consequently, microsatellites have found extensive usage in high-resolution genotyping of other apicomplexan species such as *Cryptosporidium parvum*, *C. hominis*, *T. gondii*, *Plasmodium falciparum* and *Theileria parva* (Regidor-Cerrillo et al., 2006). Using these microsatellite markers for genotyping *N. caninum* has revealed that the population is somewhat divided based on geographic location, with distinct populations identified in various countries across South America and Europe demonstrating significant genetic variability worldwide (Regidor-Cerrillo et al., 2020).

In one study, an assessment of the genetic diversity of 108 *N. caninum* isolates from different regions of the world with MS markers which utilised seven loci (MS4, MS5, MS6A, MS7, MS8, MS10, and MS12), revealed a significant level of genetic diversity (Regidor-Cerrillo et al., 2013). Another study of goats in China, which used the same seven genetic markers (MS4, MS5, MS6A, MS7, MS8, MS10, and MS12), identified two sequences at the *ITS1* region, three genotypes at the MS5 microsatellite locus, and one genotype at the MS8 locus (Qian et al., 2020).

Medina-Esparza et al. (2016) analysed *ITS1* PCR-positive brain samples from aborted foetuses in a Mexican study and performed *N. caninum* multilocus genotyping (MLG) using nine microsatellite markers (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21). The study found that all the genotypes identified were distinct from those found in other *N. caninum* populations across the globe. In addition, multilocus genotypes with varied characteristics were observed in every herd studied. The authors then concluded that their results suggested that certain multilocus genotypes might be preserved within the herd, possibly through vertical transmission of *N. caninum* in consecutive pregnancies (Medina-Esparza et al., 2016).

2.2.3. Host range

Neospora caninum primarily infects dogs and cattle but is also infective to all major domestic livestock species, companion animals, wildlife (Donahoe et al., 2015; McAllister, 2022) and captive animals, including deer, rhinoceros, rodents, rabbits, coyotes, wolves, foxes (McAllister, 2022). Neosporosis was also diagnosed in a captive Malayan tapir (*Tapirus indicus*) in Germany (Peters et al., 2017). The parasite was recently confirmed in pigs for the first time in China using a competitive-inhibition enzyme-linked immunoassay assay (cELISA) and PCR (Gui et al., 2020). Serological evidence of infection has been reported in various other species, and these include feral rats in India (*Rattus rattus*) (Dhandapani et al., 2017), non-human primates, including mandrills (*Mandrillus sphinx*), chimpanzees (*Pan troglodytes*), sun-tailed monkeys (*Cercopithecus solatus*) as well as the western gorillas (*Gorilla gorilla*) (Akue et al., 2018). However, no parasite material was detected. *Neospora caninum* has also been detected in chicken (*Gallus domesticus*) brains (Costa et al., 2008; Romero et al., 2016) and chicken meat but not in eggs (Barimani et al., 2023), sparrows (*Passer domesticus*) (Gondim et al., 2010), red foxes (*Vulpes vulpes*), and white-tailed deer (*Odocoileus virginianus*) by using a combination of serology and molecular techniques (Goodswen et al., 2013). Whilst the presence of *N. caninum* DNA in chicken brains has been reported as evidence of infection in this species (Costa et al., 2008; Romero et al., 2016), efforts to experimentally infect chickens did not yield positive results (Oliveira et al., 2018).

A review of *N. caninum* seroprevalence rates in various animal species in Iran between 2004 and 2020 revealed that the rates varied significantly among different species, with cattle exhibiting the highest infection rates, ranging from 3.8% to 76.2%, while dogs showed rates ranging from 0% to 54.6% (Gharekhani et al., 2020). Other species, such as buffaloes, horses, sheep, and cats, also exhibited moderate seroprevalence levels, with much lower rates in rodents, pigeons, and sparrows. The prevalence in hooded crows was 9.9% (Gharekhani et al., 2020). When all the species in which this parasite has been reported are considered, *N. caninum* is likely infective to all endothermic vertebrates (Goodswen et al., 2013).

2.2.4. *Neospora caninum* infections

Neosporosis is regarded as one of the most important infectious causes of endemic and epidemic abortions in cattle worldwide (Dubey & Schares, 2006; Monney & Hemphill, 2014; Reichel et al., 2013) and, to a lesser extent, sheep, goats, water buffalo, and camelids (McAllister, 2022). Cattle are the intermediate hosts most susceptible to abortion caused by *N. caninum* (Barimani et al., 2023). Infection in beef and dairy cattle causes stillbirths, foetal resorption or autolysis, abortions, or the birth of weak calves, with abortion as the chief clinical presentation (Fereig & Nishikawa, 2020). Calves born from infected cows can exhibit either acute or subclinical signs. These calves are persistently infected and capable of vertical transmission of the pathogen to their progeny (Fereig & Nishikawa, 2020; Monney et al., 2011). It is suspected that the persistently infected calves born after an epidemic are the reason why affected herds tend to end up with endemic abortions (Dubey et al., 2007). Neonatal calves born from persistently infected dams may have congenital abnormalities, nervous symptoms, low birth weight, and asthenia (Fereig & Nishikawa, 2020). These abortions commonly occur between five and six months of gestation, although they may also occur from three months (Fereig & Nishikawa, 2020). In some geographic regions, up to 42.5% of abortions have been attributed to *N. caninum* (Nematollahi et al., 2013).

Individual animal prevalence rates ranging from 2.5% to 18.9% and herd-level prevalence rates of up to 29.4% in beef cattle and 44.4% in dairy cattle have been recorded using enzyme-linked immunosorbent assays (Fereig et al., 2016; Nasir et al., 2012). In the KwaZulu Natal province of South Africa, prevalence rates of 26.82 % and 5.17% in dairy and beef cows, respectively, have been reported (Chisi et al., 2013). In another study in Henan Province of central China, overall seroprevalence rates of 49.3% and 29.3% were detected in aborting and non-aborting dairy cattle, respectively (Qian et al., 2017).

In sheep and goats, neosporosis can cause abortion, neonatal deaths, and congenital abnormalities (Fereig & Nishikawa, 2020). In addition, adult sheep may exhibit non-specific clinical signs (Fereig & Nishikawa, 2020). Transplacental transmission in sheep has also been suggested as a possibility (Fereig & Nishikawa, 2020). Within ovine and caprine populations, seroprevalence rates at the animal level, as documented in Europe and South America, demonstrate a considerable range spanning from 0.8% to 60.6% (Basso et al., 2022; Maia et al., 2021).

In dogs, *N. caninum* causes neuromuscular disease (Donahoe et al., 2015; Monney et al., 2011) and cutaneous neosporosis in immunocompromised animals (Boyd et al., 2005; Legnani et al., 2016). However, clinical signs are more common in congenitally infected puppies, including hindlimb paresis or paralysis, progressive myositis, dysphagia, jaw paralysis, skin necrosis, and muscle flaccidity (Fereig & Nishikawa, 2020; McAllister, 2022).

2.2.5. Risk factors

Since dogs are the definitive hosts (Goodswen et al., 2013; McAllister et al., 1998), the presence of farm dogs increases the risk of *N. caninum* infection in cattle (Dubey & Schares, 2011) through faecal contamination of pastures and open water sources with oocysts. In addition, a study in Canada demonstrated a higher risk of infection in properties where dogs had access to placentas and fetuses compared to properties where dogs had no access (Llano et al., 2018).

Other risk factors associated with bovine neosporosis which have so far been identified include the number of farm dogs, self-rearing of replacement heifers, stocking density and farm size, stage of pregnancy, age of cattle, breed, herd size (Bartels et al., 2006; Dubey et al., 2007b; Ghalmi et al., 2012; Otranto et al., 2003; Pare et al., 1998), production system (Moore et al., 2009), antibodies against other pathogens like bovine viral diarrhoea (Björkman et al., 2000), feeding of milk or colostrum (Davison et al., 2001), human population density (Schares et al., 2004) and the region within countries (Bartels et al., 2006).

Vertical transmission of *N. caninum* is highly effective (Regidor-Cerrillo et al., 2020), resulting in the persistent prevalence of the parasite in herds over time (De Aquino Diniz et al., 2019). A study in Colombia reported that properties that purchased replacement heifers had a higher prevalence of *N. caninum* and were at a 54% greater risk of acquiring the infection compared to properties where replacements were sourced from the same farm (Llano et al., 2018). Therefore, rearing replacement heifers on the farm is recommended to avoid introducing new infections from external sources. However, if the recipient herd already has a higher seroprevalence of *N. caninum* compared to the population from which replacement heifers are obtained, purchasing new heifers may reduce infection in the herd. This is supported by a study that found rearing own replacement

heifers to be a potential risk factor for a high seroprevalence of *N. caninum* in beef cattle calves (Barling et al., 2001; Dubey et al., 2007b)

Colostrum feeding is also a risk factor for *N. caninum* infection. In one study, calves aged up to one week were successfully infected with *N. caninum* tachyzoites fed orally in colostrum or milk replacers, but cross-suckling from seropositive cows to seronegative calves did not result in infection (Davison et al., 2001). Furthermore, *N. caninum* DNA has been demonstrated in colostrum, suggesting that the potential risk of transmitting *N. caninum* to neonatal calves by feeding colostrum exists (Moskwa et al., 2007).

Infections with other pathogens, such as bovine viral diarrhoea virus (BVDV), could be a risk factor, considering that this virus is widespread in both domestic and wildlife ruminants in Namibia (Depner et al., 1991). In Laos, exposure to BVDV was associated with rising antibody titres against *N. caninum* and *Leptospira interrogans* serovar Hardjo (Olmo et al., 2019). A similar association between BVDV and *N. caninum* was also found in Sweden (Björkman et al., 2000). In addition, a study in Colombia demonstrated that animals that tested positive for *N. caninum* antibodies were five times more likely to experience abortion than animals that tested negative (Llano et al., 2018). The intensity and the duration of the increase in anti-*N. caninum*-specific antibodies during gestation could be related to the risk of foetal infection in the case of existing latent infection in pregnant animals (Guy et al., 2001; Jensen et al., 1999; Stenlund et al., 1999).

High stocking density of beef cattle, particularly in ranches practising supplemental feeding, has been identified as a risk factor for *N. caninum* infection in Texas and the northwestern United States (Barling et al., 2001). The authors hypothesised that ranches employing hay rings to feed round bale hay might elevate calving density near feeders that are infrequently relocated, potentially heightening the exposure of beef cattle to *N. caninum* (Barling et al., 2001). This is because cattle might give birth, abort, or lose placentas in areas containing bedding and hay, which could attract definitive hosts capable of defecating in the hay, thereby amplifying the risk (Barling et al., 2001). Furthermore, a study in Brazil found that the size of farmland was inversely related to the seroprevalence of *N. caninum*, potentially due to farm dogs having greater access to infected tissue in smaller farms (Dubey et al., 2007b).

A recent review reported a positive correlation between age and seropositivity in cattle (Wei et al., 2022). The number of pregnancies and years of milk production were identified as associated risk factors for neosporosis, suggesting that age and length of milk production are risk factors for neosporosis in dairy cattle (Klauck et al., 2016). In Argentina, it was reported that the likelihood of seropositivity increased by 3.5% with each additional year of age in cattle and buffaloes (Moore et al., 2014), whilst in Colombia, cattle aged three years and older had a 70% higher chance of having had exposure to sporulated oocysts of *N. caninum* compared to younger animals (Llano et al., 2018). Increasing age elevates the risk of horizontal disease transmission because of a higher probability of exposure to sporulated oocysts from the environment.

Several studies have identified the history of abortion as a risk factor for *N. caninum* infection in cattle (Dubey et al., 2007b; Klauck et al., 2016; Moore et al., 2009; Snak et al., 2018). Cattle with a history of abortion were found more likely to be seropositive to *N. caninum* (Moore et al., 2009; Wei et al., 2022; Ying et al., 2022).

Breed is also another risk factor associated with neosporosis. Studies have shown that beef cattle tend to have lower seroprevalences than dairy cattle (Reichel et al., 2020). In Argentina, dairy herds were three times more likely to be seropositive to *N. caninum* infection than beef herds (Moore et al., 2009). A study in Colombia found that purebred dairy cattle were three times more likely to acquire infection than crossbred animals (Llano et al., 2018). However, in a recent study on indigenous Atlas Brown cattle in Northeast Algeria, no association was found between the high *N. caninum* seropositivity (36.2%) and abortion (Abdeltif et al., 2022). Therefore, it is essential to be cautious when interpreting these findings because the differences in production systems employed for various breeds may have been the cause of the observed variations rather than breed-related differences in susceptibility to infection (Dubey et al., 2007b). Among the different cattle species, yaks were found to have the highest infection rate, and yellow cattle had the lowest (Wei et al., 2022).

Female cattle have been reported to be more susceptible to neosporosis than male cattle, which was attributed to females being immunosuppressed during pregnancy, increasing their susceptibility (Wei et al., 2022).

2.2.6. Economic impact

Economic losses due to neosporosis are mainly attributable to abortion losses, costs associated with seeking veterinary assistance, diagnostic procedures, rebreeding, prolonged calving interval, additional artificial insemination, loss of milk yield, and replacing culled aborted cows (Demir et al., 2020; Dubey & Schares, 2011). In a study involving ten countries (Argentina, Australia, Brazil, Canada, Mexico, Netherlands, New Zealand, Spain, United Kingdom, and the USA), *N. caninum* infection/abortion was estimated to cost the dairy and beef industry a total of US\$842.9 million and US\$455.4 million, respectively (Reichel et al., 2013). In Turkey, the total cost of *N. caninum* infection per dairy cow was estimated to be US\$710, and the annual economic costs were estimated at US\$40.5 million (Demir et al., 2020). Globally, the total losses are estimated at US\$1.298 billion (Ying et al., 2022).

2.2.7. Zoonotic implications

Serological evidence of human exposure to *N. caninum* has been demonstrated in the USA (Tranas et al., 1999) and mainly in human immunodeficiency virus (HIV) patients in Brazil (Oshiro et al., 2015). Furthermore, human patients who were HIV-positive and those with neurological disorders have been found to have a higher prevalence of *N. caninum* antibodies than newborns and healthy individuals (Lobato et al., 2006). *Neospora caninum* genetic material and IgG antibodies to *N. caninum* have been detected in human fetuses' umbilical blood, although the parasite was not isolated (Duarte et al., 2020). In addition, *N. caninum*-IgM and *T. gondii*-IgM antibodies were also detected in women who had spontaneously aborted in Jordan, suggesting active co-exposure to both parasites (Abo-Shehadeh et al., 2021). *In-vitro* studies have shown the infection of human trophoblastic (BeWo) and uterine cervical (HeLa) cell lines. Both cell lines were found to be susceptible to *N. caninum* infection, with HeLa cells being more vulnerable (Carvalho et al., 2010). However, studies in England (McCann et al., 2008) and Northern Ireland (Graham et al., 1999) failed to demonstrate any serological evidence of *N. caninum* exposure in humans, including 76 women with a history of repeated abortions (Petersen et al., 1999). In Spain, 600 clinical samples from human patients displaying signs of toxoplasmosis but with negative *T. gondii* PCR results

were tested, and no evidence of *N. caninum* DNA was found, and the authors concluded that *N. caninum* was unlikely to be an opportunistic zoonotic agent (Calero-Bernal et al., 2019).

Therefore, thus far, the zoonotic potential of *N. caninum* cannot be ruled out, such as its risk to pregnant women (Duarte et al., 2020). In addition, other authors have concluded that *N. caninum* may have the potential to infect immunocompromised individuals, especially in the presence of the human immunodeficiency virus, and that infections may be linked to neurological conditions in man (Barratt et al., 2010). However, no irrefutable evidence exists that *N. caninum* can successfully infect humans because the parasite has not been confirmed in human tissues (Duarte et al., 2020; Dubey et al., 2007b).

2.2.8. Diagnosis

Available diagnostic tests for neosporosis include microscopy, serology and molecular tests. Several serologic tests have been developed over time, including indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assays (ELISAs), agglutination tests, immunoblotting techniques, and more recently, iscom ELISAs utilizing immunostimulating complex preparations (Björkman & Ugglä, 1999). Each assay has its strengths and weaknesses concerning sensitivity (Se) and specificity (Sp), which are paramount when considering their application in different epidemiological contexts.

ELISA-based diagnostics dominate current practices due to their adaptability for high-throughput screening (Guido et al., 2016). A comprehensive comparison by Alvarez-García et al. evaluated ten commercially available ELISAs, revealing varying degrees of Se and Sp, with some requiring re-standardization based on threshold gradient receiver operating characteristic (TG-ROC) analyses (Alvarez-García et al., 2013). Notably, HIPRA-CIVTEST, IDVET, BIOVET, and IDEXX Rum exhibited Se and Sp greater than 95%, indicating robust performance characteristics post-adjustment.

Advancements in molecular biology have led to the identification of novel antigens, such as NcSRS2 and NcSAG1, among others, that have been incorporated into newer diagnostic platforms promising enhanced accuracy (Sinnott et al., 2017). Abdelbaky et al.'s study provided insight into antigen-specific responses correlating with abortion events - a primary concern in

bovine neosporosis - suggesting that NcSAG1 and NcGRA7 could serve as potential markers indicative of abortion risk when assessed via IgG-iELISA (Abdelbaky et al., 2020).

Despite these advancements in antigen discovery and assay development, challenges persist. For instance, there remains variability between tests, leading to discrepancies; Björkman and Ugglä highlighted limitations regarding cross-reactivity amongst various host species, which complicates inter-test comparisons (Björkman & Ugglä, 1999). Cross-reactions between *N. caninum* and *T. gondii* have also been described (Gondim et al., 2017).

The histopathological diagnosis of *N. caninum* infection is an intricate process that relies on identifying characteristic tissue lesions and the presence of the parasite itself within affected tissues (Anderson et al., 2000; Duarte et al., 2020). Histopathology remains a cornerstone in diagnosing neosporosis due to its ability to provide direct visual confirmation of parasitic involvement in tissue damage.

Neospora caninum induces lesions predominantly in neural and muscular tissues, such as encephalitis, myocarditis, myositis, hepatitis, and placentitis (Anderson et al., 2000). In aborted fetuses or animals with neuromuscular symptoms suggestive of neosporosis, these lesions are often multifocal and characterized by mononuclear cell infiltrates comprising lymphocytes, macrophages, and plasma cells with associated necrosis (Da Costa et al., 2022), indicating a chronic inflammatory response against persistent infection.

Intracellular tachyzoites may be observed within these lesions using special stains like immunohistochemistry (IHC), where antibodies specific to *N. caninum* antigens are employed (Anderson et al., 2000). This technique enhances diagnostic specificity by distinguishing *N. caninum* from other morphologically similar protozoan parasites, such as *T. gondii* (Lakhdhir et al., 2020).

The quality and interpretability of histopathological findings depend heavily on sample integrity; hence, fixation artefacts or autolysis may impede accurate diagnosis. Moreover, while IHC is highly specific when positive, it requires well-preserved tissues, which are not always available, especially in field conditions where abortions might not be promptly attended.

Molecular techniques like polymerase chain reaction (PCR) have been used alongside histopathology to confirm *Neospora* DNA within affected tissues (Perotta et al., 2021), thereby increasing diagnostic confidence, particularly when typical intracellular structures cannot be definitively identified microscopically or when IHC results are equivocal. PCR can be used in aborted fetuses and has the highest sensitivity and specificity (Dubey & Schares, 2011; Kierbić et al., 2019).

The *NC-5* gene is highly sensitive and specific for detecting *N. caninum* (Kaufmann et al., 1996; Yamage et al., 1996). It has been used to detect *N. caninum* DNA using nested PCR, which targeted the *Nc-5* gene (Arbabi et al., 2016) in bovine blood (Okeoma et al., 2004), sheep brain, Mongolian gerbils (*Meriones unguatus*) and vesper mice (*Calomys callosus*) brains (Pena et al., 2007). A PCR targeting the *ITS1* sequence has been used in several studies for detecting *N. caninum* DNA in the neck muscles of cattle and sheep (Amdouni et al., 2018), in dog faeces (Li et al., 2014), abortion investigations (Bartley et al., 2019), detecting *N. caninum* DNA in wild carnivore tissues (Bartley et al., 2013), and for molecular characterisation of different isolates (Luis F.P. Gondim et al., 2004; Stenlund et al., 1997).

2.2.9. Treatment and control

Despite several *in-vivo* and *in-vitro* studies (Sánchez-Sánchez et al., 2018) having been conducted to test various treatments, there is currently no effective treatment nor vaccine against neosporosis (Novoa et al., 2020; Semango et al., 2019). Administering sulphadiazine and trimethoprim to the cattle and toltrazuril to newborn calves and dogs, together with periodic treatment of the environment with a phenolic disinfectant, reduced abortion (from 188 to 9) and *N. caninum* seroprevalence (from 68.7% to 0%) after one year of treatment (Cuteri et al., 2005).

Other remedies, including curcumin, artemether, atrazine and ponazuril, showed some *in-vitro* activity against *N. caninum*, while glyphosate, cyclophosphamide, diminazene aceturate and praziquantel were found to be ineffective (Qian et al., 2015). In addition, naphthoquinone buparvaquone showed some efficacy in reducing, and in some cases, even eliminating, fetal infection during pregnancy (Müller et al., 2015). The Thai piperaceae (*Piper betle*) plant extract

increased the clinical scores and survival rates of mice experimentally infected with *N. caninum* (Leesombun et al., 2017).

Although vaccination is considered the ideal strategy to control neosporosis (Monney & Hemphill, 2014), there is currently no effective commercial vaccine against the disease or an effective treatment (Goodswen et al., 2013; Monney et al., 2011). A killed *N. caninum* tachyzoite commercial vaccine, Bovilis NeoGuard™, was eventually withdrawn from the market due to its low efficacy and potential side effects, which resulted in productivity losses (Monney & Hemphill, 2014; Weston et al., 2012).

Several other control measures have been recommended, which include quarantine and testing of replacement and purchased cattle, prevention of transmission from dogs and other potential definitive hosts, prevention of waterborne transmission, rodent control, prevention of putative factors for disease recrudescence in congenitally infected cattle, embryo transfer of embryos from infected dams into uninfected recipients, artificial insemination of seropositive dairy cows with semen from beef bulls (these are more resistant), testing and culling of positive animals (Dubey et al., 2007b). It has been established that it is impossible to eradicate *N. caninum* in a herd if any recommended control strategy does not interrupt horizontal transmission (Häsler et al., 2006; Reichel et al., 2020).

2.2.10. Namibian context

Despite the worldwide documentation of *N. caninum* as a cause of bovine abortions and neuromuscular disease in dogs, no studies have been conducted on domestic animals in Namibia and most southern African countries. However, Seltsmann and co-workers did a serosurvey involving 12 Namibian wildlife species and positive titres were detected in only two brown hyenas (*Hyaena brunnea*) and one jackal (*Canis mesomelas*) (Seltsmann et al., 2020). However, an earlier study involving one lion (*Panthera leo*) and six cheetahs (*Acinonyx jubatus*) produced negative results (Cheadle et al., 1999).

2.3. *Toxoplasma gondii*

Toxoplasma gondii, the cause of toxoplasmosis, is a worldwide (Al-Malki, 2021) zoonotic protozoan parasite that can infect any endothermic animal, including marine mammals, birds (Dubey, 2013; Dubey et al., 2012; Dupont et al., 2012) and fish (Marino et al., 2019). It is regarded as the most successful parasite (Hiszczyńska-Sawicka et al., 2014), and it is estimated that a third of the world's human population is infected by it (Innes, 2010). In the feline definitive hosts, the global prevalence is estimated at 35% in domestic cats and 59% in wild cats (Yektaeian et al., 2021).

2.3.1. Genetic diversity

There is a correlation between disease severity and various genotypes of *T. gondii*, with differing levels of virulence exhibited among these genotypes (Du Plooy et al., 2023). *Toxoplasma gondii* strains, types I, II, and III, are classified based on their varying virulence levels in outbred mice. Type I is highly virulent, mainly found in humans and lethal to mice. Type II is the most commonly isolated strain, causing chronic infections in mice. Type III is relatively rare and displays intermediate virulence (Dardé, 2004). In some instances, there have been reports of systemic fatal toxoplasmosis in adult goats (Dubey et al., 2020), and an outbreak in dairy goats in Brazil was marked by cases of abortion, stillbirth, and the death of adult goats (Neto et al., 2018).

The severity of the disease varies depending on several factors, including host immune status, parasite genotype/strain type, route/dose/exposure time to infection, and co-infections with other pathogens (Hamilton et al., 2019b; Saraf et al., 2017). One study examined the virulence of different *T. gondii* strains in mice and found a potential association between virulence in mice and disease severity in human toxoplasmosis (Saraf et al., 2017). In one study, Hamilton *et al.* (2019) compared the virulence of *T. gondii* strains from the Caribbean to a virulent Brazilian strain and a less virulent European strain using 120 mice. The Caribbean strain with ToxoDB genotype #13 showed high virulence, similar to the Brazilian strain. The parasite burden was higher in the lungs and eyes of mice infected with these strains. Genetic analysis revealed differences in polymorphic rhoptry proteins (ROP) proteins between the virulent Caribbean and Brazilian strains, suggesting additional factors influencing virulence (Hamilton et al., 2019a).

These findings suggest that specific *T. gondii* genotypes may be associated with increased disease severity in animals. In addition to mouse models, other animal species have been used to study the link between *T. gondii* genotypes and disease severity. A study on free-ranging chickens from the Caribbean found that atypical strains of *T. gondii* dominate in South America, where they are associated with more severe disease in humans (Hamilton et al., 2019b). Similarly, a study conducted on goats during gestation showed that infection with specific genotypes of *T. gondii* resulted in reproductive loss (De Oliveira et al., 2022). A study conducted in Portugal found that type II strains were most commonly isolated from human infections (Vilares et al., 2017). Another study conducted on human cancer patients showed a high prevalence of type I strains among those infected with *T. gondii* (Cong et al., 2015).

Several techniques have been utilised for genotyping *T. gondii*, which include MS analysis focusing on specific regions such as TUB2, W35, TgM-A, B18, B17, M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, and N83; multi-locus sequence typing (MLST) targeting BTUB, SAG2, GRA6, and SAG3; PCR-RFLP focusing on SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico; RAPD-PCR targeting genomic DNA, and high-resolution melting (HRM) analysis concentrating on the *B1* gene (Du Plooy et al., 2023; Liu et al., 2015).

Toxoplasma gondii was generally regarded as having low genetic variation, and three main clonal types (I, II & III) were described, although other new genotypes described as "atypical", "unusual", "non-archetypal" or "exotic" strains have also been described (Darde, 2008). In a review paper, Darde (2008) reviewed the general distribution of various *T. gondii* genotypes across the world as follows: Europe and USA (type II), South America (type II and other genetically distinct isolates), and Africa and Asia (types I & III) (Darde, 2008). However, according to a recent review by Du Plooy et al. (2023), the following genotypes were found in different regions of Africa: 13 ToxoDB genotypes, all three clonal types (I, II & III), Africa types (1, 2, 3 & 4), various recombinant genotypes, mixed types, type II-variants, atypical types as well as some unknown types (Du Plooy et al., 2023).

In Serbia, Uzelac et al. (2021) reported that 45% of genotypes found there were variants and recombinants of clonal types II and III, and 42% were archetypes. Atypical types, Africa 4 and ToxoDB genotypes (#1 and #2) were also reported there. Nine host species were involved in this

study, and these included humans, horses (*Equus caballus*), feral pigeons (*Columba livia*), golden jackal (*Canis aureus*), red fox, the grey wolf, domestic pigs (*Sus scrofa domesticus*), chickens (*Gallus gallus domesticus*), and sheep (*Ovis aries*) (Uzelac et al., 2021).

During a study conducted in the Bahia state of Brazil, no clonal lineages I, II or III were found in cats. However, the researchers detected five atypical genotypes (De Jesus Deiró et al., 2021). In the USA, ToxoDB genotypes (#4, #127, #197) were detected in North American river otters (*Lontra canadensis*) (Cotey et al., 2022). In a review paper by Costa et al. (2022), 190 genotypes were identified by analysing 561 *T. gondii* isolates obtained from 6,356 free-range chickens. ToxoDB #59 and #2 were the prevalent genotypes in America, while #1 was the most common in Africa. Moreover, three atypical isolates from genotype ToxoDB #9 were detected in Asia (Costa et al., 2022).

Battisti et al. (2018) conducted a study in Italy where they utilised a nested PCR of 6 loci (alt.SAG2, GRA6, 5'SAG2, BTUB, C22-8 and SAG1) along with in silico RFLP. Skeletal muscle samples from the following animal species were used: cattle (*Bos taurus*), swine, fox, roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*). The study revealed a substantial prevalence of genotype I and non-canonical genotypes (Battisti et al., 2018).

According to some authors, *T. gondii* genotypes circulating in Africa and Asia are under-explored (Chaichan et al., 2017; Darde, 2008). However, a recent South African study detected genotype II in a Red-eyed Dove (*Streptopelia semitorquata*) (Lukášová et al., 2018). Another review of *T. gondii* genotype distribution in Asia revealed 36 different genotypes, 15 identified by a ToxoDB number and the other 21 atypical or unique genotypes (Chaichan et al., 2017). According to the same review, genotype ToxoDB#9 (Chinese 1) was the most common genotype found in Asia, although other clonal types I, II and II variants, and III were also commonly found (Chaichan et al., 2017). An atypical genotype (haplogroup 12, polymerase chain reaction restriction fragment length polymorphism genotype 5) common in North American wildlife caused a toxoplasmosis outbreak after the attendees to a retreat in Wisconsin consumed undercooked venison (Schumacher et al., 2021).

2.3.2. Life cycle and transmission

The wild and domestic Felidae are the only known definitive hosts of *T. gondii*, and they are the chief reservoir of this parasite (Abbas et al., 2020; Attias et al., 2020; Dubey, 2013), whilst endothermic animals are the intermediate hosts (Dubey, 2013; Marino et al., 2019). Rodents are the natural intermediate hosts for *T. gondii*, and the parasite has been shown to modify their behaviour to increase the chances of predation (Desmettre, 2020; Gil et al., 2023; Webster, 2007), thereby perpetuating its life cycle (Figure 2.2). *Toxoplasma gondii* has three infectious stages: tachyzoites, bradyzoites, and sporozoites (Attias et al., 2020; Dubey, 2013). The intermediate hosts (all mammals, birds, fish) get infected by ingestion of food or water contaminated by sporulated oocysts from cat faeces, ingestion of meat infected by tissue cysts (bradyzoites) or transplacental transmission of tachyzoites from the gravid female to the foetus (Dubey, 2013; Dubey et al., 2007a; Tenter et al., 2000). Therefore, the transmission of *T. gondii* can occur between intermediate hosts and between final hosts, from intermediate to final host and from the final host to the intermediate host (Tenter et al., 2000).

When naive felids ingest raw meat containing *T. gondii* tissue cysts, the protozoa initiates enteroepithelial replication (Dubey, 2013). The digestion of tissue cysts in the stomach and small intestine causes the bradyzoites to be released and invade the intestinal epithelium, where they undergo sexual and asexual multiplication (Attias et al., 2020; Calero-Berna & Gennari, 2019; Dubey, 2013). This releases oocysts in felid faeces, which are detectable from 3-20 days post-infection (Dubey, 2013, 1998a). Under suitable environmental conditions of temperature, moisture and UV light, the oocysts can sporulate within one to five days (Dubey, 2013; Dubey et al., 2011; Hisczyńska-Sawicka et al., 2014). Cats can excrete oocysts in their faeces more than once in their lifetime (Abbas et al., 2020; Dubey, 1995; Hisczyńska-Sawicka et al., 2014). However, other workers contend that cats usually shed oocysts once in a lifetime, even then, for a few weeks, and that repeated shedding only occurs when they are immunosuppressed (Hisczyńska-Sawicka et al., 2014; Moré, 2022) or are experimentally infected with brain homogenate (Hisczyńska-Sawicka et al., 2014). Cats can shed millions of oocysts after ingesting only a few tissue cysts (Dubey, 1995; Dubey et al., 2011). Depending on the conditions, oocysts can persist in the environment for up to 18 months (Innes, 2010). *Toxoplasma gondii* employs host

manipulation to promote the successful culmination of its life cycle. For instance, infected rodents lose their natural aversion to feline predators and instead exhibit an attraction towards cat urine (Aguirre et al., 2019).

Intermediate hosts are infected by consuming uncooked or undercooked meat containing tissue cysts (bradyzoites), feed or water contaminated with felid faeces containing sporulated oocysts, which culminates in extraintestinal multiplication and parasitaemia (Dubey, 2013, 1998a; Dubey et al., 2007a). The released bradyzoites and sporozoites invade the intestinal epithelium, where several rounds of epithelial multiplication ensue (Dubey, 2013). The produced tachyzoites then spread throughout the body (Dupont et al., 2012), presumably via the cardiovascular and lymphatic systems. This way, tachyzoites are disseminated throughout the body tissues, where they multiply inside the cells until they burst, resulting in tissue necrosis (Dubey, 2013). Transmission from the dam to offspring can also occur through milk containing tachyzoites (Tenter et al., 2000).

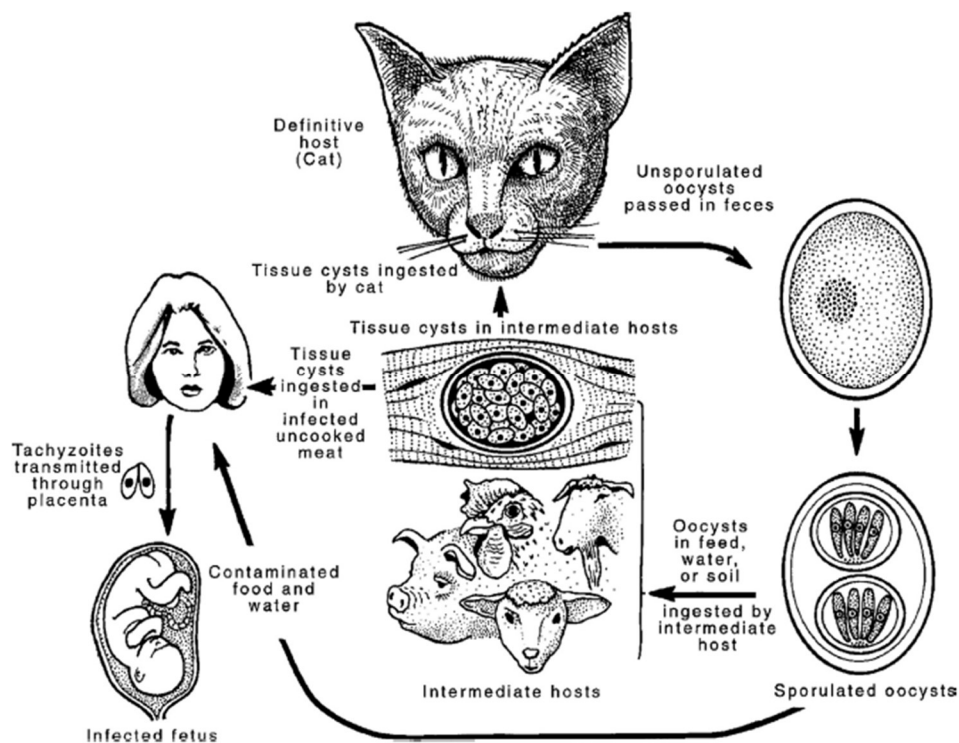


Figure 2.2: Life cycle of *Toxoplasma gondii* (Dubey, 2014); used with permission.

When the tachyzoite is predominant, immunocompromised individuals, including young animals, may die due to generalised toxoplasmosis (Dubey, 2013). Immunocompetent animals respond with a robust cell-mediated immunity to the tachyzoites and suppress the infection, causing the tachyzoites to transform into bradyzoites within tissue cysts (Dubey, 2013). Tissue cysts have a high affinity for nervous tissue, the eye and muscles (skeletal and cardiac), but they can also be found in other visceral organs like the liver, lungs and kidneys, to a lesser degree (Tenter et al., 2000). The tissue cysts can measure up to 70 μm in diameter and contain hundreds of bradyzoites in a thin, strong cyst wall (Dubey, 2013). Tissue cysts may remain viable for the intermediate host's lifespan (Dubey, 2013; Dupont et al., 2012; Tenter et al., 2000). The life cycle is completed when the felids (definitive hosts) consume infected uncooked or undercooked tissues of the intermediate hosts (Innes, 2010).

2.3.3. Animal and human infections

Tachyzoites are the chief cause of tissue damage, and host immunity is vital in limiting their dissemination in the body (Calero-Berna & Gennari, 2019; Moré, 2022). In immunocompetent animals, toxoplasmosis is commonly a subclinical disease because these animals are better equipped to suppress the spread of tachyzoites. *Toxoplasma gondii* commonly causes abortions, foetal mummification, foetal resorption, infertility, stillbirths, or births of weak offspring in small ruminants (sheep and goats), which can have profound economic implications (Al-Malki, 2021; Khan & Noordin, 2020). Pigs are occasionally affected, but cattle, buffaloes and horses are resistant (Abbas et al., 2020; Dubey, 2013). In pregnant ewes, the tachyzoites cross the placenta, causing necrosis of placental cotyledons and foetal organs and, eventually, foetal death. If they occur, other clinical signs are non-specific and include anorexia, diarrhoea, vomiting, weight loss and weakness (Ferra et al., 2020).

In dogs and cats, clinical toxoplasmosis is common in cats but rare in dogs; in the latter, the disease has been associated with immunosuppressive therapy and lack of vaccination against canine distemper (Calero-Berna & Gennari, 2019). Nervous signs observed in dogs include seizures, tremors, cranial nerve deficits, ataxia, paresis/paralysis and noise sensitivity. Other clinical signs include myositis, anterior uveitis, endophthalmitis, and chorioretinitis; cutaneous signs include epidermal nodules, pyogranulomatous and necrotising dermatitis, and panniculitis (Calero-Berna

and Gennari, 2019). Kittens infected *in-utero* can develop more severe signs, including hepatitis, cholangiohepatitis, pneumonia, encephalitis, ascites, lethargy, and dyspnea. In adult cats, hepatitis, hepatic failure, hyperplastic cholangitis, extra-intestinal enteritis, inflammatory intestinal disease, gastric wall thickening due to eosinophilic fibrosing gastritis, and regional lymphadenopathy have been described (Calero-Berna & Gennari, 2019). In addition, pneumonia and acute respiratory distress, icterus, uveitis, iridocyclitis, myocarditis, diarrhoea, and even death have also been observed (Calero-Berna & Gennari, 2019; Moré, 2022).

Toxoplasmosis in humans has been associated with spontaneous abortion (Kalantari et al., 2021), stillbirth, myocarditis, cerebral disease, epilepsy, encephalitis, blindness (Mazuz et al., 2023), congenital malformations (Kalogeropoulos et al., 2022), schizophrenia (Yolken et al., 2009), unnatural causes of death due to traffic accidents and suicide attempts (Sutterland et al., 2019). In addition, *T. gondii* also causes other ocular disorders like uveitis, retinitis and retinochoroiditis in humans (Kalogeropoulos et al., 2022). Therefore, it is unsurprising that *T. gondii* has been recognised as the most predominant parasitic zoonotic infection, resulting in the FAO and WHO declaring it a foodborne pathogen of global concern (Franco-Hernandez et al., 2016).

2.3.4. Diagnosis

Light microscopy can be used to identify *T. gondii* tachyzoites or bradyzoites in cerebrospinal fluid, amniotic fluid, aqueous humour, bronchoalveolar lavage fluid, semen, brain tissues, lymph nodes and muscles, which have been stained with haematoxylin and eosin (H&E), Giemsa or periodic acid Schiff (PAS) stains (Uddin et al., 2021). On histopathology, toxoplasmosis can cause necrotising encephalitis, and protozoal cysts may be present in the brain, lung, heart, skeletal muscle, or even adipose tissue (Fisk et al., 2023). Immunohistochemical staining can identify bradyzoites and tachyzoites in tissue sections; however, due to their morphological similarities, *T. gondii* should be distinguished from *N. caninum* and *Sarcocystis* spp. cysts (Moré, 2022).

Immunoassays are widely used to detect infections in animal sera and other tissue fluids. IgM antibodies are produced shortly after an infection, lasting no more than three months post-infection. Therefore, IgM titres are a better indicator of recent infections (Abbas et al., 2020; Dubey, 2013). On the other hand, IgG antibodies are detectable about three months post-infection

and may last for years. Therefore, paired sera are more informative when using IgG antibody titres for diagnosis (Moré, 2022). However, a study by Opsteegh and co-workers demonstrated a poor correlation between the presence of anti-*T. gondii* antibodies in cattle and detection of parasites by PCR, unlike in sheep, where the correlation is good (Opsteegh et al., 2011). Although cattle can be easily infected with *T. gondii*, they are not considered ideal hosts as they have evolved a stronger immune response to combat *T. gondii* infection than sheep. This immune response aids in eliminating *T. gondii* from tissues and results in transient antibody responses (Dong et al., 2018).

Indirect hemagglutination assay (IHA), indirect fluorescent antibody assay (IFAT), latex agglutination test (LAT), western blotting (WB), direct agglutination test (DAT), modified agglutination test (MAT) and ELISA tests are used for serological diagnosis (Ferra et al., 2020; Khan & Noordin, 2020; Moré, 2022). WB can detect both IgG and IgM antibodies, but it is time-consuming; with IFAT, antibody titres can be determined, but there can be cross-reactions; DAT and MAT have high sensitivity and specificity, can detect IgG and IgM antibodies and are easy to implement (Ferra et al., 2020). Currently, most commercial ELISAs are indirect, targeting *T. gondii*-specific IgG antibodies (Liyanage et al., 2021). CSF and aqueous humour can also be examined for tachyzoites or anti-*T. gondii* antibodies (Moré, 2022).

The antigenic structure of *T. gondii* was discovered through proteomics, and this has galvanised the development of recombinant antigens, enabling the development of newer, cheaper, and more effective diagnostic tools (Ferra et al., 2020). However, the use of recombinant antigens for serodiagnosis has not been as well developed for animals compared to humans (Ferra et al., 2020). Furthermore, the cross-reactions of *T. gondii* with other Apicomplexa-type parasites like *Neospora sp.* and *Sarcocystis sp.* have not been adequately investigated (Ferra et al., 2020), and this has limited the use of recombinant antigens for serodiagnosis in animals.

Bioassays are the most sensitive in detecting *T. gondii* cysts in animal tissues (Al-Malki, 2021). The procedure involves dissecting the suspected infected tissues, digesting them with trypsin, pepsin or acid and then inoculating the parasites into mice, which are then monitored for clinical signs or seroconversion (Al-Malki, 2021; Garcia et al., 2006). The infected tissues from mice are then examined microscopically (Dubey, 1998b; Garcia et al., 2006) or inoculated in cats, and the faecal samples are analysed for *T. gondii* oocysts one to two weeks later (Al-Malki, 2021). The cat

faeces can be subjected to flotation with NaNO₃ followed by PCR analysis (Sroka et al., 2018), or they can first concentrated using the Ritchie technique and then subjected to PCR amplification (Zamora-Vélez et al., 2020). *Toxoplasma gondii*, *N. caninum*, and *Hammondia* oocysts are physically similar and, therefore, difficult to distinguish microscopically (Schaes et al., 2005), so molecular methods are more sensitive and specific.

PCR-based methods are recommended for detecting tissue cysts because they are less time-consuming than bioassays, although they are not as sensitive because of the small amount of tissue used (Al-Malki, 2021). Conventional PCR strategies often target specific genetic markers such as the *Bl* gene, a 529-bp repeat element, bradyzoite genes, the immunodominant *P30* antigen or ribosomal DNA regions known for their high copy numbers within the *T. gondii* genome (De Barros et al., 2022; Ellis, 1998). Due to their high sensitivity and specificity, the 529-bp sequence and the *Bl* gene are the most commonly used targets, and studies have shown that the markers for the 529-bp repeat region are more sensitive than those for the multicopy *Bl* gene (De Barros et al., 2022).

Nested PCR amplification has been employed to enhance sensitivity by performing two rounds of PCR using two sets of primers (Fazel et al., 2021), while quantitative polymerase chain reaction (qPCR) allows quantification of parasite load by measuring DNA amplification during each cycle in real-time (Miura et al., 2021). Nested PCR was successfully used to detect *T. gondii* DNA from cattle tissues with varying prevalence rates across different tissue types; diaphragm tissue exhibited the highest infection rate, followed by heart and tongue (Fazel et al., 2021). This nested approach enhances detection capabilities but requires careful handling to avoid contamination, leading to false positives. On the other hand, quantitative PCR (qPCR) not only detects but also quantifies *T. gondii* oocysts shed by cats, providing valuable information on environmental contamination risks posed by definitive hosts (Miura et al., 2021). Moreover, qPCR's ability to quantify pathogen load can be instrumental in assessing infection intensity and potential transmission dynamics among intermediate hosts.

Molecular techniques have further evolved with PCR-restriction fragment length polymorphism (RFLP) analysis, enabling genotyping based on polymorphic genes like GRA6 and SAG2; this

method identified Type II strains as predominant among cattle isolates in Iran (Fazel et al., 2021). Other molecular methods include loop-mediated isothermal amplification (LAMP), microsatellite analysis (MSA), multilocus sequence typing (MLST), PCR-restriction fragment length polymorphisms (PCR-RFLPs), random amplified polymorphic DNA-PCR (RAPD-PCR), high-resolution melting (HRM), metagenomics analysis (MGA), and whole-genome sequencing (WGS) (Castro et al., 2020; Uddin et al., 2021).

In addition to these conventional approaches, recent advances highlight microRNAs' role during *T. gondii* infections, which may serve as potential biomarkers for diagnosis through non-invasive sample collection methods amenable to sensitive molecular assays like real-time PCR (Hsu et al., 2022).

Serological methods coupled with molecular diagnostics provide complementary data; however, low correlation between seropositivity and PCR results indicates that serology alone may not reflect current infection status accurately without concurrent direct pathogen detection methodologies such as PCR-based tests (Landrau-Giovannetti et al., 2022).

2.3.5. Zoonotic implications

Toxoplasma gondii is a zoonotic parasite which causes an infection which is generally asymptomatic in immunocompetent persons, with the parasite encysted mostly in cardiac and skeletal muscles, retina and brain tissue (Al-Malki, 2021). Humans get infected when they consume infected undercooked meat or oocyst-contaminated food or water (Al-Malki, 2021; Dubey, 2013). It is particularly of concern in immunocompromised persons, where it usually causes meningoencephalitis, which is caused by the reactivation of the parasite from the brain as immunity declines (Al-Malki, 2021; Dubey, 2013). In pregnant women, tachyzoites can be transmitted transplacentally and cause congenital malformations, abortions, stillbirths, neonatal deaths, mental retardation and blindness (Yektaeian et al., 2021).

In the USA, it is estimated that 10-50% of the population has been exposed to *T. gondii* (Dubey et al., 2011). Global prevalence ranges from less than 10% to more than 90%, with South America,

Eastern/Central Europe, the Middle East, Southeast Asia, and Africa having the highest prevalence (Yektaeian et al., 2021), especially in East Africa and North Africa (Hammond-Aryee et al., 2014).

In Namibia, seroprevalence rates of 2.61% (9/344) and 9% (65/725) were detected in pregnant women in Windhoek and the San community, respectively, with levels which are much lower in comparison to other developing countries (Joubert & Evans, 1997; van der Colf et al., 2020). These low levels of *T. gondii* infections in the Namibian human population have been attributed to the more rapid desiccation of the oocysts in the country's semi-arid environment (Jacobs & Mason, 1978; Joubert & Evans, 1997; Noden & Colf, 2013).

2.3.6. Treatment and prevention

Treatment of toxoplasmosis in livestock is generally not practised, although drugs like sulfadiazine/pyrimethamine combination, diaminodiphenylsulfone, atovaquone, spiramycin and clindamycin are helpful but have no effect on the bradyzoite stage (Dubey, 2013; Moré, 2022). Clindamycin is indicated for treating acute toxoplasmosis in dogs (10–40 mg/kg) and cats (25–50 mg/kg) and must be administered for 3-4 weeks (Moré, 2022). Diclazuril, ponazuril, and toltrazuril have been used to treat acute toxoplasmosis in cats and to reduce the shedding of oocysts (Moré, 2022).

The only vaccine currently registered for use in sheep is Toxovax® (MSD Animal Health) (Smith et al., 2021; Zhang et al., 2013), which is a live-attenuated tachyzoite vaccine (Smith et al., 2021; Zhang et al., 2013). It is an 'incomplete' S48 strain that does not form tissue cysts (Smith et al., 2021; Wastling et al., 1994). In addition, the antibodies produced are only against the tachyzoite stage, which has been shown to significantly reduce the number of abortions (Wastling et al., 1994). The ewes should be vaccinated once off, intramuscularly, a month before mating because the induced immunity is long-lasting (Smith et al., 2021). There is no human vaccine against toxoplasmosis (Moré, 2022).

The effectiveness of new-generation vaccines like vector-based and DNA vaccines has been explored with mixed results. Parenteral injection of a recombinant BCG strain, which produces and secretes GRA1, a major *T. gondii* antigen, into mice and sheep could only produce partial protection upon challenge (Supply et al., 1999). Hiszczyńska-Sawicka and coworkers concluded

that injecting sheep with DNA plasmids encoding for MIC3, an adhesion *T. gondii* protein, elicited a robust immune response after a booster (Hiszczyńska-Sawicka et al., 2012). In their review, Yektaeian and others concluded that naked DNA vaccines could elicit adequate humoral and cellular immune responses against *T. gondii* (Yektaeian et al., 2021). However, the immune responses induced by DNA vaccines had not yet been evaluated for their efficacy in protecting pregnant ewes against abortion (Hiszczyńska-Sawicka et al., 2014).

In order to prevent human infection, hands must be washed thoroughly with soap and water after contact with meat and other potentially contaminated materials (Moré, 2022). The USA Centres for Disease Control and Prevention (CDC) recommends the following measures to prevent human toxoplasmosis (CDC, 2018): washing fruits and vegetables thoroughly or peeling them before consumption; pasteurising goat's milk; avoiding eating raw or undercooked oysters, mussels, and clams; washing cutting boards, dishes, counters, utensils, and hands with soapy water after contact with raw meat, poultry, seafood, or unwashed fruits or vegetables; avoiding untreated water; educating children about the importance of handwashing; changing the cat litter box daily while wearing disposable gloves; feeding cats only canned, dried commercial food or well-cooked table food; covering sandpits when not in use, as cats prefer sand or loose soil for defecation; wearing gloves while gardening or wash hands thoroughly after contact with soil; pregnant or immunocompromised individuals should avoid changing cat litter, keeping cats indoors, or handling stray or new cats; cooking ground meat, whole cuts, and poultry to at least 71°C, 63°C, and 74°C, respectively.

Healthcare professionals should also be at the forefront of educating the public on toxoplasmosis, particularly during pre-natal and post-natal care visits and educating immunocompromised individuals (Smith et al., 2021). In addition, animal health practitioners (AHP) should also do their part in educating the farming community and cat owners. However, a recent study in Namibia has shown that AHPs hardly ever discuss toxoplasmosis with cat owners (Samkange et al., 2023). Sadly, this might well be the scenario with most animal health practitioners around the world.

To deactivate *T. gondii* cysts in meat for human consumption, freezing to –12°C, cooking to an internal temperature of 67°C, and gamma irradiation (0.5 kGy) were adequate to destroy tissue cysts (Dubey, 1996). El-Nawawi and co-workers (2008) also observed that heating meat infected

by *T. gondii* at 60°C or 100°C for 10 minutes, freezing it at -10°C for three days or -20°C for two days, or irradiation at 75 or 100 krad was adequate to inactivate the tissue cysts. However, cooking in a microwave and chilling at 5°C for five days were insufficient to destroy tissue cysts (El-Nawawi et al., 2008).

2.4. Knowledge, attitudes and practices concerning neosporosis and toxoplasmosis

The measurement of knowledge, attitudes and practices (KAP) of a given population is essentially a diagnosis of its education level concerning a given disease or subject (Kaliyaperumal, 2004; Sarria-Guzmán et al., 2021). KAP studies were developed in the 1950s and have since been refined to the extent that they are now widely used for determining human behaviour regarding given diseases because they are cheap to conduct and provide invaluable information (Rav-Marathe et al., 2016).

The literature has a paucity of information on KAP studies on *N. caninum*. In the only neosporosis KAP study found in the literature, Gaber and co-workers (2021) found 0% (N=41) knowledge of neosporosis amongst livestock farmers. The same study further noted that no attention was paid to neosporosis. The authors then concluded that neosporosis was a neglected disease in Egypt (Gaber et al., 2021), which is probably the case in most African countries.

Toxoplasmosis has been the subject of more KAP studies worldwide than neosporosis, with mixed results. Studies in Saudi Arabia, Malaysia and Ethiopia showed a very low level of toxoplasmosis awareness among farmers, pregnant women and female students, ranging from 5.9% to 20.9% (Desta, 2015; Mahfouz et al., 2019; Sadiq et al., 2021). However, 84% of Italian women claimed to have heard about toxoplasmosis, although their level of knowledge was deemed superficial and incomplete (Martini et al., 2020). Gaps in knowledge on toxoplasmosis among medical, biology and veterinary students at a Moroccan university were also found (Ait Hamou et al., 2021). In Senegal, none of the cattle farmers interviewed mentioned toxoplasmosis as a zoonosis, even though it had been reported in that country (Tebug et al., 2015). Cattle farmers in Zimbabwe were also unaware of toxoplasmosis (Chikerema et al., 2013) and, most likely, neosporosis as well.

A toxoplasmosis KAP study in Italy involving medical professionals (medical doctors, nurses, midwives, and laboratory technicians) found that 81.25% knew its aetiology, 64.5% knew the

definitive host, 57.29% knew the symptoms of the disease in immunocompetent persons, over 50% knew the transmission routes and only 14.58% knew about the *Toxoplasma* IgG avidity test (Laboudi et al., 2020). The *Toxoplasma* IgG avidity test is used to estimate the time when the infection was acquired (Findal et al., 2015).

In one American survey of veterinarians and physicians, it was found that a very low proportion of veterinarians (25.97% or 47/181) and physicians (33.33% or 67/201) were able to correctly identify the zoonotic pathogens of greatest importance to immunocompromised individuals, including *T. gondii*, virtually no communication between veterinarians and physicians concerning zoonotic diseases (Hill et al., 2012). This is why a One Health approach has been strongly advocated, for example, with zoonotic diseases like toxoplasmosis (Aguirre et al., 2019; Wilkes et al., 2019).

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Chapter 3: Evaluation of knowledge, attitudes and practices regarding neosporosis and toxoplasmosis among farmers and animal health practitioners in Namibia

(Adapted from: Samkange, A., Chitanga, S., Neves, L., Matjila, T., 2023a. Evaluation of knowledge, attitudes and practices regarding neosporosis and toxoplasmosis among farmers and animal health practitioners in Namibia. *Tropical Animal Health and Production*. 55, 28. <https://doi.org/10.1007/s11250-022-03441-y>)

3.1. Abstract

This study assessed the knowledge, attitudes and practices of livestock farmers in Namibia's Khomas region and animal health practitioners (veterinarians and animal health technicians) in the whole country concerning neosporosis and toxoplasmosis.

Structured questionnaires were used, and 63 farmers and 51 animal health practitioners responded out of an estimated 560 farmers in the Khomas region and 300 veterinarians and veterinary technicians in the country.

Only 15.9% (10/63) of the livestock farmers had heard about neosporosis or toxoplasmosis or knew how animals get infected ($p < 0.0001$). Only 5% of the farmers knew the risks associated with keeping dogs and cats concerning neosporosis and toxoplasmosis, respectively ($p < 0.0001$). None of the 51 animal health practitioners routinely requested for *Neospora caninum* or *Toxoplasma gondii* laboratory tests in cases of cattle, sheep or goat abortions. Although all animal health practitioners indicated they routinely interacted with livestock farmers, none regularly discussed neosporosis or toxoplasmosis. Very few animal health practitioners (3.9%) indicated that they had ever discussed either neosporosis or toxoplasmosis at a farmers' gathering ($p < 0.0001$), with only 21.6% having talked to at least one cat owner about toxoplasmosis in the previous 12 months ($p < 0.0001$).

The study concluded that farmers in the Khomas region were generally unaware of neosporosis and toxoplasmosis but could change their attitudes and practices if educated. The animal health

practitioners lacked a deeper understanding and appreciation of the two diseases, which is required to cultivate enough confidence to educate farmers. Sharing these research findings and other relevant information on the two diseases at forums used by both groups to share and acquire new information, such as farmers' meetings, veterinary congresses, journals, and newsletters, could help educate farmers and animal health practitioners.

3.2. Introduction

Namibia's economy heavily relies on agriculture, with approximately 70% of its population dependent on the sector, primarily through livestock production (Humavindu & Stage, 2013; Meat Board of Namibia, 2015; Simasiku & Sheefeni, 2017). Consequently, livestock farming contributes significantly to the country's gross domestic product (GDP), accounting for around 4.3% (BDO Namibia, 2019; Meat Board of Namibia, 2019), and employs about 30% of the nation's labour force (Humavindu & Stage, 2013; Simasiku & Sheefeni, 2017). However, livestock diseases pose a significant risk to the economy, potentially causing reduced production and severe economic repercussions.

Many infectious causes of abortions have been reported in the country. These include, among others, brucellosis (Madzingira et al., 2020), chlamydiosis (Samkange et al., 2010), coxiellosis (Bishi et al., 2018), Schmallenberg virus (Molini et al., 2018), Rift Valley Fever (Cosseddu et al., 2020) and bovine viral diarrhoea (Depner et al., 1991). Among the pathogens known to cause reproductive losses are *Neospora caninum* and *Toxoplasma gondii*, although they have not been reported in livestock in Namibia. However, *N. caninum* and *T. gondii* have recently been reported in Namibian wildlife species like cheetahs (*Acinonyx jubatus*), African lions (*Panthera leo*), blue wildebeest (*Connochaetes taurinus*), brown hyenas (*Hyaena brunnea*), and black-backed jackals (*Canis mesomelas*) (Seltmann et al., 2020). Therefore, livestock species like cattle and small ruminants (sheep and goats) could be at risk due to their proximity to wildlife in commercial farming establishments.

Neosporosis caused by *N. caninum* and toxoplasmosis caused by *T. gondii* are important worldwide protozoal infections that negatively impact livestock productivity through abortions, stillbirths, infertility and the birth of weak offspring (Al-Malki, 2021; Dubey & Schares, 2011;

Khan & Noordin, 2020; Monney et al., 2011). Globally, annual losses due to *N. caninum*-induced abortions have been estimated between US\$633.4 million and US\$2.380 billion (Reichel et al., 2013). In parts of South America, Europe and Australia, these have been estimated between US\$1.4 million and US\$50 million (Bennett & Ijpelaar, 2005; Freyre et al., 1997; Stelzer et al., 2019). These figures clearly illustrate the significant economic impact of neosporosis and toxoplasmosis on the livestock industry worldwide.

To a great extent, livestock neosporosis and toxoplasmosis have generally been overlooked in sub-Saharan Africa, including Namibia. As a result, fewer studies have been conducted on toxoplasmosis and neosporosis, prompting their consideration as neglected diseases in the region (Gaber et al., 2021; Tegegne et al., 2016). The neglect of neosporosis and toxoplasmosis could be due to a lack of awareness caused by limited research.

Despite the evident negative impact of the two infections on livestock production, very little has been done to assess the KAP of critical players in the livestock industry. To my knowledge, there is only one KAP study on *N. caninum*, which was done in Egypt (Gaber et al., 2021). Although several *T. gondii* KAP studies have been done around the world, including Morocco (Ait Hamou et al., 2021; Laboudi et al., 2020), Zimbabwe (Chikerema et al., 2013), Saudi Arabia (Mahfouz et al., 2019), Malaysia (Sadiq et al., 2021), the USA (Hill et al., 2012) and Senegal (Tebug et al., 2015), these studies were only from a zoonotic perspective with no reference to the livestock industry. In addition, no KAP survey of the two diseases has been done in southern Africa, including Namibia.

This study aimed to assess the knowledge, attitudes and practices of the livestock farmers in Namibia's Khomas region and the animal health practitioners (veterinarians, animal health technicians and other para-veterinary professionals) in the country concerning neosporosis and toxoplasmosis.

3.3. Materials and methods

3.3.1. Study areas

The livestock producers enrolled on this study were drawn from the Khomas region of Namibia. The animal health practitioners (veterinarians, animal health technicians and private para-veterinary professionals) were drawn from the whole country. The Khomas region was selected for this study because of its central location and its easier accessibility to the researcher. The Khomas region is one of Namibia's fourteen regions, covers approximately 36,964 km² (Namibia Statistics Agency, 2011), and has approximately 560 commercial farms (Directorate of Veterinary Services, 2018). The region is situated in the central part of the country and is sandwiched between four other regions: Erongo to the west, Otjozondjupa to the north, Omaheke to the east, and Hardap to the south (Figure 3.1).

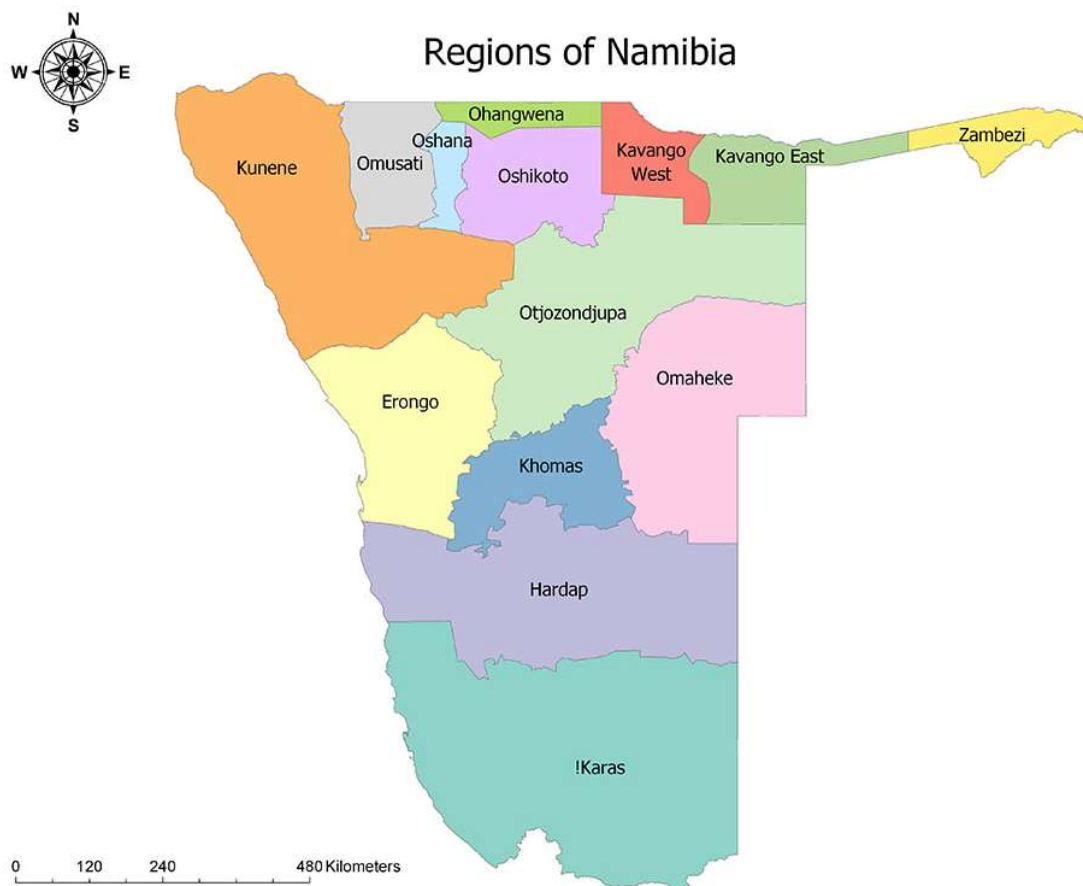


Figure 3.1: Map showing the regions of Namibia with the Khomas region at the centre and surrounded by Erongo, Otjozondjupa, Omaheke and Hardap regions. (Source: <https://www.mappr.co/counties/namibia/>; used with permission).

3.3.2. Study subjects

The KAP study population included all 560 livestock producers in the Khomas region and all animal health practitioners (approximately 85 veterinarians and over 200 animal health technicians) in Namibia who are involved in production animal practice. Namibia has over one million registered livestock producers compared to less than 300 animal health practitioners, and therefore, sampling the farmers from the whole country would have been more costly and tedious than animal health practitioners.

3.3.3. Sample size determination

The sample size was determined using the following sample size formula (Pfeiffer, 2002):

$$n = 1.96^2 \times P(1-P)/d^2$$

Where n = sample size; d = precision; P = estimated population portion.

Applying this formula using an estimated 20% population proportion of farmers and AHP who were expected to score well in the KAP assessment and 10% precision, the sample size was calculated as follows:

$$n = 1.962 \times 0.2 (1-0.2)/(0.1)^2 = 61$$

The sample size was therefore calculated at 61 each for farmers and animal health practitioners.

3.3.4. Data collection

This study was carried out between May 1 and May 31, 2022. Two structured questionnaires, one for the farmers and another for the animal health practitioners, were used to collect information on their knowledge, attitudes, and practices concerning neosporosis and toxoplasmosis. Prior to that, I developed the questionnaires and pretested them during a previous pilot research project. The

questions were then modified to ensure that they were clear and could be answered within a reasonably short time.

A convenience sampling strategy was employed because the questionnaires were only sent out to farmers and animal health practitioners for whom contact details were accessible or could be met in person. The farmer questionnaires were sent through emails, and the farmers would complete and email them back. Others were completed during farmers' meetings and through face-to-face and telephonic interviews by the first author; no other interviewers were employed. The questionnaire for animal health practitioners was disseminated electronically via email, either directly to the AHP or indirectly through the Veterinary Association of Namibia, which maintains direct contact with the majority of practising veterinarians in Namibia. Additionally, in certain instances, physical distribution of the questionnaires was undertaken.

The farmers' questionnaire solicited information on their educational level and experience in farming, their knowledge of causes of abortions, their specific knowledge of neosporosis and toxoplasmosis and the role of cats and dogs in their transmission. Questions on farmers' attitudes centred on their perspectives on livestock abortions, what they would do if neosporosis and toxoplasmosis were diagnosed on their farms, and how important abortions were on their farms. Finally, questions on the farmers' practices gathered information on the vaccines used at the farm, sources of replacement stock, what they would do if abortions occurred on their farm, what they did with aborted animals, and how much livestock production contributed to their livelihoods.

The animal health practitioner questionnaire assessed their knowledge of neosporosis and toxoplasmosis concerning transmission, clinical signs, availability of vaccines and zoonotic implications. In addition, questions on attitudes pertained to whether or not they thought the two diseases occurred in Namibia and whether they thought that the veterinary fraternity in Namibia was well informed concerning *N. caninum* and *T. gondii*. Finally, the questions on practices asked if the animal health practitioners routinely requested laboratory tests for the two diseases in abortion cases and whether they regularly educate their clients on these diseases.

3.3.5. Data analysis

The questionnaire data were captured in Microsoft Excel 2013. The data were first collated, similar questions were grouped, and the responses summarised in a manner easily captured and analysed. However, no data was removed as outliers since the data were categorical. Statistical analysis calculations were done using the one-sample z-test for a population proportion calculator (<https://epitools.ausvet.com.au/ztestone>). The questionnaire data for farmers and animal health practitioners were analysed separately since they had different questionnaires.

3.4. Results

3.4.1. The knowledge, attitudes and practices of farmers

The majority [93.7%; 59/63] of the interviewed farmers had high literacy levels and more than ten years of experience [71.3%; 45/63] in livestock production (see Table 3.1). Sixty-one point nine per cent (61.9%) of the respondents had experienced abortions in their livestock and thought they knew the causes ($p=0.0589$). Only 1.6% (1/63) of the farmers listed neosporosis and toxoplasmosis among the causes of abortions they knew, and 4.8% (3/63) were aware that toxoplasmosis was a zoonosis ($p<0.0001$).

Table 3.1: Summary of the knowledge of farmers regarding neosporosis and toxoplasmosis

KNOWLEDGE	YES	NO	% YES	P value
Secondary school education level or higher	59	4	93.7	<0.0001
At least ten years of farming experience?	45	18	71.4	0.0007
Known causes of abortions include neosporosis & toxoplasmosis	1	62	1.6	<0.0001
Have you ever experienced abortions on the farm & do you know the causes?	39	24	61.9	0.0589
Knowledge of zoonotic causes of livestock abortions includes toxoplasmosis?	3	60	4.8	<0.0001
Knowledge of how animals get infected by abortion-causing agents	23	40	36.5	0.0321
Knowledge of how to prevent livestock abortions	37	26	58.7	0.1673
Have you heard of neosporosis or toxoplasmosis?	10	53	15.9	<0.0001
Know how animals are infected by neosporosis	9	54	14.3	<0.0001
Know how animals are infected by toxoplasmosis	10	53	15.9	<0.0001
Knowledge of the risks of keeping dogs	5	58	7.9	<0.0001
Knowledge of the risks of keeping cats	5	58	7.9	<0.0001

Fifteen point nine per cent (15.9%; 10/63) of the respondents had heard about neosporosis or toxoplasmosis or knew how animals get infected ($p<0.0001$). Only 5% (5/63) of the farmers were aware of the risks associated with keeping dogs and cats concerning neosporosis and toxoplasmosis ($p<0.0001$). Most of the farmers (87.3%) indicated that they would be worried if abortions occurred in their livestock, and 79.4% would take corrective measures ($p<0.0001$) (Table 3.2). About 69.8% correctly indicated that they would consult their veterinarian if their animals tested positive for neosporosis or toxoplasmosis, and 66.7% indicated that they would not purchase an animal with a history of abortion ($p=0.008$). Less than half of the respondents (41.3%) listed abortion among the causes of livestock losses in their livestock ($p=0.167$). Most respondents (69.8%) indicated that they preferred well-cooked meat ($p=0.0017$), and only 17.5% of the respondents thought that other livestock farmers had ever heard of neosporosis or toxoplasmosis ($p<0.0001$).

Table 3.2: Summary table of the attitudes of farmers concerning neosporosis and toxoplasmosis

ATTITUDES	%			
	YES	NO	YES	P value
They are worried that abortions are occurring.	55	8	87.3	<0.0001
Are abortions taken seriously if they occur?	50	13	79.4	<0.0001
Correct measures in response if animals test positive for neosporosis or toxoplasmosis	44	19	69.8	<0.0001
Appropriate attitude towards the purchase of an animal with a history of abortion	42	21	66.7	0.008
Ranking of important causes of livestock losses includes abortions?	26	37	41.3	0.1673
Correct attitude about eating undercooked meat	44	19	69.8	0.0017
Whether they think other farmers have heard of neosporosis or toxoplasmosis	11	52	17.5	<0.0001

About 76.2% of the respondents indicated that livestock production contributed at least 50% of their livelihoods ($p<0.0001$) (Table 3.3). However, most of the farmers sold no more than 30 sheep (23.8%) or goats (14.3%) per year ($p<0.0001$), and 57.1% of them sold more than 20 cattle per year ($p>0.05$).

In addition, none of the farmers included *T. gondii* in their vaccination programmes. 49.2% of the farmers indicated that they would cull animals that aborted more than once ($p=0.8989$), and only 36.5% gathered animal health information concerning the farm from which they sourced

replacement stock ($p=0.0321$). Fifty-eight point seven per cent (58.7%) of the respondents preferred their grilled meat to be well-done ($p=0.1673$).

Table 3.3: Summary of farmer practices regarding neosporosis and toxoplasmosis

PRACTICES			%	P value
	YES	NO	YES	
Does livestock production contribute at least 50% to livelihood?	48	15	76.2	<0.0001
More than 20 cattle are sold per year	36	27	57.1	0.2597
More than 30 sheep are sold per year	15	48	23.8	<0.0001
More than 30 goats sold per year	9	54	14.3	<0.0001
Vaccines administered include <i>T. gondii</i> ?	0	63	0.0	<0.0001
Cull animals that abort more than once?	16	47	25.4	<0.0001
Replacement stock sourced elsewhere	31	32	49.2	0.8989
Do you gather information on the farm from which replacement stock is sourced	23	40	36.5	0.0321
Grilled meat preferred well-done?	37	26	58.7	0.1673

3.4.2. The knowledge, attitudes and practices of animal health practitioners

The KAP of animal health practitioners (Table 3.4) showed that only 9.8% (5/46) of the respondents listed neosporosis and toxoplasmosis among the common causes of livestock abortions ($p<0.0001$).

However, on most key questions, the respondents had good knowledge scores of the two parasitic infections, ranging from 60.8% to 90.2% and averaging 68%. Low scores were on questions relating to the zoonotic implications of neosporosis (41.2%; $p=0.2088$) and the risk of toxoplasmosis posed by veterinary-inspected meat (23.5%; $p=0.0002$).

Fifty-two point nine per cent (52.9%) of the animal health practitioners thought that neosporosis was present in Namibia (Table 3.5), and 56.9% thought that this disease was important for the country and that farmers should be worried about it ($p>0.05$).

Table 3.4: Summary of the knowledge of animal health practitioners regarding neosporosis and toxoplasmosis

KNOWLEDGE	YES	NO	% YES	P value
Knowledge of common causes of abortions in cattle includes <i>N. caninum</i>	5	46	9.8	<0.0001
Knowledge of common causes of abortions in sheep & goats includes <i>T. gondii</i>	5	46	9.8	<0.0001
Average or higher level of knowledge of <i>N. caninum</i>	30	21	58.8	0.2088
Knowledge of <i>N. caninum</i> transmission	35	16	68.6	0.0079
Knowledge of final hosts of <i>N. caninum</i>	34	17	66.7	0.0171
Knowledge of clinical signs of neosporosis in canids	21	30	41.2	0.2088
Ability to name at least one host of <i>N. caninum</i>	46	5	90.2	<0.0001
Knowledge of the main problem caused by <i>N. caninum</i> in cattle	36	15	70.6	0.0033
Knowledge of zoonotic implications of <i>N. caninum</i>	21	30	41.2	0.2088
Knowledge about <i>N. caninum</i> vaccination	35	16	68.6	0.0079
Average or higher level of knowledge of <i>T. gondii</i>	30	21	58.8	0.2088
Knowledge of <i>T. gondii</i> transmission	35	16	68.6	0.0079
Knowledge of <i>T. gondii</i> definitive host	31	20	60.8	0.1229
Knowledge of <i>T. gondii</i> clinical signs in cats	14	37	27.5	0.0013
Knowledge of animals affected by <i>T. gondii</i>	34	17	66.7	0.0079
Knowledge of the main problem caused by <i>T. gondii</i> in goats & sheep	36	15	70.6	0.0033
Knowledge of zoonotic implications of <i>T. gondii</i>	38	13	74.5	0.0006
Knowledge of how humans get infected by <i>T. gondii</i>	34	17	66.7	0.0079
Knowledge about <i>T. gondii</i> vaccination	7	44	13.7	<0.0001
Knowledge of whether inspected meat poses the risk of toxoplasmosis	12	39	23.5	0.0002

Furthermore, close to half of the respondents (47.1%) indicated that they would recommend routine vaccination against *N. caninum* and *T. gondii* if effective vaccines were available ($p>0.05$). On the other hand, only 33.3% and 13.7% of the respondents thought other animal health practitioners were well informed about toxoplasmosis and neosporosis, respectively ($p<0.05$). Ninety point two percent (90.2%) felt that it was necessary to educate farmers on these two diseases ($p<0.0001$), and 76.5% believed that *N. caninum* and *T. gondii* should be included in the abortion screening panel used at the central veterinary laboratory in Namibia ($p=0.0002$).

Table 3.5: Summary of the attitudes of the animal health practitioners regarding neosporosis and toxoplasmosis

ATTITUDES	%			
	YES	NO	YES	P value
Do you think <i>N. caninum</i> is present in Namibia?	27	24	52.9	0.6787
Is <i>N. caninum</i> important for Namibia?	29	22	56.9	0.3244
Should farmers be worried about <i>N. caninum</i> ?	29	22	56.9	0.3244
Whether they would recommend <i>N. caninum</i> vaccination by farmers if it were available	24	27	47.1	0.6787
If they thought the veterinary fraternity was well-informed about toxoplasmosis	17	34	33.3	0.0171
If they thought the veterinary fraternity was well-informed about neosporosis	7	44	13.7	<0.0001
Whether they thought it necessary to educate farmers on toxoplasmosis & neosporosis	46	5	90.2	<0.0001
I would recommend routine toxoplasmosis vaccination if it were available	24	27	47.1	0.6787
Would like <i>T. gondii</i> & <i>N. caninum</i> included in routine lab abortion screening.	39	12	76.5	0.0002

The practices of the Namibian animal health practitioners are summarised in Table 3.6. None of the 51 respondents routinely requested *N. caninum* or *T. gondii* tests in cattle, sheep or goat abortion cases.

Table 3.6: Summary of the practices of the animal health practitioners regarding neosporosis and toxoplasmosis

PRACTICES	%			
	YES	NO	YES	P value
Lab tests routinely requested in cattle abortion cases include <i>N. caninum</i>	0	51	0.0	<0.0001
Lab tests routinely requested for sheep & goat abortion cases include <i>T. gondii</i>	0	51	0.0	<0.0001
Have you ever requested lab tests for <i>N. caninum</i> or <i>T. gondii</i> ?	1	50	2.0	<0.0001
Do they usually interact with livestock farmers?	51	0	100.0	<0.0001
Whether they usually have farmers' meetings	19	32	37.3	0.0697
Topics generally discussed at farmers' meetings include <i>N. caninum</i> & <i>T. gondii</i>	0	51	0.0	<0.0001
Whether neosporosis or toxoplasmosis was ever discussed at any farmer gathering	2	49	3.9	<0.0001
Whether they talked to any cat owner about toxoplasmosis in the previous 12mo	11	40	21.6	<0.0001

Only one respondent (2% [n=51]) ever requested laboratory testing for *N. caninum* or *T. gondii* ($p<0.0001$). Although all respondents indicated that they routinely interact with livestock farmers, none of them normally discussed neosporosis or toxoplasmosis with them. Only 3.9% (2/51)

indicated that they had ever discussed either neosporosis or toxoplasmosis at a farmers' meeting ($p < 0.0001$), and only 21.6% (11/40) had talked to at least one cat owner about toxoplasmosis in the previous 12 months ($p < 0.0001$).

3.5. Discussion

To the author's knowledge, this is the first KAP study regarding neosporosis and toxoplasmosis in southern Africa. Although the results showed high literacy levels and many years of experience in livestock farming among most farmers, 84.1% (53/63) of them had never heard of neosporosis or toxoplasmosis and how animals get infected. These findings are similar to one Egyptian study where none of the 41 farmers interviewed knew about neosporosis (Gaber et al., 2021). In addition, studies in Saudi Arabia, Malaysia and Ethiopia showed a very low level of toxoplasmosis awareness among farmers, pregnant women and female students, ranging from 5.9% to 20.9% (Desta, 2015; Mahfouz et al., 2019; Sadiq et al., 2021). However, in another study, 84% of Italian women claimed to have heard about toxoplasmosis, although their level of knowledge was deemed superficial and incomplete (Martini et al., 2020).

Given that most farmers had never heard of neosporosis or toxoplasmosis, it is not surprising that an even higher proportion (92% [$n=63$]) were unaware of the risks posed by dogs and cats as definitive hosts of *N. caninum* and *T. gondii*, respectively. The farmers' attitudes concerning these two diseases further reflected this, as most (82.5%) thought other farmers were unaware of neosporosis and toxoplasmosis. Also unsurprising was that none of the respondents routinely vaccinated against *T. gondii*. However, it should be noted that there is only one registered vaccine against *T. gondii* in the world, Toxovax[®], which is a live attenuated S48 strain licensed for sheep (Ducournau et al., 2020; Monney et al., 2011). There is currently no commercial vaccine against *N. caninum* (Marugan-Hernandez, 2017). Bovilis Neoguard[®] was discontinued because it could only protect against horizontal transmission but not against vertical transmission of *N. caninum* (Monney & Hemphill, 2014).

It was encouraging, however, that most farmers indicated that they would be worried if abortions occurred in their livestock and were prepared to take remedial measures if neosporosis or toxoplasmosis was diagnosed in their animals. This shows that most farmers might be inclined to

change their attitudes and practices concerning the two diseases and adopt preventive measures like vaccination, keeping closed herds, and prior quarantining and testing all replacement stock. However, of concern was that only a small percentage of animal health practitioners (3.9% [n=51]) had ever discussed these two diseases with livestock producers. There is, therefore, a need for animal health practitioners to sensitise livestock producers about neosporosis and toxoplasmosis.

The fact that 76.2% (n=63) of the farmers indicated that livestock production contributed at least 50% of their livelihoods shows a very high reliance on livestock production. This further collaborates with other studies, which estimated that about 70% of Namibia's population depends directly or indirectly on agriculture, of which livestock production accounts for 80% (Humavindu & Stage, 2013; Meat Board of Namibia, 2015; Simasiku & Sheefeni, 2017). This high dependency on livestock production makes this industry sensitive to the effects of epizootics (Heath, 2008), including neosporosis or toxoplasmosis, if they are not controlled, which could significantly impact the farmers' livelihoods.

Although the number of animal health practitioners who participated (n=51) in the survey fell short of the calculated sample size of 61, it is important to note that despite these limitations, the data collected still provided invaluable insights. The animal health practitioners generally displayed a fair understanding of neosporosis and toxoplasmosis, as expected. However, only a small percentage (9.8%) listed *N. caninum* and *T. gondii* among the common causes of abortions they were aware of. In addition, many of them were unaware that veterinary-inspected meat still poses a risk of toxoplasmosis (Franco-Hernandez, Acosta, Cortés-vecino, & Gomez-Marin, 2015) if it is not adequately cooked. Furthermore, none of them routinely requested the central veterinary laboratory to test for *N. caninum* or *T. gondii* in abortion cases. This shows a lack of a deeper understanding of these two diseases among Namibian animal health practitioners. This probably explains why none of the respondents routinely discussed these diseases with farmers. Only a tiny percentage (3.9%) had ever discussed neosporosis or toxoplasmosis at any farmers' gathering, even though all the respondents routinely interacted with livestock farmers.

This study has demonstrated that the farming community in the Khomas region of Namibia is generally unaware of neosporosis and toxoplasmosis. However, there are indications that they might be willing to change their attitudes and practices if they were educated. On the other hand,

animal health practitioners had a basic knowledge of the two diseases. However, they lacked the more profound understanding and appreciation required to cultivate enough confidence to educate livestock farmers. Therefore, the failure of animal health practitioners to educate livestock producers about neosporosis and toxoplasmosis during their routine interactions with them presents a missed opportunity which should be utilised.

The limited depth of understanding among farmers and animal health practitioners could be attributed to the low prevalence of these pathogens due to the arid conditions found in Namibia, where animals are primarily raised under extensive farming conditions.

3.6. References

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Chapter 4: Seroprevalence and associated risk factors for bovine neosporosis in the Khomas region of Namibia

(Adapted from: Samkange, A., Chitanga, S., Tjipura-Zaire, G., Mutjavikua, V.G., Smith, J.W., Neves, L., Matjila, T., 2023b. Seroprevalence and associated risk factors of bovine neosporosis in the Khomas region of Namibia. Onderstepoort J. Vet. Res. 90, a2077. <https://doi.org/10.4102/ojvr.v90i1.2077>)

4.1. Abstract

Neospora caninum, an indirectly transmitted coccidian parasite, occurs worldwide and is one of the most important causes of abortion, especially in cattle. However, no studies have been done in Namibia to determine the *N. caninum* status in livestock. Therefore, this study aimed to determine the seroprevalence of *N. caninum* in cattle and the associated risk factors in the Khomas region of Namibia. Seven hundred thirty-six sera were collected from cows in 32 farming establishments. These comprised 698 beef and 38 dairy cattle sera, which were tested for anti-*N. caninum* IgG antibodies using a commercial ELISA kit. Questionnaires were administered concurrently to determine possible risk factors associated with *N. caninum* seropositivity. Forty-two beef cattle sera were positive, giving an animal-level seroprevalence rate of 5.7%. Eight of the 32 establishments had at least one positive animal, giving a herd-level seroprevalence of 25%. There was no significant association between seropositivity and the presence of dogs, jackals, history of abortions, farm size, number of cattle or average annual rainfall. The establishments with moderate to high numbers of Feliformia were 9.8 times more likely to be seropositive to *N. caninum* than those with none to low levels of the former ($p=0.0245$). The authors concluded that the seroprevalence level of *N. caninum* in the Khomas region was relatively low compared to other parts of the world and that the role of Feliformia in the epidemiology of bovine neosporosis needed to be further investigated.

4.2. Introduction

Neospora caninum is an obligate intracellular coccidian parasite of the Apicomplexa phylum and Toxoplasmatidae family, which occurs worldwide (Dubey et al., 1988; Gharekhani et al., 2021;

Goodswen et al., 2013). Natural infections have been recorded primarily in all major domestic livestock species, companion animals, chickens, and sparrows, and also in wildlife and captive animals, including deer, rhinoceros, rodents, rabbits, coyotes, dingos, wolves and foxes (Donahoe et al., 2015; Fereig and Nishikawa, 2020; McAllister, 2022). The canids are considered the definitive hosts.

The hosts mainly affected by neosporosis are dogs and cattle. It causes polyradiculoneuritis and polymyositis in young dogs, with dermatitis and neurological manifestations being characteristic in adult dogs (Decôme et al., 2019; Fereig & Nishikawa, 2020). Neosporosis is of significant economic importance in livestock, particularly cattle, in which infection is characterised by abortions, stillbirths, the birth of weak neonates, congenital malformation, increased numbers of culled cows and decreased milk yield (Gharekhani et al., 2020; Kierbić et al., 2019), with associated annual losses running into hundreds of millions of United States dollars (Demir et al., 2020; Reichel et al., 2013). Infection in these intermediate hosts is through ingestion of food and water contaminated with sporulated oocyst (Dubey et al., 2007; McAllister, 2022), with the subsequent vertical transmission in infected herds playing a more significant role (Gharekhani et al., 2020; Lefkaditis et al., 2020; Sinnott et al., 2017), hence the abortions. Several risk factors have been identified to be associated with infection in livestock, and these include the presence and number of farm dogs (Dubey & Schares, 2011), farm production systems and practices (Bartels et al., 2006; Dubey et al., 2007; Ghalimi et al., 2012; Otranto et al., 2003; Pare et al., 1998), antibodies against other pathogens like BVD (Björkman et al., 2000), human population density (Schares et al., 2004) and the region within countries (Bartels et al., 2006).

Whilst *N. caninum* is presumed to occur worldwide (Reichel et al, 2020), there is wide variation in infection prevalence across countries and regions, with some countries not having any data. Within the southern African region, evidence of bovine neosporosis has been reported in South Africa and Zimbabwe, with varied prevalence (Adesiyun et al., 2020; Jardine & Last, 1993, 1995; Jardine & Wells, 1995; Njiro et al., 2011). Other hosts which have shown evidence of infection in southern Africa include dogs (Jardine & Dubey, 1992), birds (Lukášová et al., 2018) and wildlife (Seltmann et al., 2020). Despite the importance of the livestock sector in Namibia

and the possible impact of *Neospora* infection on livestock reproductive health, no study has determined the prevalence, distribution and potential risk factors associated with infection in cattle production. Therefore, this study aimed to determine the seroprevalence of *N. caninum* infection in cows and heifers and the associated risk factors.

4.3. Materials and methods

4.3.1. Study population and setting

The study animals were the cows and heifers in the Khomas region of Namibia. This region is located in the central part of the country (Figure 3.1, Chapter 3), and it was chosen as a follow-up to a previous KAP study that had been done in the same region (Chapter 3). Namibia's sub-tropical climate varies from arid to semi-arid, and it is the driest country in sub-Saharan Africa (Mwazi & Shamathe, 2007). The country's central highlands receive an annual rainfall of between 300 mm and 400 mm and have an altitude of up to 1,900 metres (Kandiwa et al., 2017). The vegetation is predominantly shrub-veld, and ambient temperatures range from 7 °C in winter to 33 °C in summer (Kandiwa et al., 2019). The Khomas region has about 556 farming establishments, with approximately 44,000 primarily commercial beef cattle (Directorate of Veterinary Services, 2018). Additionally, there are a few resettlement farms and communal settlements.

4.3.2. Study design

This research was a cross-sectional study in which 32 farming establishments in the Khomas region were selected. These comprised 26 commercial beef farms, three communal beef herds, two dairy establishments, and one resettlement farm.

4.3.3. Sampling and data collection

Sample size calculations were done according to Pfeiffer's methods (Pfeiffer, 2002). For farm selection, estimated herd-level and individual animal-level prevalence rates of 20% and 10%, respectively, were used (Fereig et al., 2016; Nasir et al., 2012). A multistage sampling strategy was used to select the farming establishments included in this study. The region was first divided

into clusters ranging from three to seven farming units. Seven clusters were chosen using convenience sampling, especially targeting farms that had previously reported abortions.

The sample size formula for detecting disease in finite populations was used to determine the number of farms to be sampled:

$$n = [1 - (1 - \beta)^{1/d}] [(N - d/2) + 1/2]$$

Where β = confidence level (as a proportion); N = population size; n = sample size; d = number of diseased. When this formula was applied using an estimated farm-level prevalence of 10%, as estimated from the literature (Fereig et al., 2016; Nasir et al., 2012), the minimum number of farms required was calculated at 28, using the tables provided (Pfeiffer, 2002).

For calculating the number of individual animals to be sampled, the sample size formula for determining disease prevalence was applied using the following sample size formula (Pfeiffer, 2002):

$$n = 1.96^2 \times P(1-P)/d^2$$

Where n = sample size; d = precision; P = estimated prevalence.

When this formula was applied using a 50% prevalence to maximise the number of animals and a precision of 5%, the sample size was calculated as follows:

$$n = 1.962 \times 0.5 (1-0.5)/(0.05)^2 = 385$$

Therefore, a minimum of 385 cattle were required.

Subsequently, a stratified random sampling technique was used to select individual animals to be sampled at farm level. A total of 736 cows and heifers were selected. At least ten cattle were sampled from each of the 32 farming establishments. Blood was collected from the coccygeal or jugular veins using 20-gauge needles into plain vacutainer® blood tubes. During the animal sampling, a questionnaire was administered to determine the possible risk factors of *N. caninum* in the Khomas region.

4.3.4. Serological analysis

In the laboratory, sera were extracted from the clotted blood by centrifugation at 6,000 rpm for 10 minutes and stored at -20°C until testing. An indirect enzyme-linked immunosorbent assay (ELISA) (IDEXX *Neospora* X2[®] (IDEXX Laboratories, Inc, Maine 04092, USA) was used to detect specific anti-*N. caninum* IgG antibodies in the bovine sera according to the manufacturer's instructions. The ELISA kit used has a sensitivity and specificity of 100% and 93.3%, respectively (Wu et al., 2002).

All reagents were brought to 18-26°C before use and then mixed. Undiluted positive and negative controls and diluted test sera were added to antigen-coated plates and incubated. The solution was then removed, and the wells were washed four times. Conjugate was added to all the wells, followed by incubation and further washing. A substrate was then added, followed by a 15-minute incubation and the addition of a Stop Solution. The assays were duplicated, with absorbance values measured at 650nm. The sample-to-positive absorbance ratio (S/P) was used to determine the presence or absence of anti-*N. caninum* IgG antibodies. An S/P ratio of less than 0.50 was interpreted as negative, and a value of at least 0.50 was positive.

4.3.5. Data analysis

The questionnaire and serology results were captured in a Microsoft Excel 2013 spreadsheet. The captured data were analysed using descriptive statistics, chi-square test, odds ratios and multiple regression analysis at a 95% confidence level. Regression analysis was used on quantitative data to analyse the relationship between the dependent variable (number of *N. caninum* seropositive cases per establishment) and the independent variables (number of cattle and dogs per establishment, farm size and average annual rainfall). The chi-square test and odds ratios were used to analyse the relationship between categorical data (the history of abortions, sightings of stray dogs, number of jackals, number of Feliforma and rain scores over the previous three seasons) and the *N. caninum* status of each establishment. Finally, descriptive statistics were used to calculate the seroprevalence rates. All the statistical analyses were done using Microsoft Excel 2013.

4.4. Results

In the study, 736 cattle (698 beef and 38 dairy) were sampled from 32 farming establishments, 30 of which were beef herds, and the remaining two were dairy. Eight of the 32 establishments had at least one seropositive animal, giving an overall herd-level prevalence of 25%, and they were widely distributed across the sampling sites (Figure 4.1). An overall animal-level prevalence of 5.7% (42/736) was observed, which varied widely across the farming establishments. For the eight seropositive establishments, animal-level seroprevalence rates ranged from 3.0% (1/33) to 81% (17/21) (Table 4.1). The herd-level and animal-level seroprevalences for the beef herds were 26.7% (8/30) and 6.0% (42/698), respectively. All the seropositive establishments were beef-producing commercial farms. The herd-level and animal-level seroprevalences for the two dairy farms sampled were 0% (0/2 & 0/38). Additionally, all three communal establishments and one resettlement farm sampled (all beef) were seronegative.

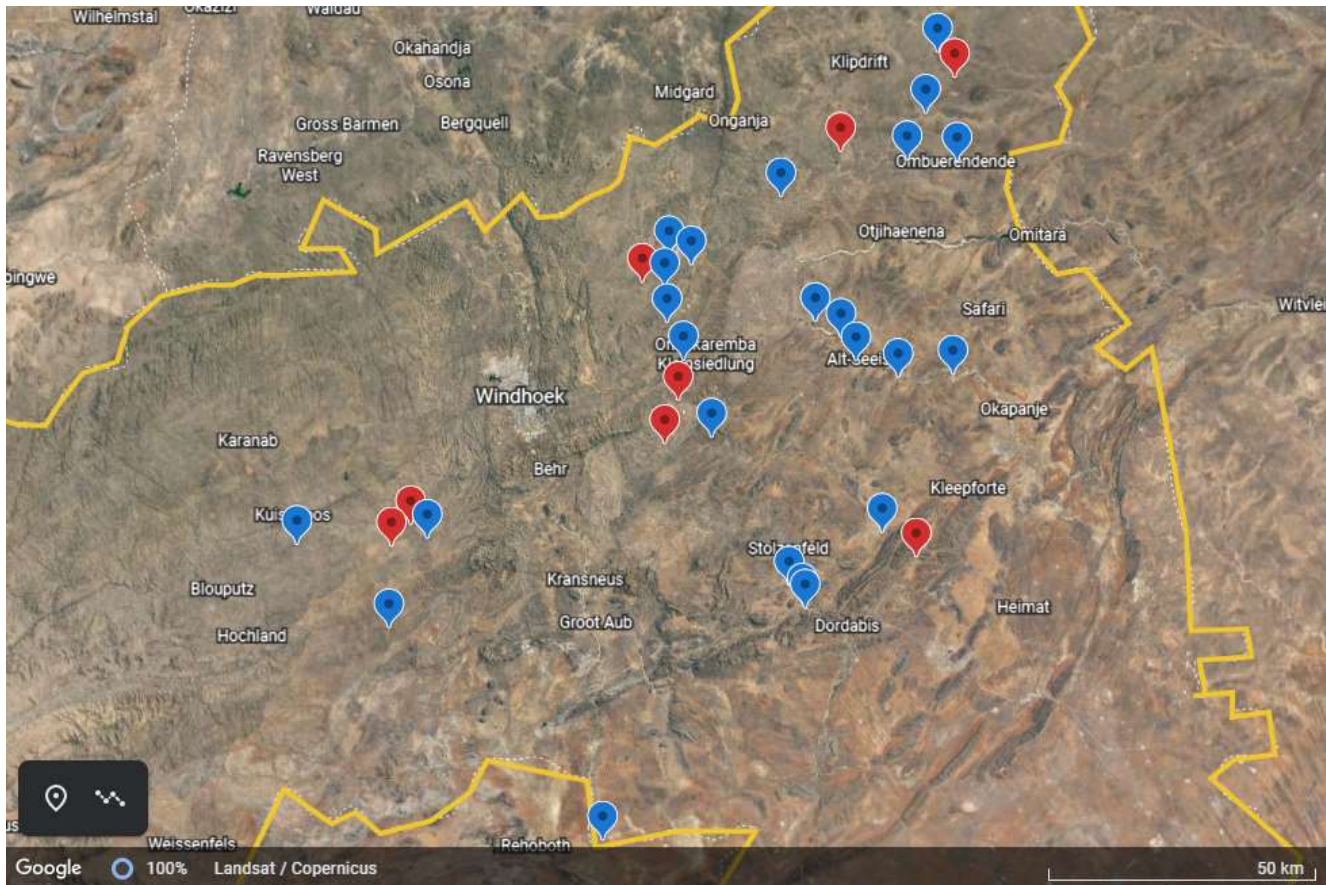


Figure 4.1: Google Earth map showing the distribution of the cattle farming establishments sampled in the Khomas region of Namibia, which is delineated in yellow. The eight positive farms are marked red, and the negative ones in blue.

Table 4.1: A summary of the *N. caninum* ELISA test results for cattle sampled in the Khomas region of Namibia

Farming establishment no.	Type of establishment	No. of cattle sera tested	No. seropositive	Prevalence (%)
1	Beef commercial	12	0	0.00
2	Resettlement	32	0	0.00
3	Beef commercial	30	0	0.00
4	Beef commercial	18	0	0.00
5	Dairy	25	0	0.00
6	Beef commercial	39	0	0.00
7	Beef commercial	30	0	0.00
8	Beef commercial	43	3	6.98
9	Beef commercial	23	0	0.00
10	Beef commercial	33	1	3.03
11	Beef commercial	24	0	0.00
12	Beef commercial	40	0	0.00
13	Beef commercial	26	0	0.00
14	Beef commercial	25	0	0.00
15	Beef commercial	27	0	0.00
16	Dairy	13	0	0.00
17	Beef commercial	15	0	0.00
18	Beef commercial	25	0	0.00
19	Beef commercial	24	7	29.17
20	Beef commercial	18	0	0.00
21	Beef commercial	20	10	50.00
22	Beef commercial	21	17	80.95
23	Beef commercial	22	0	0.00
24	Beef commercial	20	2	10.00
25	Beef commercial	23	0	0.00
26	Communal	13	0	0.00
27	Communal	11	0	0.00
28	Communal	5	0	0.00
29	Beef commercial	18	1	5.56
30	Beef commercial	12	0	0.00
31	Beef commercial	26	1	3.85
32	Beef commercial	23	0	0.00
TOTALS		736	42	5.71

The results of possible risk factors for *N. caninum* seropositivity in cattle investigated using a questionnaire are shown in Table 4.2. The table summarises the results of the statistical analyses of the putative risk factors for seropositivity.

Eighteen of the 32 establishments (56.25%) sampled had a history of abortions during the previous five years, ranging from low levels of less than 10% (score 1), moderate levels of 10% to 15% (score 2) to at least one overt abortion outbreak (score 3) in the same period (Figure 4.2). Eleven of the eighteen farms that reported at least one incident of abortions in the previous five years reported at least one incident of moderate to high abortion levels (scores between 2 and 3). Five of the eight farming establishments with at least one *N. caninum* seropositive animal had a history of previous abortions. However, this was not statistically significant on the chi-square test ($p=0.497$).

The annual rainfall received per farming establishment averaged 344 mm per year, ranging from 224 mm to 600 mm annually. The average annual rainfall for 72% (23/32) of the establishments ranged between 301mm and 400mm per year, whilst 25% (8/32) of the establishments received 201mm to 300mm annually (Figure 4.3). However, the average rainfall per establishment was not significantly associated with *N. caninum* seropositivity on multiple regression analysis ($p=0.143$).

Table 4.2: summary of the statistical analyses results performed on the possible risk factors associated with *Neospora caninum* seropositivity

POSSIBLE RISK FACTOR	ANALYSIS TOOL	P-VALUE	ODDS RATIO	CI-INTERVALS
Total no. of cattle	Multiple regression	0.946	-	-0.005-0.005
No. of dogs	Multiple regression	0.433	-	-0.255-0.112
Farm size	Multiple regression	0.713	-	-0.0003-0.0004
Average annual rainfall	Multiple regression	0.1428	-	-0.005-0.034
No. of Feliformia	Chi-square	0.024	9.8	0.061-4.504
Sighting of stray dogs	Chi-square	0.838	0.8	-1.769-1.435
History of abortions	Chi-square	0.497	0.7	-1.985-1.297
No. of jackals	Chi-square	0.854	0.6	-2.438-1.416

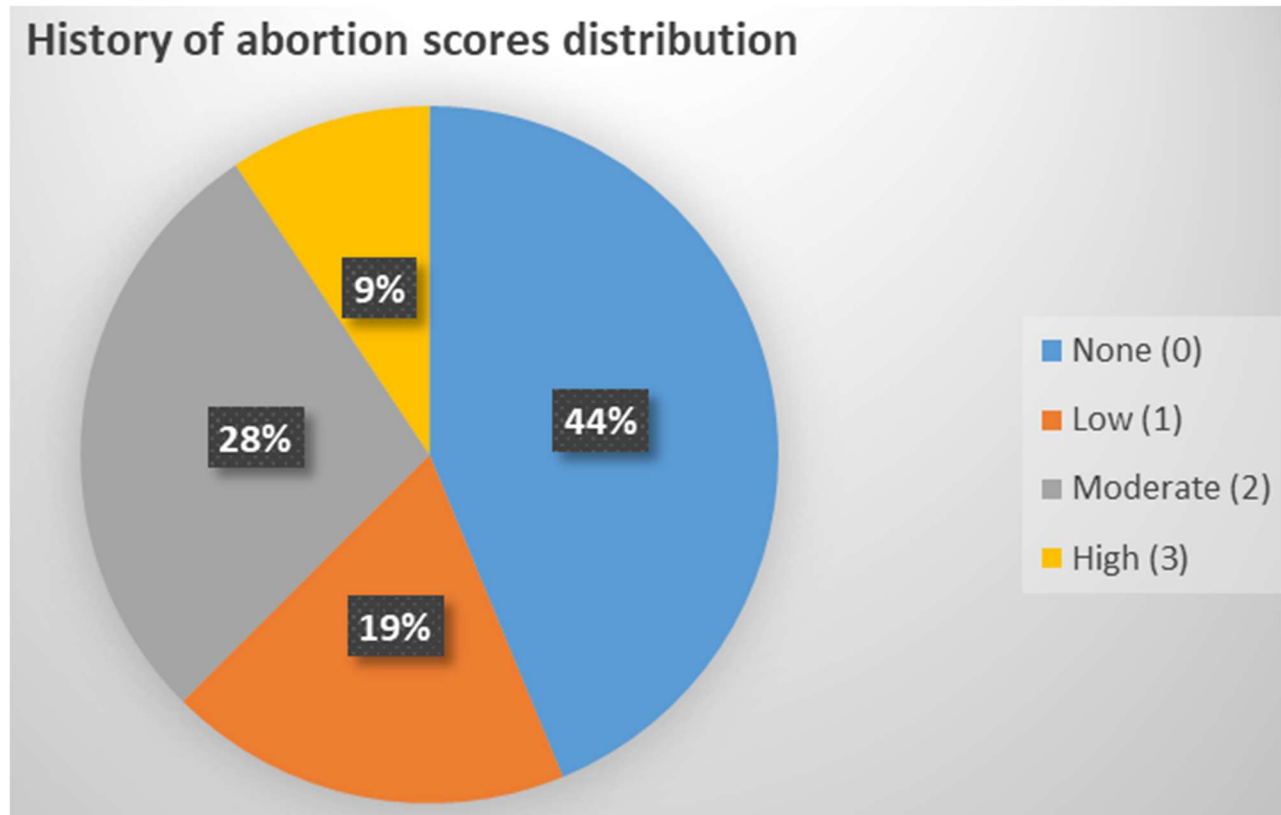


Figure 4.2: Distribution of abortion of scores among sampled establishments

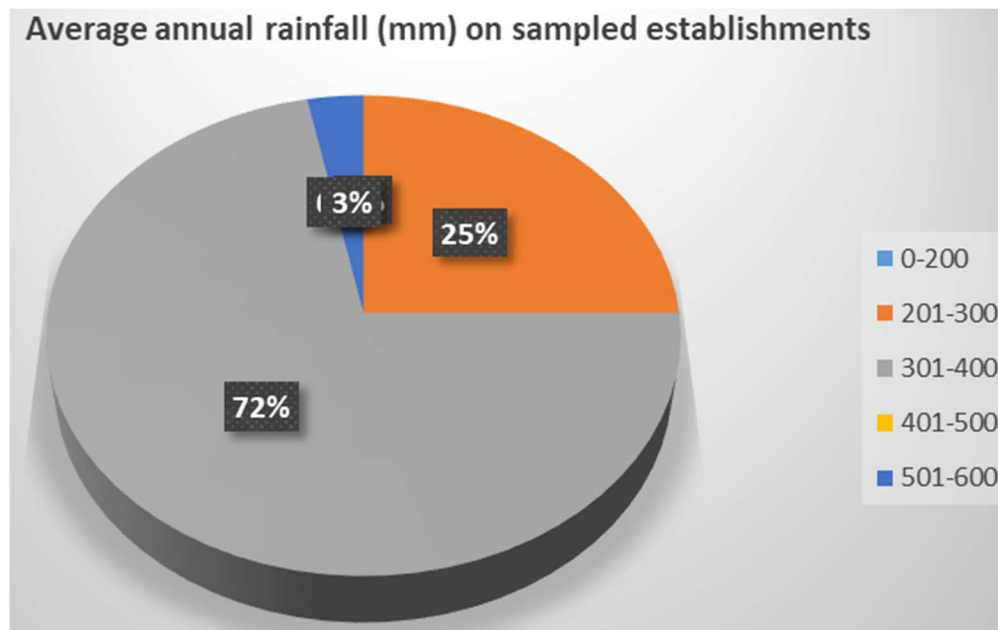


Figure 4.3: Average annual rainfall distribution among the sampled farming establishments

The sighting of Feliformia (primarily hyenas, cheetahs and leopards) at the farming establishments was significantly associated with *N. caninum* seropositivity amongst the cattle on the chi-square test and odds ratio analysis. The establishments with moderate to high numbers of Feliformia on their properties were 9.8 times more likely (OR = 9.8; 95% CI 0.061-4.504) to be seropositive to *N. caninum* than those with none to low levels ($p=0.0245$) (Figure 4.4).

Except for one, all the other 31 establishments had at least one dog, ranging from one to 41, with the latter being at a resettlement farm with many livestock owners and households (Figure 4.5).

The average number of dogs per establishment sampled was six. The presence of dogs in the establishments was not significantly associated with seropositivity on multiple regression analysis ($p=0.433$). On chi-square analysis, no significant association was found between *N. caninum* seropositivity and sightings of stray dogs in sampled establishments ($p=0.838$).

Distribution of Feliformia in sampled establishments

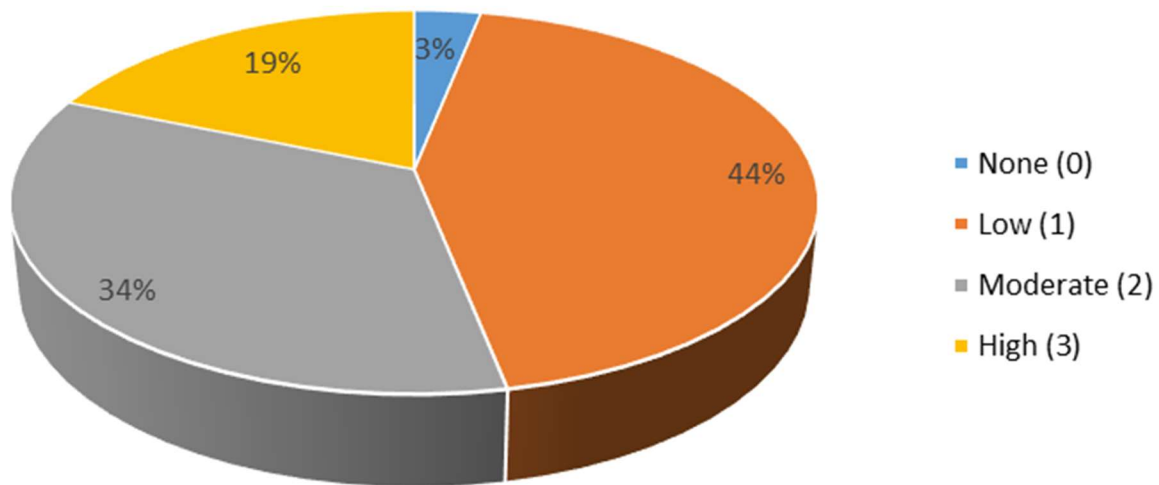


Figure 4.4: Feliformia score distribution among sampled establishments

Distribution of dogs on sampled establishments

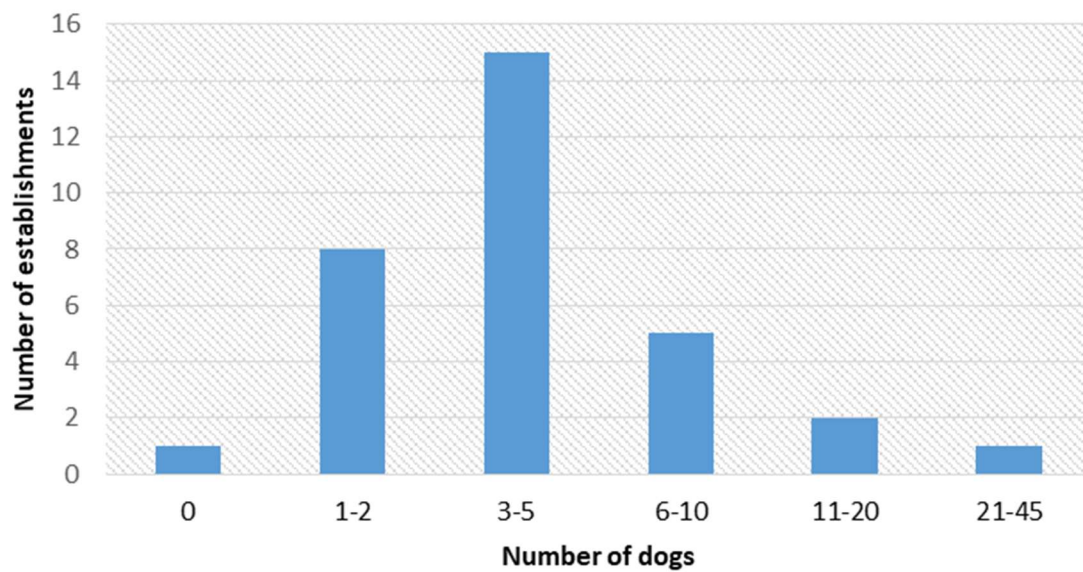


Figure 4.5: Distribution of dogs in sampled farming establishments

All the farming establishments reported observing high numbers of jackals on their properties (score 3); this was, however, not significantly associated with *N. caninum* seropositivity on chi-square analysis ($p=0.854$).

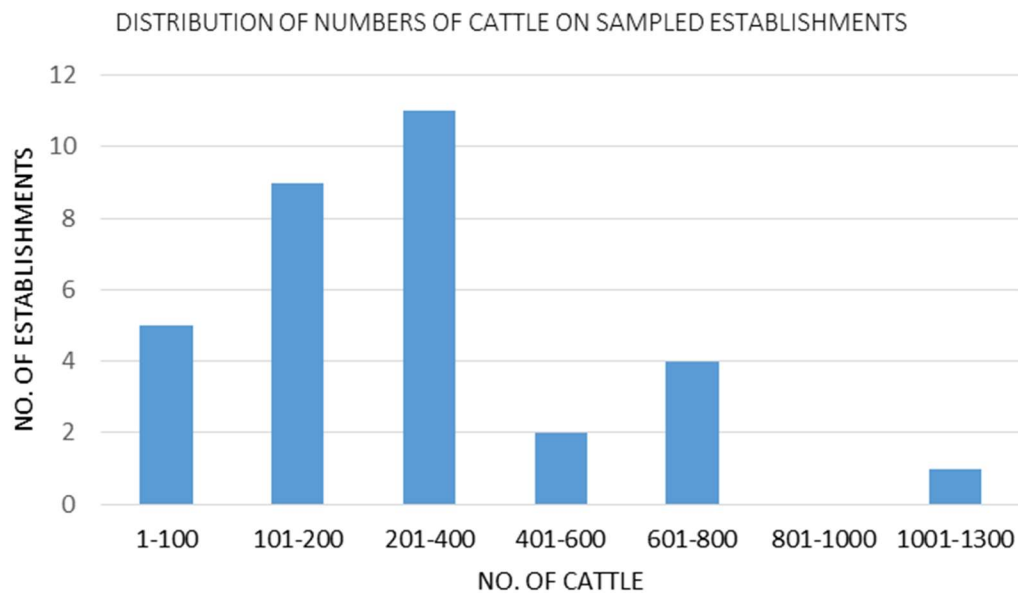


Figure 4.6: Distribution of the numbers of cattle in sampled cattle herds

The average number of cattle per sampled establishment was 301, ranging from 13 to 1205 animals. The modal range was between 201 and 400 cattle (Figure 4.6). On multiple regression analysis, there was no significant association between cattle numbers per establishment and seropositivity ($p=0.946$). There was also no significant association between farm size and seropositivity on multiple regression analysis ($p=0.713$).

4.5. Discussion

In this study, female cattle were targeted because vertical transmission is the most important in the epidemiology of *N. caninum* (De Aquino Diniz et al., 2019; Dubey et al., 2007). In addition, Wei and coworkers found that female cattle had a higher seroprevalence rate than males (Wei et al., 2022). Therefore, targeting females increased the probability of detecting positive animals.

The current study reports an overall animal-level seroprevalence of 5.7%, which is low. To the author's knowledge, this is the first study on *N. caninum* seroprevalence in cattle reported from Namibia. This observed prevalence is comparable to that reported in neighbouring South Africa, where a 5.2% seroprevalence was reported in beef cattle (Chisi et al., 2013). A similarly low seroprevalence rate was also detected in Iran (3.8%), where it was attributed to a combination of warm and dry climate followed by cold and dry conditions (Noori et al., 2019). These climatic conditions were deemed unfavourable for the survival of *N. caninum* oocysts in the environment (Noori et al., 2019).

On the other hand, humid conditions coupled with mild to warm environmental temperatures are ideal for the sporulation and survival of oocysts (Dubey et al., 2007) and have also been associated with higher incidences of *N. caninum* abortions (Wouda et al., 1999). Similar findings were also made in Northwest China, which has a generally hot and dry climate with very little rainfall in the summer months compared to the other parts of the country, and the area has been found to have the lowest *N. caninum* seroprevalence rate of 9.4% in the country (Wei et al., 2022). These scenarios perfectly mirror the semi-arid conditions of the Khomas region of Namibia, where the current study was conducted, and it could explain why the seroprevalence was low.

A study in northern Tanzania (Arusha region) determined a seroprevalence rate of 21.5% (Semango et al., 2019), which is much higher than the current study. The semi-arid nature of Namibia's climate could affect the survival and sporulation of the *N. caninum* oocysts in the environment (Dubey et al., 2007; Noori et al., 2019) and, therefore, reduce transmission. On the other hand, northern Tanzania is more humid and has a higher average annual rainfall (Kimaro et al., 2018) compared to Namibia, which provides a more conducive environment for the sporulation of *N. caninum* oocysts. This explains the higher prevalence rates in other countries like Tanzania (Kimaro et al., 2018; Semango et al., 2019), the northern part of Algeria (Ghalmi et al., 2012), Argentina (Moore et al., 2003), China (Qian et al., 2017), Colombia (Llano et al., 2018), North and Central America, Asia, Europe and India (Hebbar et al., 2022).

The low animal-level *N. caninum* seroprevalence rate in the current study could also be attributed to the fact that 95% (698/736) of the samples tested were from beef cattle compared to

only 5% (38/736) from dairy cattle. Some studies have found that beef cattle are less susceptible to *N. caninum* than dairy cattle and therefore tend to have lower seroprevalence rates (Fort et al., 2015; Gharekhani et al., 2020; Haddad et al., 2005; Quintanilla-Gozalo et al., 1999; Ribeiro et al., 2019).

All communal, dairy and resettlement establishments were negative, possibly because the number of animals sampled from this category was small. Therefore, a larger sample size might have yielded a different result. In communal setups, the dog population is expected to increase with the increased human population (Butler & Bingham, 2000), thereby increasing the probability of *N. caninum* infection in these herds. However, that did not seem to be the case in this study. Interestingly, one study in South Africa reported an animal-level seroprevalence of 26.8% in dairy cattle in KwaZulu Natal province (Chisi et al., 2013), unlike the findings of the current study where the two small-scale dairy herds sampled were both negative. These results could be because the dairy industry in Namibia is still in its infancy, with an estimated herd of only 3000 cattle (Bieldt, 2005). In addition, the largest dairy farm in the country was not sampled because it fell outside of the study area.

The current study found no significant association between the number of dogs at the farming establishments and *N. caninum* seropositivity, similar to findings in Tanzania (Mathew, 2017; Semango et al., 2019). However, these findings are somewhat surprising since dogs are the definitive hosts of *N. caninum* (Goodswen et al., 2013; McAllister et al., 1998). Other studies have indeed confirmed that the presence of farm dogs increases the risk of *N. caninum* infection in cattle (Abdeltif et al., 2022; Dubey et al., 2007; Fávero et al., 2017; Robbe et al., 2016) and goats (Rodrigues et al., 2020), most likely through faecal contamination of pastures and open water sources with oocysts. The lack of significant association with the number of dogs found in the present study might have been caused by the fact that most of the farm dogs were confined to the homesteads and, therefore, did not have easy access to livestock pastures. This, in turn, meant that the risk of pasture contamination, regardless of the number of dogs at the farming establishment, was markedly reduced. In their study, Guimaraes and coworkers found a positive correlation in *N. caninum* seroprevalence between cattle and dogs (Guimaraes et al., 2004). Therefore, the low prevalence in cattle could have been a reflection of low infection in dogs that

were confined to homes because they did not have access to carcasses. Often, dogs that are confined to homes are probably well looked after and fed commercial food. However, such an inference can only be speculative since our study did not test the farm dogs.

The *N. caninum* positive status was significantly associated ($p < 0.05$) with the presence of Feliformia (brown hyenas, leopards, cheetahs) but not black-backed jackals, despite most farmers reporting many sightings of jackals on their properties. Domestic dogs (*Canis familiaris*) and black-backed jackals (*Canis mesomelas*) look physically similar; therefore, the possibility of mistaking these two species in places where the presence of Feliformia was reported cannot be entirely disregarded. In a recent Namibian study, brown hyenas (*Hyaena brunnea*) and black-backed jackals (*Canis mesomelas*) were found to be seropositive to *N. caninum* (Seltmann et al., 2020); however, the significance of these findings in light of the results of the current study need to be further investigated. Furthermore, workers in Tanzania have also suggested the possible involvement of wildlife in the epidemiology of *N. caninum* after finding no association between dog ownership and cattle seropositivity (Semango et al., 2019).

In one study, self-rearing of replacement heifers was associated with an increased risk of bovine neosporosis (Otranto et al., 2003). However, the current study found no such association, despite all the establishments indicating that they self-reared replacement heifers. This discrepancy may be attributed to the unique context of the Khomas region, where our study was conducted. Specifically, the notably low seroprevalence of *N. caninum* in this area likely reduces the likelihood of vertical parasite transmission. Consequently, the diminished presence of the parasite within the region may have mitigated the anticipated association between self-rearing practices and neosporosis incidence.

This study found no significant association between abortion history and *N. caninum* seropositivity. One study in Northeast Algeria made similar findings, and the authors concluded that those cows were resistant to *N. caninum* abortions (Abdeltif et al., 2022). However, further investigations would be needed, given the extreme biological importance of the latter findings. The same scenario could also be at play in the Khomas region of Namibia, especially considering that significant variations in seropositivity have been found between countries, within countries, regions in the same country, and breeds (Dubey et al., 2007). Another study in Southeastern Iran

found no association between *N. caninum* seropositivity and a history of abortions (Noori et al., 2019). However, other studies have demonstrated a significant association between *N. caninum* seropositivity and a history of abortions (Ghalimi et al., 2012; Llano et al., 2018; Moore et al., 2009). As earlier noted, this discrepancy could be due to variations between countries or regions.

The findings in this study were generally in agreement with some studies in other parts of the world. However, the seroprevalence level of *N. caninum* in the Khomas region is much lower than in most other parts of the world. Furthermore, the role of *Feliformia* in the epidemiology of bovine neosporosis needs to be further investigated.

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Chapter 5: Seroprevalence and associated risk factors of *Toxoplasma gondii* in goats and sheep in the Khomas region of Namibia

(Adapted from: Samkange, A., Chitanga, S., Tjipura-Zaire, G.N., Mutjavikua, V.G., Smith, J.W., Neves, L., Matjila, T., 2023c. Seroprevalence and associated risk factors for *Toxoplasma gondii* infection of goats and sheep in the Khomas region of Namibia. J. South African Vet. Assoc. 94, 123–129. <https://doi.org/10.36303/JSAVA.548>)

5.1. Abstract

This study aimed to determine the seroprevalence levels of *Toxoplasma gondii* in sheep and goats and the associated risk factors in the Khomas region of Namibia. Two hundred ninety-nine and 345 sheep and goat sera, respectively, from 22 farming establishments were tested. An IDEXX Toxotest Ab®, a commercial ELISA kit, was used to screen for IgG antibodies to *T. gondii*. Overall, 3.68 % (11/299) of the sheep sera were positive, and 61.54 % (8/13) of the sheep flocks tested had at least one positive animal. Only one of the 345 goat sera from 19 flocks was positive, giving animal-level and herd-level prevalences of 0.29 % and 5.26 %, respectively. Sheep flocks had significantly greater animal-level and flock-level prevalences than goats ($p < 0.05$) and were 13.14 times more likely to be seropositive (OR = 13.14; CI 95 %: 1.686 - 102.382) than goat flocks. A questionnaire was also administered to identify any putative risk factors associated with seropositivity. Eight risk factors were evaluated, including the total number of goats, total number of sheep, farm size, average rainfall, presence of wild Felidae (African lions, caracals, cheetahs and leopards), presence of domesticated and stray cats and history of abortions in the flocks. Seropositivity to *T. gondii* in sheep was positively associated with the total number at the farming establishment, history of abortions and farm size ($p < 0.05$), but not with goats. The study determined that sheep in the Khomas region were probably more exposed to *T. gondii* infection than goats. It also found *T. gondii* seroprevalences that were much lower than those in similar studies from other countries in the sub-region and elsewhere.

5.2. Introduction

Toxoplasma gondii is an intracellular protozoan pathogen presumed to occur worldwide (Al-Malki, 2021). It is associated with livestock reproduction losses through abortions, stillbirths, mummifications, infertility, early embryonic death and foetal resorption (Al-Malki, 2021; Omonijo et al., 2022; Tagwireyi et al., 2019), especially in sheep and goats (Abbas et al., 2020). Sheep are the most susceptible to *T. gondii* infection among livestock (Cenci-Goga et al., 2013), whilst buffalo, cattle and horses are relatively resistant (Abbas et al., 2020; Belluco et al., 2016). The protozoan is considered an important zoonosis, and it is estimated that one-third of the world's human population is infected with *T. gondii* (Abbas et al., 2020; Innes, 2010; Kolören & Dubey, 2020; Tegegne et al., 2016; Zhang et al., 2013). The infection is usually subclinical in humans (Kalogeropoulos et al., 2022), with occasional acute toxoplasmosis outbreaks (Dubey, 2021; Kolören & Dubey, 2020) and severe toxoplasmic complications in immunocompromised individuals and congenital disabilities in fetuses if women are infected for the first time during pregnancy (Kalogeropoulos et al., 2022).

Toxoplasma gondii has a complex life cycle which involves the felids (domestic and wild cats) as definitive shedding oocysts into the environment through faeces (Belluco et al., 2016; Dubey, 1998), and the oocysts may take 2-3 days to sporulate and become infective (Dubey et al., 2011). The intermediate hosts, virtually all warm-blooded animals, including humans, get infected when they ingest food or water contaminated with sporulated oocysts (Pinto-Ferreira et al., 2019), infected raw or undercooked meat and other tissues, unpasteurised milk and milk products like cheese (Al-Malki, 2021; Pinto-Ferreira et al., 2019) and during pregnancy, by vertical transmission (Dubey, 1996). The definitive host gets infected when it consumes the tissues of the intermediate hosts (Tenter et al., 2000).

Varying *T. gondii* seroprevalence rates in sheep and goats have been reported in different parts of the world, for example, 12-36% in sheep in the Nordic-Baltic region (Olsen et al., 2019), 42.47% in goats in India (Bachan et al., 2018), 48.6% in sheep and 30.7% in goats in Greece (Tzanidakis et al., 2012), 41.1% in sheep in Costa Rica (Villagra-Blanco et al., 2019), and 26.1% in sheep in

Israel (Mazuz et al., 2023). In southern Africa, the seroprevalence rates reported in sheep and goats in Zimbabwe were 8.8% (n=216) and 7.1% (n=156), respectively, and in South Africa, the figures ranged from 4.3% (n=600) to 64.5% (n=121) in sheep and 53.9% (n=128) in goats (Omonijo et al., 2022; Tagwireyi et al., 2019). However, in Namibia, the only documented studies regarding *T. gondii* infection involved wildlife and humans (Joubert & Evans, 1997; Selmann et al., 2020; van der Colf et al., 2020) but not in livestock.

The risk factors linked to the infection in other countries include the presence of felid definitive hosts, flock size, age, geographical factors, farm management factors and biosecurity (Caballero-Ortega et al., 2008; Stelzer et al., 2019). Therefore, this study aimed to determine the seroprevalence levels of *T. gondii* in goats and sheep and the associated risk factors in the Khomas region of Namibia.

5.3. Materials and methods

5.3.1. Study area

The study area was Namibia's Khomas region, located in the central part of the country (Figure 5.1). Namibia's sub-tropical climate varies from arid to semi-arid, and it is the driest country in sub-Saharan Africa (Mwazi & Shamathe, 2007). The country's central highlands receive an annual rainfall range of 300 - 400 mm and have an altitude of 1,900 m (Kandiwa et al., 2017). The vegetation is predominantly shrub-veld, and ambient temperatures range from 7 °C in winter and up to 33 °C in summer (Kandiwa et al., 2019). The Khomas region has approximately 560 livestock farming units, mainly commercial, with some resettlement farms and communal settlements (Directorate of Veterinary Services, 2018).

5.3.2. Study animals

The study animals were adult goats and sheep. This region has approximately 91,000 sheep and 20,000 goats distributed among some of the estimated 560 farming establishments (Directorate of Veterinary Services, 2018) that primarily farm beef cattle. All the animals were extensively reared.

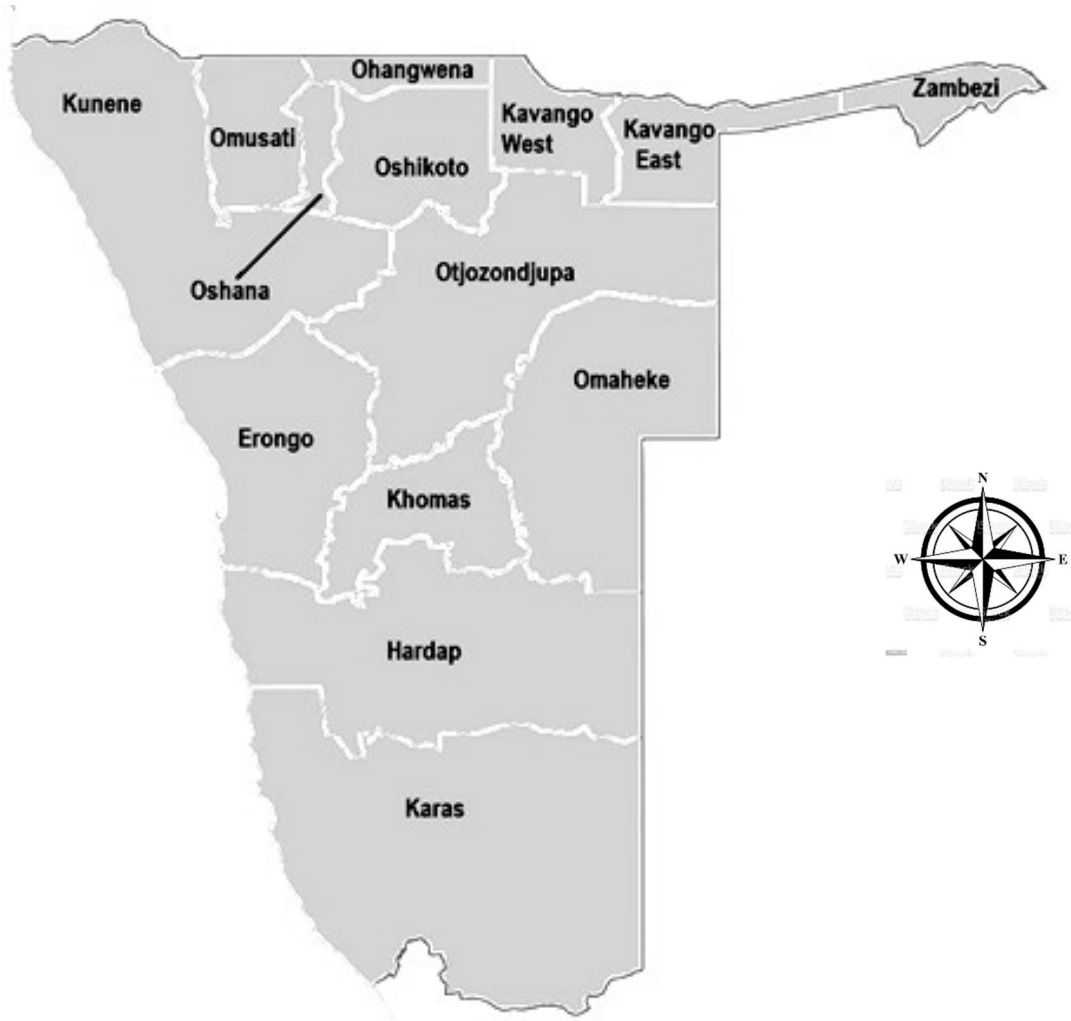


Figure 5.1: Map of Namibia showing its fourteen regions. The Kunene region is located centrally, as shown on the map. Source: <https://www.hauniversity.org/en/Namibia-Regions.shtml> The map was used with permission.

5.3.3. Sampling and data collection

Sample size determination was done according to Pfeiffer (2002), using estimated herd-level and animal-level prevalences of 20% and 10%, respectively (Omonijo et al., 2022; Tagwireyi et al., 2019), at 95% confidence level and 5% precision.

The sample size formula for detecting disease in finite populations was used to determine the number of sheep and goat farms to be sampled:

$$n = [1-(1-\beta)^{1/d}][(N-d/2)+1/2]$$

Where β = confidence level (as a proportion); N = population size; n = sample size; d = number of diseased. When this formula was applied using an estimated farm-level prevalence of 20%, as estimated from the literature (Omonijo et al., 2022; Tagwireyi et al., 2019), the minimum number of farms required was calculated at 14, using the tables provided (Pfeiffer, 2002).

For calculating the number of individual animals to be sampled, the sample size formula for determining disease prevalence was applied using the following sample size formula (Pfeiffer, 2002):

$$n = 1.96^2 \times P(1-P)/d^2$$

Where n = sample size; d = precision; P = estimated prevalence.

When this formula was applied using a 50% prevalence to maximise the number of animals and a precision of 5%, the sample size was calculated as follows:

$$n = 1.962 \times 0.5 (1-0.5)/(0.05)^2 = 385$$

Therefore, a minimum of 385 sheep and goats were required.

Convenience sampling was used to select the farms because few farming establishments rear sheep and goats in the Khomas region. At the farm level, multistage sampling was employed, where adult sheep and goats were segregated into distinct groups within each flock. These separate groups were then assigned individual numbers, and a random number generator was utilised to select the specific numbers for sampling according to the pre-determined sample size for each flock. Blood was collected from the jugular veins into plain Vacutainer® blood tubes using 20-gauge needles. In the laboratory, the clotted blood samples were centrifuged at 1,957 x g for 10 min to separate the sera. Next, sera were pipetted into 2ml cryovials (Labocare™, EREZ Medical UK, London), which were then stored at -20°C until they were tested.

A questionnaire was administered during sample collection to determine the possible risk factors for *T. gondii* infection. The questionnaire solicited information on the following risk factors: total number of sheep and goats on the farm, farm size, average rainfall, presence of sylvatic Felidae, history of abortions, presence of domesticated cats, and the presence of stray cats. The selection of these risk factors was based on available literature sources in light of the prevailing conditions in the Khomas region of Namibia. The questionnaire was first validated by administering it to a small group of six participants as a pilot test before its use in the main study after making the necessary modifications.

5.3.4. Serological analysis

The IDEXX Toxotest[®] (IDEXX Laboratories, Inc, Maine 04092, USA) test kit, an indirect enzyme-linked immunosorbent assay (ELISA), was used to test for specific anti-*T. gondii* IgG antibodies in sheep and goat sera. The ELISA test has a sensitivity and specificity of 98.8% and 92.8%, respectively (Patel et al., 2017).

The test was performed according to the manufacturer's instructions. All reagents were brought to 18 - 26°C and mixed. First, diluted positive and negative controls and diluted test sera were added to antigen-coated plates, mixed and then incubated at 37° C for 1 hr. After incubation, the excess solution was discarded, and the wells were washed thrice with Wash Solution. Conjugate was then added to all the wells, after which the plates were tightly sealed and subsequently incubated for an additional one hour at 37°C. Again, the excess conjugate solution was discarded, and the wells were washed thrice with Wash Solution. Next, a TMB Substrate was added to each well, and the plates were incubated at 18 – 26 °C for 15 min. A Stop Solution was then added to each well. Finally, the results were read at a wavelength of 450 nm using an ELISA reader within 2 hr after adding the Stop Solution.

The positive and negative controls were then validated, and ratios of a sample to positive control (S/P ratios) were interpreted as follows: a ratio of less than 20% was interpreted as negative; a ratio of at least 20% but less than 30% was suspect; a ratio of at least 30% but less than 100% was interpreted as weak positive. Finally, a ratio of at least 100% was interpreted as positive.

5.3.5. Data analysis

The serology and questionnaire data were captured in a Microsoft Excel 2013 spreadsheet, in which all the statistical calculations were done. Descriptive statistics were then used to calculate herd-level and animal-level prevalence (P) rates. The z-test for comparing two proportions and odds ratios (OR) were used to compare the prevalence rates between sheep and goats. Finally, linear regression analysis and the Chi-square test were used to evaluate the risk factors. Linear regression analysis was used to evaluate continuous risk factor variables, where the dependent variable was seropositivity, whilst the independent variables were farm size, average annual rainfall, and the number of sheep and goats. The Chi-square test was used to evaluate the following categorical risk factor variables: the presence of sylvatic Felidae, history of abortions, the presence of domesticated cats, and the presence of stray cats.

5.4. Results

Eleven of the 299 sheep sera from 13 farming establishments were positive, giving an animal-level prevalence of 3.7% (Confidence Interval [CI] 95%: 2.07-6.47) (Table 5.1). Another five sheep (P 1.7 %; CI 95 %: 0.72 - 3.85) were suspicious but were not retested for confirmation. Eight of the thirteen establishments tested had at least one positive sheep, giving a herd-level prevalence of 61.5% (CI 95 %: 35.52 - 82.29) (Figure 5.2). Of the 345 goat sera tested from 19 farming establishments, only one was positive, giving an animal-level prevalence of 0.3 % (CI 95 %: 0.05 - 1.62) and a herd-level prevalence of 5.3 % (CI 95 %: 0.90 - 24.60) (Table 5.2). Seven goats (2.0 %) were suspicious but were not retested for confirmation.

Sheep had a significantly greater individual-level prevalence than goats ($p = 0.0015$) and were 13.14 times more likely to be positive (OR = 13.14; CI 95 %: 1.686 - 102.382) than goats. In addition, the sheep's herd-level prevalence was significantly greater than for goats ($p = 0.0005$), and sheep farming establishments were 28.8 times more likely to have at least one positive animal than goat flocks (OR = 28.8; CI 95 %: 2.879 - 288.103).

Table 5.1: Sheep *Toxoplasma gondii* IgG antibody ELISA results for 13 farm units, Khomas region.

Farm no.	No. of sheep sera	No. positive	No. suspect	% positive	% suspect
1	15	2	1	13.33	6.67
2	32	0	0	0.00	0.00
3	30	3	0	10.00	0.00
4	29	1	1	3.45	3.45
5	30	1	0	3.33	0.00
6	31	1	0	3.23	0.00
7	21	0	1	0.00	4.76
8	24	0	0	0.00	0.00
9	18	1	2	5.56	11.11
10	16	1	0	6.25	0.00
11	2	0	0	0.00	0.00
12	5	1	0	20.00	0.00
13	46	0	0	0.00	0.00
TOTALS	299	11	5	3.68	1.67

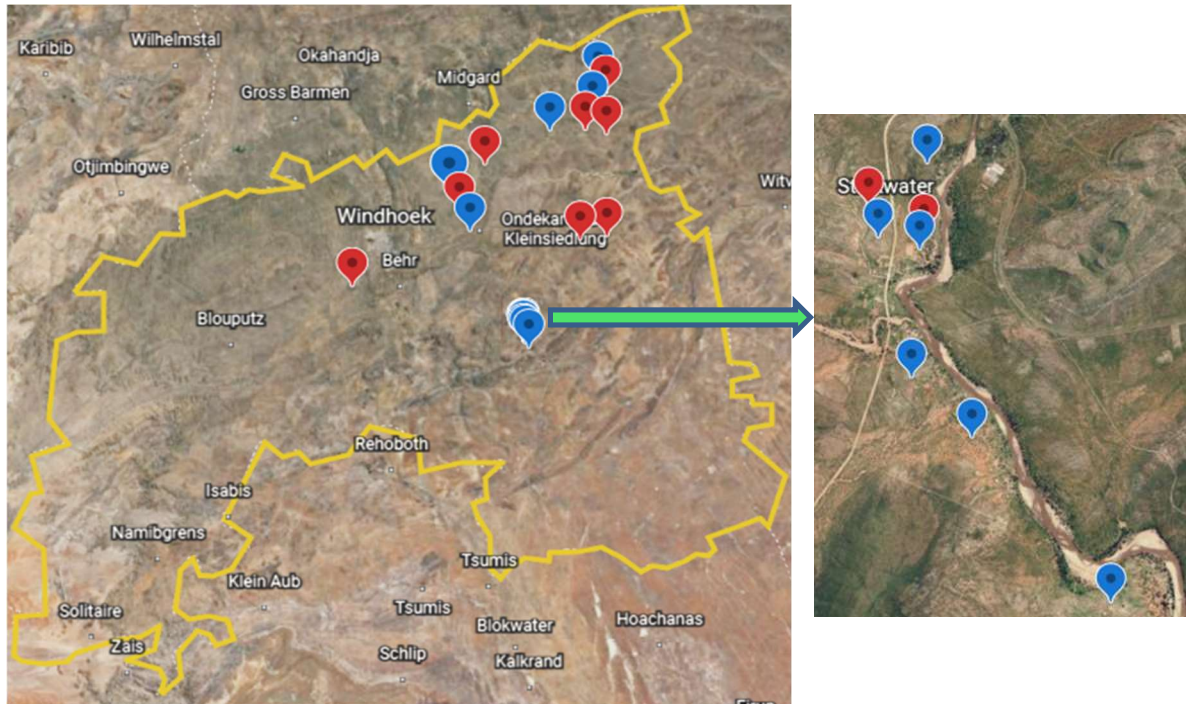


Figure 5.2: Google map showing the location of *T. gondii* positive and suspicious (red markers) and negative (blue) farming establishments for sheep and goats in the Khomas region. The map on the right is a magnified view of one cluster, as shown by the green arrow.

Table 5.3 shows the list of eight potential risk factors investigated using questionnaires. Seropositivity to *T. gondii* was positively associated with the total number of sheep at the farming establishment, a history of abortions and farm size ($p < 0.05$). However, there was no significant association between seropositivity and the number of goats at the establishment, the presence of Felidae (African lions, caracals, cheetahs, leopards), the presence of domesticated cats, the presence of stray cats and the average rainfall ($p > 0.05$).

Table 5.2: Goat *Toxoplasma gondii* IgG antibody ELISA results for 19 farm units, Khomas region.

Farm no.	No. of goat sera	No. positive	No. suspect	% positive	% suspect
1	12	0	1	0.00	8.33
2	15	0	3	0.00	20.00
3	20	0	0	0.00	0.00
4	14	0	0	0.00	0.00
5	30	0	0	0.00	0.00
6	19	0	1	0.00	5.26
7	21	1	0	4.76	0.00
8	19	0	0	0.00	0.00
9	22	0	0	0.00	0.00
10	18	0	1	0.00	5.56
11	17	0	0	0.00	0.00
12	11	0	0	0.00	0.00
13	15	0	0	0.00	0.00
14	11	0	0	0.00	0.00
15	2	0	0	0.00	0.00
16	15	0	0	0.00	0.00
17	3	0	0	0.00	0.00
18	18	0	0	0.00	0.00
19	63	0	1	0.00	1.59
TOTALS	345	1	7	0.29	2.03

Table 5.3: Summary of the significance of association (p-value) between the dependent variable (seropositivity) and the list of eight independent variables (risk factors) in the Khomas region

Risk factor	Statistic used	P-value
Total no. of goats	Linear regression	0.4382
Total no. of sheep	Linear regression	0.0209
Farm size	Linear regression	0.0011
Average rainfall	Linear regression	0.9662
Presence of sylvatic Felidae	Chi-square	0.8047
History of abortions	Chi-square	0.0301
Presence of cats	Chi-square	0.3384
Presence of stray cats	Chi-square	0.6646

5.5. Discussion

To the author's knowledge, this is Namibia's first study on *T. gondii* infection in sheep and goats. The study determined animal-level prevalence rates of 3.68 % and 0.29 % in sheep and goats, respectively, which are lower compared to South Africa, India, Iran and Italy, where seroprevalence rates ranged from 27 % to 64.46 % (Bachan et al., 2018; Gazzinos et al., 2015; Sharif et al., 2015; Tagwireyi et al., 2019). These low prevalence rates in the Khomas region of Namibia might be due to its harsh climate, which is predominantly hot and dry and characterised by infrequent and unpredictable rainfall. As a result, almost 92% of its land area falls under very-arid, arid, or semi-arid categories, ranking the country as the second driest region after the Sahara Desert (The World Bank Group, 2021).

Exposure to extreme temperatures and UV radiation has been shown to inactivate *T. gondii* oocysts. For instance, *T. gondii* oocysts can survive 32 days at 35°C but only nine days at 40°C (Robert-Gangneux & Darde, 2012). In addition, continuous exposure to UV irradiation has been shown to inactivate >99.9% of the *T. gondii* oocysts exposed (Dumetre et al., 2008). These conditions are common in the Namibian climate (Samkange et al., 2020; The World Bank Group, 2021), creating an unfavourable environment for the *T. gondii* oocysts to sporulate (Yan et al., 2016). Therefore, *T. gondii* infections tend to be high in warm and humid environments but low in hot and dry climates (Meerburg & Kijlstra, 2009).

Some studies have shown that animals reared under extensive conditions have lower seroprevalence rates than those reared under intensive or semi-intensive conditions (Tagwireyi et al., 2019; Tzanidakis et al., 2012). In contrast, a study conducted by van der Puije and colleagues showed opposite findings, indicating that sheep raised under extensive conditions had a significantly higher seroprevalence than those raised semi-intensively (van der Puije et al., 2000). The conditions in which animals are raised, fed, and watered in different production systems can affect the risk of contamination with *T. gondii* oocysts and exposure (Stelzer et al., 2019). For example, intensive farming systems, which often involve high levels of confinement, may have lower exposure to oocysts than extensive or other systems that allow outdoor access for animals (Stelzer et al., 2019). However, if feed supplements are stored in places where they may attract domestic or feral cats, which then go into these feed stores in search of rodent prey, the feed could

end up being contaminated with cat faeces and, therefore, *T. gondii* oocysts. This potentially explains why intensive farming is not always associated with lower toxoplasmosis prevalence (Stelzer et al., 2019). However, all small ruminants in the current study were grazed extensively, which meant less exposure to viable *T. gondii* oocysts, most likely due to adverse weather conditions.

Similarly low seroprevalences were found recently among pregnant women in Windhoek (2.6%; n = 344) (van der Colf et al., 2020), which is in the Khomas region of Namibia, and earlier among blood donors from the central part of the country (0.96%; n=312) (van der Colf et al., 2014), figures that were lower compared to other developing countries, and the former authors attributed this to geographical and climatic factors. Humans can acquire *T. gondii* infection by consuming undercooked or raw meat (Tonouhewa et al., 2017), particularly mutton and goat meat that contains tissue cysts (Al-Kappany et al., 2018; Al Hamada et al., 2019). Additionally, unpasteurised goat milk can also be a source of infection for humans (Jones et al., 2009). However, beef and cow milk are not significant sources of infection because cattle can effectively eliminate *T. gondii* (Esteban-Redondo & Innes, 1997), although other authors contend that the exact impact of beef on the epidemiology of *T. gondii* infections in humans remains uncertain (Dubey et al., 2020b). Another way humans can acquire *T. gondii* infection is by consuming fresh produce contaminated with oocyst-infected cat faeces. Fresh fruits, raw vegetables used in salads, and similar products can be contaminated with these oocysts, which can survive for months in the environment (Lass et al., 2012; Marques et al., 2020). The low seroprevalence rates found in sheep and goats in this study imply that the risk of human exposure to *T. gondii* tissue cysts from small ruminant tissues and milk products and oocysts from the environment could be low. Some authors have described sheep and goats as biological indicators for *T. gondii* oocysts in the environment (Al-Kappany et al., 2018), which possibly explains why human infections are also low. Hence, the findings of the current study are in agreement with previous human studies conducted in Namibia, indicating low seroprevalence rates and demonstrating that *T. gondii* infections in sheep, goats and humans in the Khomas region are very low compared to the rest of the world.

Recent reviews have compared worldwide seroprevalences in sheep and goats using different serological tests. The seroprevalence rates in sheep ranged from 0 to 98.9%, whereas in goats, they

ranged from 1 to 95.2% (Dubey et al., 2020; Dubey et al., 2020a), and the serological results were not affected by the type of test used. The current study found that sheep had significantly higher animal-level and herd-level prevalences than goats. Similar trends have been reported in many parts of the world, including Northern Italy (Gazzinos et al., 2015), Africa, the Caribbean Islands, Greece, Portugal, Spain, Poland (Moskwa et al., 2018; Stelzer et al., 2019), Lebanon (Khalife et al., 2022) and Colombia (Martínez-Rodríguez et al., 2020). In addition, studies in Iran and South Africa also found higher seroprevalences in sheep than in goats, although they were not statistically significant (Bahreh et al., 2021; Tagwireyi et al., 2019). However, studies in the USA and Egypt recorded a higher seroprevalence in goats than sheep (Al-Kappany et al., 2018; Mishra et al., 2016). Therefore, with few exceptions, the current study agrees with reports from most studies from other parts of the world, which indicate that sheep are more infected than goats. The explanation could be that sheep are typical grazers, whereas goats are browsers (Yisehak et al., 2016), which makes the sheep more exposed to *T. gondii* oocysts than goats.

Toxoplasmosis is a significant cause of abortion for sheep and goats (Abbas et al., 2020; Dubey & Jones, 2008). The significant association between seropositivity to *T. gondii* and a history of abortions found in the current study is in agreement with several studies done elsewhere: Northern Iraq (Al Hamada et al., 2019); Central Ethiopia (Gebremedhin et al., 2013) and a review by Stelzer et al. (2019). However, Abdelbaset et al. (2020) did not find such an association in Egypt.

The total number of sheep at the farming establishment is another significant risk factor determined in the current study. Since sheep appear more at risk of *T. gondii* infection than goats, as established in this study, it is logical that the higher the number of sheep at an establishment, the greater the risk of toxoplasmosis. An alternative explanation could be that when the level of environmental contamination is minimal, having more animals increases the probability of contracting the infection compared to having fewer animals. However, this disagrees with an Italian study where seropositivity decreased with increasing flock size (Cenci-Goga et al., 2013). It is worth noting, however, that this Italian research studied large flock sizes numbering 300-400 sheep, unlike the current study in which flock sizes were much smaller. Additionally, climatic conditions in

Tuscany, Italy, are very different from the semi-arid Namibian conditions, which could affect the epidemiology of the disease.

Farm size was also positively associated with *T. gondii* seropositivity, implying that larger farming establishments had a significantly higher seropositivity rate in the Khomas region. A similar relationship was also observed in southern Italy (Vesco et al., 2007). Additionally, larger flock sizes have been associated with higher seroprevalences (Caballero-Ortega et al., 2008; Stelzer et al., 2019), which might be indirectly related to farm size. Some larger farms in Namibia at the livestock/wildlife interface would be expected to have more wildlife Felidae in certain places, which have been reported to be seropositive to *T. gondii* (Seltmann et al., 2020). Therefore, the chances of such felids contaminating the pastures would be higher on larger farms.

The small ruminants in the Khomas region have been exposed to *T. gondii* infection, although the seroprevalence is lower than that of other countries in the region and elsewhere. Sheep, which are grazers, appear more exposed to *T. gondii* infections than goats, which tend to feed on browse. The author recommends *T. gondii* screening for all ruminant abortion samples, including cattle, submitted to the Central Veterinary Laboratory. In addition, the country's toxoplasmosis status should be continuously monitored.

5.6. References

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Chapter 6: Molecular analysis of *Neospora caninum* in cattle and *Toxoplasma gondii* in sheep and goats in Namibia

6.1. Abstract

This study sought to investigate the presence of genetic profiles of *N. caninum* in cattle and *T. gondii* in sheep and goats within the Khomas region of Namibia and to characterise any detected DNA associated with these parasites. A total of 199 bovine samples were collected, including 110 brain specimens, 75 heart tissues, and 14 blood samples. The samples were assayed with conventional PCR targeting the *pNc5* gene, and all the samples were negative for *Neospora* DNA. In addition, 174 brain and heart samples from healthy sheep and goats intended for human consumption at three abattoirs in the Khomas region were also tested for the presence of *T. gondii* DNA. Of these, 16.7% tested positive for *T. gondii* DNA using nested PCR targeting the B1 gene. In sheep, the heart exhibited a higher molecular prevalence (20.3%) than the brain (14.6%), with no significant difference. Overall, sheep had a 17.4% molecular prevalence, while goats showed 7.7%. All the *T. gondii* isolates detected were closely aligned to the *T. gondii* type III genotype. This study detected a significant prevalence of *T. gondii* in seemingly healthy sheep and goat meat in the Khomas region, suggesting that thorough cooking is advisable to mitigate human transmission. Routine vaccination of sheep and goats is also recommended to reduce abortion in small ruminants and human exposure risks. These findings underscore the importance of surveillance, monitoring and preventive measures for *T. gondii* infections in livestock designated for human consumption in Namibia.

Keywords: cattle; sheep; goats; *N. caninum*; *T. gondii*; molecular; Namibia

6.2. Introduction

Neospora caninum and *Toxoplasma gondii* are obligate intracellular Apicomplexan parasites with an indirect life cycle. Cats are the definitive hosts for *T. gondii* (Dubey, 1995), while dogs

and other canids are the definitive hosts for *N. caninum* (Hebbar et al., 2022; McAllister et al., 1998). Infection by *N. caninum* and *T. gondii* causes neosporosis and toxoplasmosis, respectively, which predominantly results in reproductive losses and the consequent economic losses in cattle and small ruminants (sheep and goats), respectively (Gharekhani et al., 2021; Reichel et al., 2013; Stelzer et al., 2019).

At least one-third of the human population is thought to be infected with the intracellular coccidian parasite *Toxoplasma gondii* (Marques et al., 2020; Mumtaz et al., 2022). All warm-blooded animals, especially food animals like cattle, sheep, goats, pigs, poultry, and camels, are prone to toxoplasmosis (Tonouhewa et al., 2017). In the European Union, 80% of the population has reportedly been exposed to *T. gondii*, according to Marino and colleagues (Marino et al., 2017). Furthermore, immunosuppressed patients are more likely to get clinical toxoplasmosis (Kalogeropoulos et al., 2022).

In small ruminants, *T. gondii* infection causes reproductive losses through early embryonic deaths, resorption, mummification, neonatal deaths, stillbirths, and abortions (Mumtaz et al., 2022). This results in longer lambing/kidding intervals, higher culling rates, reduced milk production, and reduced value of breeding animals, leading to reduced productivity and significant economic losses (Mumtaz et al., 2022). These losses have been estimated at US\$10-15 million annually in the UK and Uruguay combined (Stelzer et al., 2019).

Toxoplasmosis in humans has been associated with spontaneous abortion (Kalantari et al., 2021), stillbirth, myocarditis, cerebral disease, epilepsy, encephalitis, blindness (Mazuz et al., 2023), congenital malformations (Kalogeropoulos et al., 2022), schizophrenia (Yolken et al., 2009), unnatural causes of death due to traffic accidents and suicide attempts (Sutterland et al., 2019). In addition, *T. gondii* also causes other ocular disorders like uveitis, retinitis and retinochoroiditis in humans (Kalogeropoulos et al., 2022).

Across the world, molecular surveys in meat for human consumption have been carried out on goats, sheep, and cattle in Iran, Israel, Australia, and the UK using PCR assays. The prevalence rates reported in these studies exhibited considerable variation, ranging from 7.5% in Israeli cattle and 26.1% in Israeli sheep (Mazuz et al., 2023) to a range of 17.3% to 32% in Iranian sheep and 16% to 24% in Iranian goats (Yousefvand et al., 2021). Additionally, a prevalence of

43% was observed in Australian lamb (Dawson et al., 2020), while 67% of lamb cuts in the UK were affected (Aspinall et al., 2002). Belluco et al. (2016) conducted a review that encompassed studies from the USA, New Zealand, the Czech Republic, Portugal, Turkey, Colombia, Costa Rica, and Switzerland. The review yielded pooled prevalences in the meat of 2.6%, 14.7%, and 12.3% in cattle, sheep, and pigs, respectively (Belluco et al., 2016). It has been noted that the prevalence of *T. gondii* infection tends to be higher in small ruminants, mainly sheep, compared to cattle, which are considered poor hosts of this parasite (Belluco et al., 2016; Dong et al., 2018). As such, small ruminants possibly play a more significant role in the epidemiology of *T. gondii* (Dawson et al., 2020).

Genotyping of *T. gondii* is done to unravel this parasite's genetic diversity and population structure (Galeh et al., 2022; Sharif et al., 2017). This process is crucial for epidemiological studies, enabling the examination of strain distribution and prevalence across different geographic regions (Fernández-Escobar et al., 2022). Genotyping also facilitates source tracking, helping researchers trace the origins of infections and implement effective control measures. Understanding the diverse strains aids in clinical correlations, revealing potential associations between specific genotypes and severe disease outcomes (Su et al., 2010). Moreover, genotyping contributes to studies on drug resistance, vaccine development, and the evolutionary characteristics of *T. gondii*, providing essential insights for diagnosing, treating, and preventing toxoplasmosis (Chu & Quan, 2021).

Diverse methodologies have been enlisted for the genotypic characterisation of *T. gondii*. Notably, in the context of genotyping, genomic sequencing manifests a compromised sensitivity relative to sequences characterised by high repetition (Su et al., 2010). The genotyping techniques with better sensitivity and better discrimination power include multilocus PCR-RFLP (n-PCR-RFLP), multiplex multilocus PCR-RFLP (Mn-PCR-RFLP), multiplex PCR of microsatellite markers (Ajzenberg et al., 2010; Etougbétché et al., 2022; Galal et al., 2018; Galeh et al., 2022; Hamidovic et al., 2021; Rezende et al., 2021; Sharif et al., 2017; Su et al., 2010; Zhang et al., 2021), and whole-genome sequencing (Galal et al., 2018).

Bovine neosporosis, caused by *N. caninum*, is a significant cattle disease chiefly characterised by abortion, stillbirths, mummifications, foetal resorption, the birth of weak calves and infertility,

and there is no effective treatment or vaccine (Al-Qassab et al., 2010; Pereira et al., 2022). Furthermore, infection with *N. caninum* may lead to the birth of apparently healthy but persistently infected calves, which can transmit the infection to their offspring through vertical transmission (Al-Qassab et al., 2010; Müller et al., 2015). Costs associated with neosporosis are in the order of billions of US dollars worldwide annually (Reichel et al., 2013).

Dogs, wolves, dingoes, and coyotes are definitive hosts of *N. caninum*, while intermediate hosts include cattle, sheep, goats, birds, wildlife and other species (Al-Qassab et al., 2010). *Neospora caninum* mainly localises in the brains of intermediate hosts, although the DNA has also been detected in the placenta, blood, serum, milk, colostrum, and even in semen (Gharekhani et al., 2022). In cattle, the *N. caninum* DNA has been detected in the brain (amygdala & hippocampus), spinal cord, heart, lung, diaphragm, and skeletal muscle (Nishimura et al., 2013).

In Tunisia, molecular prevalences of 22% in cows and 10.6% in ewes were determined through conventional PCR and nested PCR targeting the V1-V3 regions and the *ITS1* gene (Amdouni et al., 2018). Conversely, in Iran, nested PCR revealed prevalences ranging from 1.9% in sparrows to 35.4% in aborted cow foetuses across various hosts, including cattle, dogs, rodents, cats, and birds (Gharekhani et al., 2022). A study in Central China revealed a 2.8% prevalence of *N. caninum* DNA in slaughtered goats' brain samples, along with the identification of two distinct sequences in the *ITS1* region and multiple genotypes at microsatellite loci (Qian et al., 2020). In Egypt, *N. caninum* DNA was detected in a bulk bovine milk sample using PCR targeting a 337bp segment in the pNc5 gene (Fereig et al., 2022). A study in Italy demonstrated a correlation between the genetic distance of *N. caninum* MLMG and the spatial distance of sampling sites, suggesting the presence of local subpopulations (Villa et al., 2021). Furthermore, diverse genetic profiles among *N. caninum* isolates worldwide have been observed through multilocus microsatellite analyses (Al-Qassab et al., 2010).

In southern Africa, there is a notable lack of studies on latent infections and genetic characterisation of both *T. gondii* (Galal et al., 2018; Omonijo et al., 2022) and *N. caninum* in food animals, with no such research conducted in Namibia. This is significant because livestock production is vital to Namibia's economy (Humavindu & Stage, 2013), and the common practice

of serving undercooked red meat in the country raises public health concerns about the prevalence of *T. gondii* infections. Addressing this knowledge gap is crucial.

Since molecular assays have high sensitivity and specificity to detect latent infections, the principal aim of this study is to employ molecular methodologies to detect and characterise latent *T. gondii* infections in ostensibly healthy sheep and goats intended for human consumption, given the concomitant human health risks. Similarly, the investigation seeks to examine the presence of *N. caninum* DNA within the tissues of cattle in the Khomas region of Namibia and subsequently genetically profile this pathogen.

6.3. Materials and methods

6.3.1. Study animals

The study animals for the *T. gondii* investigation were apparently healthy sheep and goats presented for slaughter and human consumption at three slaughterhouses in the Khomas region of Namibia. One of these slaughterhouses was based on a farm and only sourced animals for slaughter from the same farming establishment. However, the other two abattoirs sourced animals for slaughter from farms and auctions across the country, south of the country's Veterinary Cordon Fence.

The target population for detecting *N. caninum* latent infections comprised all clinically healthy cattle presented for slaughter at four abattoirs in the Khomas region in and around the capital city, Windhoek. Furthermore, blood samples were collected from some of the animals which were *N. caninum* seropositive during the seroprevalence study (see Chapter 4). The cattle slaughterhouses had varying throughputs, with some processing one to 10 cattle per week while others processed more than 100 per week. All the slaughtered animals underwent antemortem and post-mortem inspections done by veterinarians or environmental health practitioners.

6.3.2. Sample size and sampling strategy

The sample size formula for detecting disease in infinite populations was used to determine the number of abattoir samples collected from cattle and small ruminants (sheep and goats):

$$n = (\log (1- \beta)) / [\log (1-d/N)]$$

Where β = confidence level (as a proportion); N = population size; n = sample size; d = number of diseased animals.

When this formula was applied using an estimated prevalence of 2%, as derived from previous studies (see Chapters 4 & 5), the minimum number of abattoir samples required was calculated at 149 each for cattle and small ruminants, using the tables provided, at 5% confidence level (Pfeiffer, 2002).

A convenience sampling strategy was employed during the collection of abattoir samples due to practical constraints. The operational pace of abattoir slaughter lines and the relatively small number of slaughtered animals at a time rendered random sampling unfeasible.

6.3.3. Sample collection

The sampling period spanned from July 2022 to February 2023. Sterile scissors, forceps and a scalpel were used to incise sections of the myocardium, and a spoon was used to scoop the brain samples through the foramen magnum. Heart and brain tissue samples were preferred for analysis because they are the predilection sites of *T. gondii* infection in these animals (Benavides et al., 2017; Mazuz et al., 2023). Approximately 1.5 cm² of the heart muscle and brain tissue were collected from sheep and goat carcasses at three abattoirs and stored in sterile cryotubes. One hundred and seventy-four (174) tissue samples were collected from different animals, comprising 82 sheep brain samples, 79 sheep hearts, 10 goat brains, and three goat hearts (Table 6.1).

Table 6.1: A summary of abattoir samples collected according to species and organ type

Species	Hearts	Brains	Totals
Sheep	79	82	161
Goats	10	3	13
TOTALS	89	85	174

Tissue samples from cattle measuring approximately 2 cm² were collected from the heart muscle and brain tissue of cattle carcasses obtained from four different abattoirs, following the same protocol for small ruminant samples. In addition, 7 ml of whole blood samples preserved in EDTA (ethylenediamine tetraacetic acid) were collected from selected seropositive animals at the farms investigated in the previous study (see Chapter 4). Blood samples were obtained from the coccygeal vein using a 20-gauge needle attached to a holder and then collected into a 7 ml vacutainer tube with EDTA. A total of 199 samples were collected from different animals, comprising 110 brain samples, 75 heart muscle samples, and 14 whole blood samples.

The collected blood and tissue samples were subsequently preserved by freezing them at -20 °C until they were analysed.

6.3.4. DNA extraction

DNA was extracted from the blood, heart and brain samples using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, USA) following the manufacturer's instructions. Proteinase K Storage Buffer was initially added to each tube containing 20 mg of Proteinase K, resulting in a final 20 mg/ml concentration. A mixture of 95 µl water, 95 µl Solid Tissue Buffer, and 10 µl Proteinase K was added to each sample (≤ 25 mg) in a microcentrifuge tube. The tube was then vortexed for 10-15 sec and incubated overnight at 55°C on a heating block. After incubation, the mixture was centrifuged, and the resulting supernatant was transferred to a new microcentrifuge tube. Two volumes of Genomic Binding Buffer were added to the supernatant, and the mixture was transferred to a spin column in a collection tube and centrifuged. The flow-through and collection tube were discarded, leaving an empty spin column, which was then transferred to a new collection tube. Subsequently, 400 µl DNA Pre-Wash Buffer was added to the spin column, followed by centrifugation at 12,000 x g for 1 min. The collection tube was emptied. Next, 700 µl g-DNA Wash Buffer was added to the spin column and centrifuged at 12,000 x g for 1 min. Again, the collection tube was emptied. Then, 200 µl g-DNA Wash Buffer was added to the spin column and centrifuged at $\geq 12,000$ x g for 1 min. The collection tube with the flow-through was discarded, and the spin column was transferred to a new sterile microcentrifuge tube. Fifty microlitres of DNA Elution Buffer were added to the spin column matrix to elute the DNA,

followed by incubation at room temperature for 5 min. The tube was then centrifuged for one minute to extract the DNA. Finally, the DNA was stored at -20°C until further use.

6.3.5. Polymerase chain reaction (PCR)

6.3.5.1. *Toxoplasma gondii*

A nested PCR was used to test for *Toxoplasma* DNA in the previously extracted and stored eluted DNA material. Pooled samples, each containing genomic DNA from ten samples, underwent initial screening using nested PCR. Subsequently, individual genomic DNA samples from the positive pools were tested individually to identify the positive samples.

Two sets of specific primers (external and internal) were used to target the B1 gene: B1^{1st}F and B1^{1st}R external primer set, which multiplies the 580bp segment, were used in the first round, and the B1^{2nd}F and B1^{2nd}R internal primer set, which amplifies the 530bp section was used in the second round. The two primer pairs and cycling conditions have been previously described (Uddin et al., 2021). The sequences of the primers used in the study are shown in Table 6.2.

Table 6.2: A tabulation of the primers used in the nested PCR to detect *Toxoplasma* DNA.

Target gene	PCR round	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
B1	1 ST	TGTTCTGTCCTATCGCAACG	ACGGATGCAGTTCCTTTCTG
	2 nd	TCTTCCCAGACGTGGATTTC	CTCGACAATACGCTGCTTGA

A reaction mixture of 6.9 µl was prepared for each sample for the initial round of nested PCR. The mixture consisted of 1 µl of genomic DNA, 0.6 µl of each external primer, 3 µl of One Taq® master mix (containing Taq DNA polymerase, dNTPs, MgCl₂, and a buffer), and 1.7 µl of ultrapure nuclease-free distilled water. The PCR cyclic conditions used to detect *T. gondii* DNA are summarised in Table 6.3.

One microlitre of the PCR product from the first reaction for each sample was used for the second amplification reaction. Therefore, each reaction mixture totalled 6.9 µl consisting of 1 µl

primary PCR product, 0.6 µl of each internal primer (10 pmol/µl) (Iqaba Biotec, South Africa), 3 µl One Taq® master mix (Inqaba Biotec, South Africa), and 1.7 µl of ultrapure nuclease-free distilled water. The PCR cyclic conditions used were the same as for the first round (Table 6.3).

Table 6.3: A summary of the cyclic conditions used for *T. gondii* DNA detection for the first and second rounds of the nested PCR reaction.

STEP	TEMPERATURE	DURATION	CYCLES
Initial denaturation	95°C	2 mins	One cycle
Denaturation	95°C	15 sec	Thirty cycles
Annealing	50°C	15 sec	
Extension	72°C	30 sec	
Final extension	72°C	5 min	One cycle
Hold	4°C	∞	

A positive control containing *Toxoplasma gondii* (Nicolle and Manceaux) Nicolle and Manceaux (ATCC 50174) and a negative control containing nuclease-free water were included in each reaction. The nested PCR products were electrophoresed on a 1.5% (w/v) agarose gel with 0.05% ethidium bromide in Tris-Acetate-EDTA buffer at 110 V for 20 min. Amplicons were visualised using an ultraviolet transilluminator. The *T. gondii* positive amplicons were removed from the agarose gel and stored at -20°C until further processing. The unused PCR products from positive samples were also stored similarly.

6.3.5.2. *Neospora caninum*

To detect *N. caninum* DNA, a conventional PCR was performed using the Np6 (forward primer, 5'-CAGTCAACCTACGTCTTC-3') and Np21 (reverse primer, 5'-GTGCGTCCAATCCTGTAA-3') primers which targeted the ≈340 bp product of the pNc5 gene (Karavavuk et al., 2021). The PCR reaction was conducted using a 12.5 µl mixture consisting of 2 µl of genomic DNA, 6.2 µl of One Taq® master mix (containing *Taq* DNA polymerase,

dNTPs, MgCl₂ and a buffer), 1.2 µl of each primer (10 pmol/µl) (Inqaba Biotec, South Africa), and 1.9 µl of nuclease-free water. The PCR cyclic conditions used are summarised in Table 6.4

A positive control consisting of *N. caninum* DNA (Field strain, Italy) and a negative control containing nuclease-free water were incorporated into each reaction. Electrophoresis (110 V for 20 min) was conducted using a 1.5% (w/v) agarose gel supplemented with 0.05% ethidium bromide in Tris-Acetate-EDTA buffer to visualise the amplicons using an ultraviolet transilluminator.

Table 6.4: A summary of the PCR cyclic conditions used for *N. caninum* DNA detection.

STEP	TEMPERATURE	DURATION	CYCLES
Initial denaturation	95°C	2 mins	One cycle
Denaturation	95°C	15 sec	Thirty-five cycles
Annealing	46°C	15 sec	
Extension	72°C	30 sec	
Final extension	72°C	5 min	One cycle
Hold	4°C	∞	

6.3.6. Sequencing

The PCR products from the nested PCR reaction targeting the *T. gondii* DNA were sequenced using the Sanger method. The amplicons were subjected to bidirectional sequencing using the two sets of external and internal primer pairs employed earlier during the amplification process. The PCR amplicons were then visualised for integrity on 1% agarose gel stained with EZ-vision® Bluelight DNA Dye. Following PCR amplification, the PCR products were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific), adhering to the manufacturer's instructions. Subsequently, the fragments were sequenced using the BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 (Nimagen®), per the manufacturer's recommended protocols. The labelled products were then cleaned with the ZR-96 DNA Sequencing Cleanup Kit (Zymo Research). The purified products were injected into an Applied Biosystems ABI 3500XL Genetic Analyser with a 50 cm array using the Polymer One

Populational Seven (POP7). The FinchTV analysis software was used to analyse the sequence chromatogram.

6.3.7. Data analysis

The data were captured on a Microsoft Excel spreadsheet, and descriptive statistics were used to calculate molecular prevalence. The z-test for comparing two proportions was used to compare the molecular prevalences in different species and organs (<https://epitools.ausvet.com.au>). The *T. gondii* molecular data were subjected to analysis using Genious Prime[®], MEGA11[®], and NCBI-BLAST software applications.

6.3.8. Phylogenetic analysis

The DNA sequence data in FASTA format were analysed using the BLAST software hosted on the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). This was done to identify other homologous sequences on the GenBank database hosted by NCBI. The identified sequences were subsequently used for comparison in constructing the phylogenetic tree. Multiple sequence alignment was then performed among the selected sequences using the ClustalW method for pairwise and multiple alignments in the MEGA11 programme. The generated data was then saved in the MEGA format and used to generate a phylogenetic tree. The neighbour-joining algorithm was utilised for the analysis, and the phylogeny test was conducted using the bootstrap method set at 1000 replications.

6.4. Results

An overall prevalence of 16.7% (29/174) was recorded in this study (Figure 6.1). The prevalence in sheep was 17.4% (28/161), whilst that in goats was 7.7% (1/13). This observed difference was not statistically significant ($p = 0.3826$). When comparing the two organs sampled in sheep, the heart tissues had a higher molecular prevalence of 20.3% (16/79) compared to brain tissues, which had a prevalence of 14.6% (12/82) (Table 6.5 and Table 6.6). However, this difference was not statistically significant ($p = 0.403$). In goats, only one brain sample ($n = 3$) tested positive, but all ten heart samples were negative (Tables 6.5 and 6.6).

Table 6.5: A summary of the nested PCR test results for *T. gondii* DNA in heart samples

SPECIES	No. of heart samples	No. Positive	% Positive
Sheep	79	16	20.3
Goats	10	0	0.0
TOTALS	89	16	18.0

Table 6.6: A summary of the nested PCR test results for *T. gondii* DNA in brain samples

SPECIES	No. of brain samples	No. Positive	% Positive
Sheep	82	12	14.6
Goats	3	1	33.3
TOTALS	85	13	15.3

Regarding *Neospora*, none of the samples screened by PCR tested positive for the parasite DNA. The gel electrophoresis results, as read in the ultraviolet transilluminator, are shown in Figure 6.2.

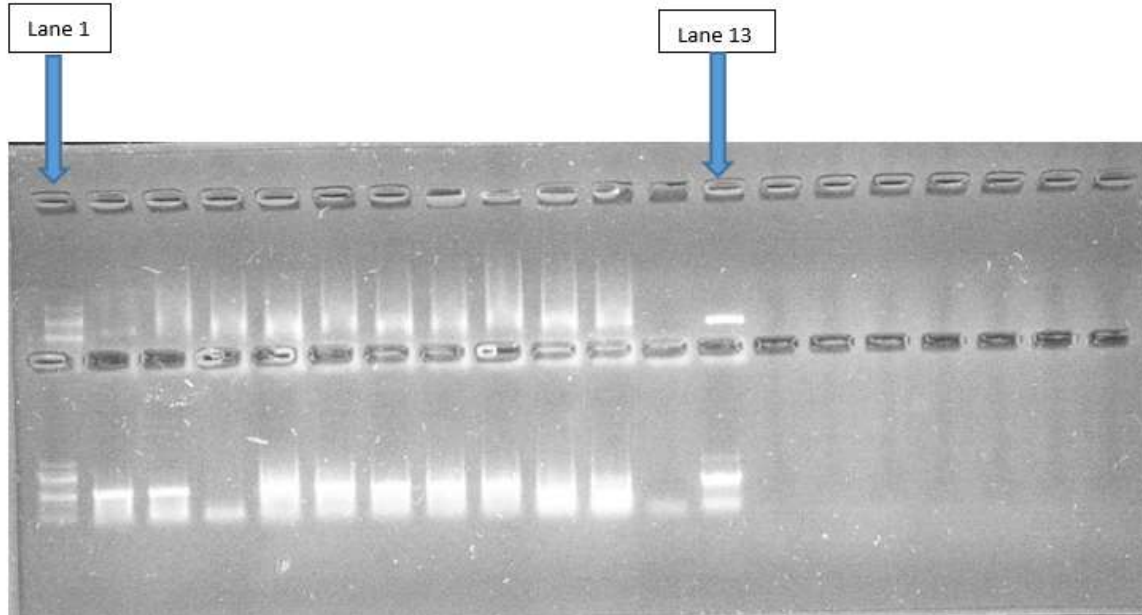


Figure 6.1: Picture showing the results of 10 samples (lanes 2 to 11) tested with nested PCR, seven of which are positive. The results of rounds one and two are on top and bottom of the picture, respectively. Lane 1 shows the two ladders of the two rounds, and Lanes 12 and 13 are negative and positive controls for the two rounds, respectively.

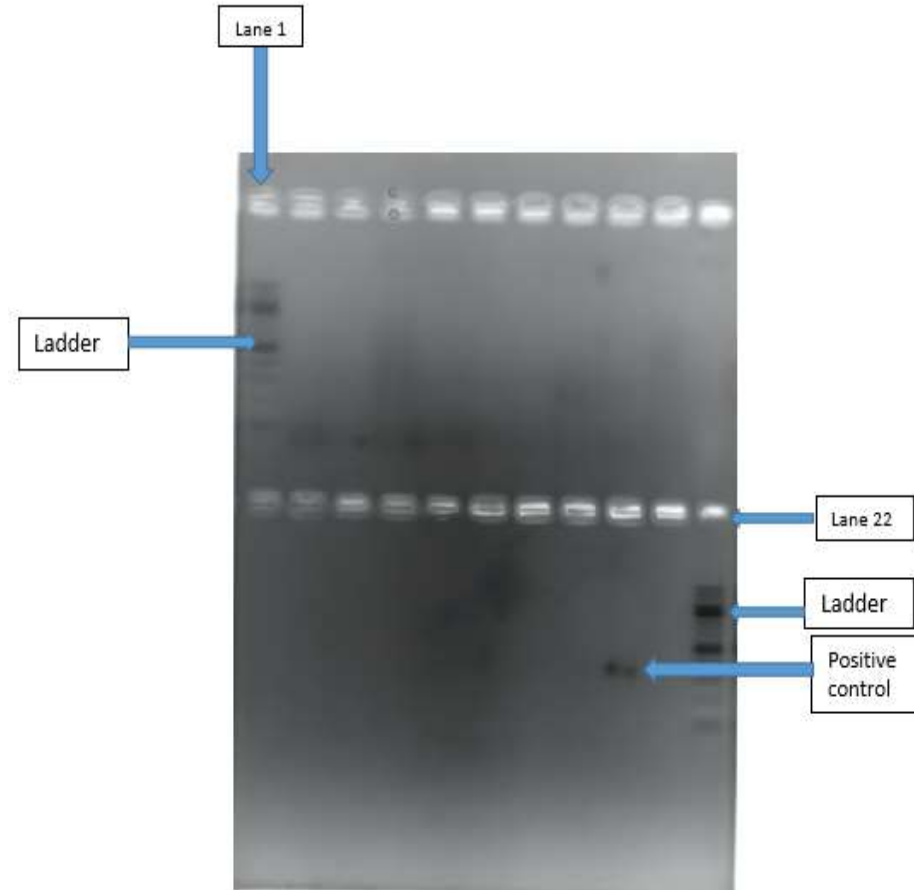


Figure 6.2: Picture showing the gel electrophoresis of PCR products after 20 minutes. Lanes 1 and 22 were laden with a 1000bp ladder. Lanes 20 and 21 were positive and negative controls, respectively. Lanes 2 to 19 had sample PCR products and were all negative.

6.4.1. Genetic characterisation

To identify the *T. gondii* genotypes, eight of the 29 positive samples were randomly selected for Sanger sequencing, and the resultant sequences were identified through NCBI-BLAST[®] (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Only eight samples were sequenced due to budgetary constraints. The eight sequences reported in the current study had the highest alignment scores, ranging from 96.44% to 100% on BLAST[®] (Table 6.7). The isolates comprised homologous *T.*

gondii type III strains, exhibiting genetic relatedness to strains from distant countries, including Australia (OM522325 & KT881314), the USA (MH744807, MH744807 & MH744805), South Korea (MW063447), India (KY514164), and Iran (MN275909).

Table 6.7: Nucleotide identity percentages of *T. gondii* from the Khomas region of Namibia compared with reference sequences from around the world

Abattoir	Animal Species	Organ	DNA ID	Target gene	GenBank Accession Number	Origin Of Reference Sequence	Nucleotide Sequence Identity
Neudamm	Caprine	Brain	CD10	B1	OM522325.1	Australia	96.44%
Neudamm	Ovine	Brain	OD64	B1	MH744807.1	USA	99.59%
Neudamm	Ovine	Brain	OD67	B1	KT881314.1	Australia	100%
Brakwater	Ovine	Heart	OD71	B1	MH744807.1	USA	99.40%
Brakwater	Ovine	Heart	OD73	B1	MH744805.1	USA	99.20%
Brakwater	Ovine	Heart	OD76	B1	MW063447.1	South Korea	99.20%
Brakwater	Ovine	Heart	OD77	B1	KY514164.1	India	99.20%
Brakwater	Ovine	Heart	OD80	B1	MN275909.1	Iran	99.00%

6.4.2. Phylogenetic analysis

In this investigation, eight query sequences were combined with 34 related sequences obtained from the GenBank database. A phylogenetic tree was constructed with this data set using the MEGA11 software (Figure 6.3). Notably, the singular sequence (NAMCD10B12F2) obtained from a goat brain sample formed a distinct cluster alongside an Australian strain (OM52232), which in turn displayed complete alignment with the *T. gondii* VEG type III strain (LN714499). Two other sequences from the current study (NAMOD71B12F1 & NAMOD76B12F6) also formed a cluster with an Indian (KY514164) and two South Korean (MW063447 & MW063448) strains, all with 100% alignment with VEG type III. Additionally, the rest of the sequences from this study also clustered with VEG type III (Figure 6.3).

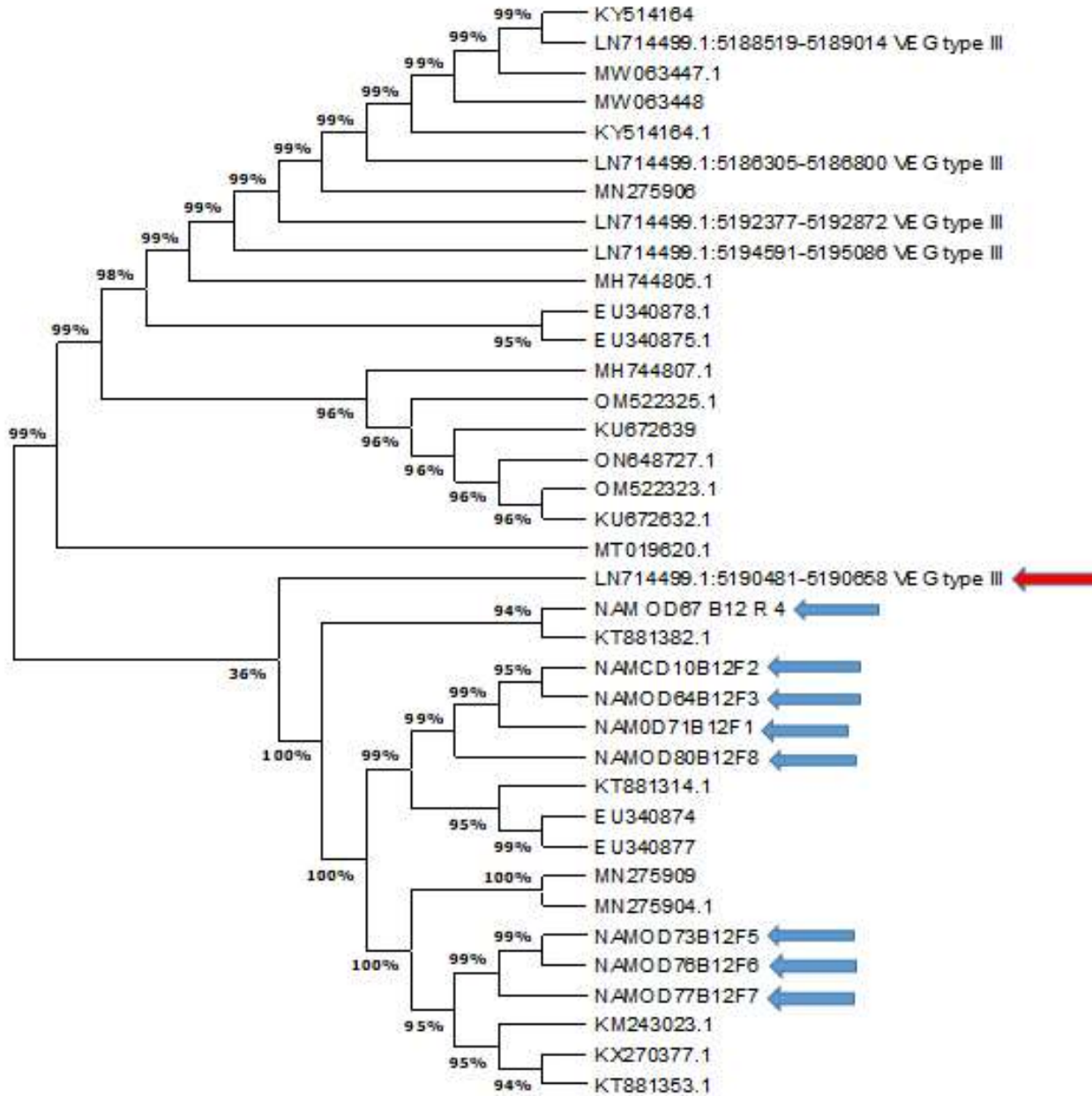


Figure 6.3: A phylogenetic tree showing all eight Namibian *T. gondii* isolates (blue arrows) in relation to other isolates from around the world and clustered with the VEG type III strain (red arrow).

6.5. Discussion

To the author's knowledge, this is the first study in southern Africa to identify and genetically characterise *T. gondii* DNA in the abattoir tissues of sheep and goats intended for human consumption. The number of goat samples was small ($n = 13$) due to the absence of a commercial goat abattoir in Namibia, which posed a challenge in acquiring goat samples.

The overall molecular prevalences found in the current study align with findings in molecular studies from other regions of the world: 2.7% in South Africa (Lukášová et al., 2018), 17.65% in Egypt (Ibrahim et al., 2017), 1.52% in India (Kalambhe et al., 2017), 8.7% in China (Zhang et al., 2021), and 51.65% in Ethiopia (Gebremedhin et al., 2014a). The findings from Iran (Amouei et al., 2022; Deljavan et al., 2022), North India (Kalambhe et al., 2017), the review by Sharif et al. (2015), the Ethiopian study by Gebremedhin et al. (2014a), and the current research demonstrate a lack of statistically significant difference in the molecular prevalence of *T. gondii* DNA between sheep and goat tissues. The comparable prevalence rates highlight the similar epidemiological significance of sheep and goats in the transmission dynamics of this parasite.

According to a meta-analysis review by Hajimohammadi et al. (2022), the world prevalence of *T. gondii* in livestock and poultry was 28.3%, ranging from a low of 1.26% to a high of 89.8% in Asia, whilst in African livestock it was estimated at 29% (Hajimohammadi et al., 2022). Overall, the molecular prevalence found in the current study was lower than the African average. This could be attributed to the harsh semi-arid to arid Namibian climate (Kgabi et al., 2016), which limits the viability and, therefore, sporulation and availability of viable *T. gondii* oocysts thereby reducing the infection of the herbivore intermediate hosts.

In the current study, the identified isolates clustered with *T. gondii* strains from diverse global regions, including Australia, India, Iran, South Korea, and the USA, among many others. This observation may be attributed to the high conservation of the *BL* gene. While the *BL* gene effectively distinguishes major genotypic groups (like types I, II, and III), its limited variability may not be sufficient for discerning closely related strains within these groups (Galal et al., 2018; Su et al., 2010). For a more detailed exploration of fine-scale genetic variations and clonal lineages, employing more variable genetic markers or conducting a comprehensive examination

of the entire genome is advisable. Enhanced resolution in determining clonal lineages can be achieved through the utilisation of more polymorphic markers, such as microsatellites or multilocus genotyping, offering a finer-scale perspective on genetic diversity within a given type or genotype (Galal et al., 2018; Su et al., 2010).

The *BI* gene, which was the focus of this study, displays a diminished level of polymorphism and is highly conserved, precluding the determination of clonal lineages across distinct isolates (Kalambe et al., 2017). As was undertaken in the present study, employing partial genomic sequencing for genotyping exhibits compromised sensitivity compared to highly repetitive sequences (Su et al., 2010). Other genotyping techniques with better sensitivity and better discrimination power could have been used to get a clearer picture, and these include multilocus PCR-RFLP (n-PCR-RFLP), multiplex multilocus PCR-RFLP (Mn-PCR-RFLP), multiplex PCR of microsatellite markers (Ajzenberg et al., 2010; Etougbéché et al., 2022; Galal et al., 2018; Galeh et al., 2022; Hamidovic et al., 2021; Rezende et al., 2021; Sharif et al., 2017; Su et al., 2010; Zhang et al., 2021), and whole-genome sequencing (Galal et al., 2018). However, the latter is costly and cumbersome, which makes it unsuitable for routine use.

Using genomic sequencing, all eight isolates in the current study were closely related to *T. gondii* type III. This strain is relatively rare and can be non-virulent or of intermediate virulence (Dardé, 2004; Galal et al., 2019; Su et al., 2010). Concluding the presence or absence of other genotypes in Namibia would be premature without employing more sensitive genotyping techniques on a larger sample size encompassing a broader geographic representation of the country. However, other genotypes that have been found in Africa include types II and III, Africa 1, Africa 2, Africa 3, and atypical strains (Etougbéché et al., 2022; Galal et al., 2018; Hamidovic et al., 2021).

In a study conducted in Ethiopia, it was discovered that 87.9% (29/33) of *T. gondii* isolates from heart samples of sheep and goats intended for human consumption were identified as type II, while 9% (3/33) were type III, and 3% (1/33) were characterised as atypical genotypes (Gebremedhin et al., 2014b). The findings of the present study contrast with the findings of this Ethiopian study, where three genotypes were found. A bigger sample size of isolates could probably have uncovered more genotypes. These variations underscore the importance of

considering regional differences in *T. gondii* genotypes and their potential implications for virulence and public health.

The investigation of latent infections of *N. caninum* in cattle in the current study was a follow-up to a previous serosurvey and risk factor evaluation study in the Khomas region of Namibia (see Chapter 4). In the latter study, an animal-level seroprevalence of 5.7% (n=736) in cattle was determined, notably lower than reported from other countries (Samkange et al., 2023a). However, PCR testing for all 199 bovine samples in the present research yielded negative results, including 110 brain samples, 75 heart samples, and 14 whole blood samples. Molecular studies regarding the prevalence of *N. caninum* in tissues sourced from slaughtered cattle are generally scarce (Amdouni et al., 2018). Consequently, the prevailing trend in most available studies is the detection of *N. caninum* DNA in aborted cows and fetuses (Nayeri et al., 2022). Globally, the molecular prevalence of *N. caninum* has been estimated at 47% and 43% in aborted cows and aborted fetuses, respectively (Nayeri et al., 2022).

At country level, *N. caninum* molecular prevalence rates of 13.6% (n=118) and 20.5% (n=78) have been reported in bovine aborted fetuses in Iran (Gharekhani et al., 2022; Salehi et al., 2021), 22% (n=150) of meat samples from cattle in Tunisia (Amdouni et al., 2018), 12.36% (n=327) of blood samples in Iraq (Al-Gharban et al., 2017), 65% (n = 106) of blood samples from aborted cows in Iraq (Al-Kaabi et al., 2023), and 13.5% (n=170) of blood samples in Colombia (Correa-Castro et al., 2021). These prevalence rates differ significantly from the findings of the current study. This discrepancy could be partially attributed to the population targeted for the present study. The current study mainly targeted tissues (hearts and brains) from slaughtered cattle earmarked for human consumption. Such animals have a lower probability of testing positive compared to targeting aborted cows and fetuses (Al-Kaabi et al., 2023; Nayeri et al., 2022). This is attributed to neosporosis being a prominent cause of abortion in cattle worldwide (Demir et al., 2020), thereby increasing the likelihood of obtaining positive results from aborted cows and fetuses (Nayeri et al., 2022).

The other reason for getting negative results in the brain samples could be related to the anatomical areas of the brain sampled in the current research. The amygdala and hippocampus are the most appropriate areas for detecting *Neospora* DNA in the bovine brain (Nishimura et al.,

2013). However, in the current study, the brain samples could only be scooped through the foramen magnum, which made it difficult to pinpoint the amygdala and hippocampus for sampling. The failure to correctly sample these specific parts of the brain could also have contributed to the negative results obtained.

The country's harsh climate might be another factor which contributed to the inability to detect *Neospora* DNA. Namibia is classified as a semi-arid country characterised by consistently low and considerably fluctuating precipitation patterns, whereby the hot and arid climatic conditions predominate. Namibia's northwest, central and southern regions are particularly arid, while the northern regions experience more rainfall and have a slightly more tropical climate (Kgabi et al., 2016). Consequently, droughts are common in the country, and the worst drought recorded in the last 100 years was in 2019 (Shikangalah, 2020). Coccidian oocysts subjected to high temperatures (25°C and 30°C) and low relative humidity (53% RH and 62% RH) have been found to lose viability very quickly and die within 24 hours. Even at 75% RH, oocysts died within 24-60 hours at temperatures ranging from 30°C to 20°C (Langkjær & Roepstorff, 2008).

Central Namibia, where this study was carried out, has a predominantly arid climate, creating harsh environmental conditions worse than these. Therefore, these unfavourable climatic conditions are unsuitable for the survival and sporulation of *N. caninum* oocysts in the environment. These harsh climatic conditions reduce the risk of environmental contamination by oocysts and, hence, the chance of being ingested by the intermediate herbivore hosts. This scenario might explain why detecting *Neospora* DNA in the tissue samples collected was problematic. However, the same factors would have been expected to affect the infection levels of *T. gondii* found in this study, which was not the case. This discrepancy indicates a more intricate interplay of biotic and abiotic variables that extends beyond the scope of climatic conditions alone. For instance, in a study conducted in Benin, it was observed that the molecular prevalence of *T. gondii*, which shares similar environmental requirements for its life cycle as *N. caninum*, did not show significant influence from environmental parameters (Etougché et al., 2022). This demonstrates the complex nature of this parasite's biology and epidemiology. Consequently, further research is warranted to understand these variables better.

As reported in an earlier study (Samkange et al., 2023a), the low prevalence of neosporosis in the Khomas region of Namibia could have also contributed to the failure to detect *Neospora* DNA in all 199 tested samples. Therefore, a larger sample size to detect positive animals would be needed to detect the positive animals, given that these were apparently healthy animals intended for slaughter. Alternatively, targeting a higher-risk group, like cows that had abortions, as well as aborted fetuses, would increase the probability of detecting positive cases (Nayeri et al., 2022).

All the fourteen blood samples tested from seropositive cows were negative on PCR. These results differ from the 12.36% and 13.5% determined for blood samples in Iraq and Colombia (Al-Gharban et al., 2017; Correa-Castro et al., 2021). The difference may be attributed to the relatively smaller sample size in the current study. Prevalence rates in blood samples are typically low, necessitating a larger sample size. However, the authors could only collect fourteen samples, as these were the only seropositive cattle that could be traced and for whom the farmers cooperated.

The commonly used molecular markers for *Neospora* identification are the 18S rRNA and *ITS1* regions within the small subunit ribosomal RNA (ssuRNA), along with *pNc5* (Khan et al., 2020). These markers are preferred due to their repetitive nature, making them more effective for polymerase chain reaction (PCR) detection than single-copy genes (Khan et al., 2020). However, although *pNc5*, a multi-copy gene, is widely employed for detecting *Neospora* infection in various hosts, it lacks exquisite specificity as its primers have been reported to amplify rodent-specific DNA (Khan et al., 2020). The current study targeted the *pNc5* gene, and the lack of specificity of its primers may also have contributed to the negative results in this research. The 18S rRNA and *ITS1* markers have been successfully used in other studies with better results (Al-Kaabi et al., 2023; Amdouni et al., 2018). Therefore, the 18S rRNA and *ITS1* markers should preferably be targeted in future studies.

This study's failure to detect latent *N. caninum* infections in cattle lays a foundation for future research into the molecular detection of *Neospora* DNA in Namibian cattle. Given the presumed low prevalence of bovine neosporosis in the Khomas region, future studies focusing on aborted cows and fetuses are expected to yield more successful results in the molecular detection of *N.*

caninum in Namibian cattle. To ensure an adequate sample size, conducting such a study over an extended period would be advisable.

In conclusion, the present study showcased a significant prevalence of *T. gondii* type III infections in seemingly healthy sheep and goat meat destined for human consumption in Namibia's Khomas region and possibly in other parts of the nation. Consequently, it is advisable to ensure the thorough cooking of sheep and goat meat to avert the potential transmission of toxoplasmosis infection to humans. Additionally, the regular vaccination for sheep and goats against toxoplasmosis in Namibia should be contemplated to diminish animal infections further, mitigate occurrences of small ruminant abortions, and minimise human exposure risks. Employing a One Health approach, whereby animal health practitioners, human health practitioners, and environmental health practitioners collaborate synergistically, offers the sole sustainable avenue for efficiently managing toxoplasmosis in animals and humans.

6.6. References

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Chapter 7: General discussion, recommendations and conclusions

7.1. General Discussion

The primary aim of this study was to gain an understanding of the epidemiology of *N. caninum* and *T. gondii* in livestock in the Khomas region of Namibia and to evaluate the knowledge, attitudes, and practices (KAP) of livestock producers and animal health practitioners in the country. To accomplish this, the study focussed on the following four main objectives in the Khomas region:

1. Assess the KAP of livestock farmers and animal health practitioners regarding neosporosis and toxoplasmosis.
2. Determine the *N. caninum* seroprevalence in cattle and the associated risk factors.
3. Determine the *T. gondii* seroprevalence in sheep and goats and the associated risk factors.
4. Detect and characterise the *N. caninum* DNA in cattle and *T. gondii* DNA in small ruminants (sheep and goats).

The following is a summary and discussion of the main findings on each of the above objectives:

7.1.1. Assessment of the KAP of livestock farmers and animal health practitioners regarding neosporosis and toxoplasmosis

The results of the KAP study revealed that most livestock producers in the Khomas region of Namibia were largely unaware of neosporosis and toxoplasmosis, as well as the methods of transmission (Samkange et al., 2023a). Conversely, animal health practitioners (AHP) demonstrated a general awareness of both diseases. However, it was found that none of them regularly requested the Central Veterinary Laboratory to conduct tests for *N. caninum* or *T. gondii* in cases of abortions. Moreover, AHPs typically did not regularly discuss these two diseases with farmers or pet owners (Samkange et al., 2023a). This scenario underscores a significant knowledge gap among livestock producers concerning neosporosis and toxoplasmosis, alongside a communication gap between the AHPs and farmers.

7.1.2. *Neospora caninum* seroprevalence in cattle and the associated risk factors

Of the 736 cow sera from 32 farming establishments, 5.7% and 25% were positive, respectively. Data from the questionnaire, which was concurrently administered to determine possible risk factors, showed that there was no significant association between seropositivity and the presence of dogs, jackals, history of abortions, farm size, number of cattle and average annual rainfall. The establishments with moderate to high numbers of Feliformia were 9.8 times more likely to be seropositive to *N. caninum* than those with none to low levels of the former (Samkange et al., 2023b). The study concluded that the seroprevalence level of *N. caninum* in cattle in the Khomas region was relatively low compared to other parts of the world and that the role of Feliformia in the epidemiology of bovine neosporosis needed to be further investigated.

7.1.3. *Toxoplasma gondii* seroprevalence in sheep and goats and the associated risk factors

In this study, a total of 299 and 345 sheep and goat sera, respectively, from 22 farming establishments were tested. Overall, 3.68 % and 0.29% of sheep and goat sera were positive, respectively. Sheep flocks had significantly greater animal-level and flock-level prevalences than goats and were 13.14 times more likely to be seropositive than goat flocks. Seropositivity to *T. gondii* in sheep was positively associated with the total number of sheep at the farming establishment, history of abortions and farm size, but not with goats. The study determined that sheep in the Khomas region were probably more exposed to *T. gondii* infection than goats. It also found *T. gondii* seroprevalences that were much lower than those in similar studies from other countries in the sub-region and elsewhere (Samkange et al., 2023c). The low seroprevalence levels were attributed to the harsh semi-arid climatic conditions prevalent in the country, which create an unsuitable environment for the long-term viability of *T. gondii* oocysts.

7.1.4. Detection and characterisation of *N. caninum* DNA in cattle and *T. gondii* DNA in sheep and goats

During this phase of the study, an analysis was undertaken to detect the presence of *N. caninum* DNA in a sample collection encompassing 110 bovine brain tissues, 75 cardiac muscle samples, and 14 blood specimens obtained from cattle. A conventional PCR targeting the 340bp product of the Nc5 gene was used. All the samples tested negative for *N. caninum* DNA (Chapter 6). It was

concluded that the negative results could be attributed to the low prevalence of neosporosis determined in the previous serosurvey (Samkange et al., 2023b). A future study targeting aborted foetuses over a more extended period was recommended in order to increase the probability of detecting *N. caninum* DNA.

The study also explored the presence of *T. gondii* DNA in the tissues of apparently healthy sheep and goats designated for human consumption. A total of 174 tissue samples were collected, consisting of 82 sheep brain samples, 79 sheep heart tissues, 10 goat brain samples, and three goat heart samples. The nested PCR analysis revealed that 16.7% of these samples were positive for *T. gondii* DNA. The overall molecular prevalence for sheep was 17.4% compared to 7.7% in goats. Although the prevalence of *T. gondii* DNA was higher in sheep than in goats, this difference was not statistically significant (Chapter 6). All the *T. gondii* isolates detected and sequenced were type III genotypes, and the sequences closely aligned with strains from several regions of the world, including Australia, India, Iran, South Korea, and the USA. The alignment scores for the sequenced Namibian isolates ranged from 96.44% to 100%.

The presence of type III genotypes of *T. gondii* in Namibia, with high sequence alignment scores to strains from diverse global locations, underscores this parasite's widespread distribution and genetic diversity. Furthermore, this study underlines the noteworthy prevalence of *T. gondii* infections in seemingly healthy sheep and goat meat intended for human consumption in Namibia's Khomas region, with significant public health and food safety implications, possibly for the entire nation.

7.1.5. Discussion and opportunities for further research

The results of the KAP study (Samkange et al., 2023a) are disconcerting, particularly when considering the serological evidence of neosporosis (Samkange et al., 2023b) and the demonstrated presence of both serological (Samkange et al., 2023c) and molecular evidence of toxoplasmosis in Namibian livestock (Chapter 6). It is, therefore, imperative to educate and raise awareness among livestock farmers about neosporosis and toxoplasmosis. To this end, targeted public awareness campaigns should be initiated to educate livestock producers about neosporosis and toxoplasmosis, including their potential impacts and preventive measures.

To reach a wider audience, a range of channels such as workshops, educational materials, farmers' magazines, and online platforms can be effectively utilised. Moreover, promoting regular communication between AHPs, livestock producers, and pet owners is essential to ensure the continuous sharing of information about these diseases. This could entail training AHPs in effective communication strategies and encouraging open dialogues with farmers to address concerns and share knowledge. Organised forums, discussion sessions, and platforms can facilitate the exchange of stakeholders' experiences, concerns, and best practices related to animal disease control.

The importance of community involvement and ownership in disease control programmes cannot be overstated. Engaging local communities, farmers, and livestock producers is crucial. By doing so, a sense of ownership and responsibility can be nurtured among the stakeholders, leading to increased dedication and adherence to disease prevention measures. When the community feels personally invested in these efforts, they are more likely to participate and take proactive steps to implement preventive measures. With community members at its core, this collaborative approach can significantly enhance the efficacy of disease control programmes and create a sustainable impact on the overall health and well-being of animals and humans.

Animal health practitioners (AHPs) need additional training and education to enhance the understanding and significance of neosporosis and toxoplasmosis. This can be achieved through specialized training and continuous education, which will keep them updated on the latest advancements in research, diagnosis, and control strategies for these diseases. Encouraging AHPs to regularly request testing for *N. caninum* and *T. gondii* in cases of abortions is crucial, as it will aid in monitoring the prevalence of these diseases. Efficient reporting mechanisms should be established to track disease incidence and potentially implement appropriate control measures to facilitate this process.

Establishing clear protocols for disease testing and diagnosis is of utmost importance. Standardized testing procedures can play a significant role in identifying the prevalence and spread of neosporosis and toxoplasmosis, thus enabling the implementation of more targeted control measures. Overall, providing ongoing education and efficient testing mechanisms will equip AHPs to combat these diseases better and contribute to their effective management.

It is crucial to foster collaboration between government veterinary services, research institutions, and private veterinary practitioners to enhance disease control efforts. This collaborative approach will facilitate the development of comprehensive disease control programmes and guidelines, ensuring a unified and effective strategy in addressing neosporosis and toxoplasmosis.

Since toxoplasmosis is an important zoonosis that poses public health risks, adopting a One Health approach to tackle this disease is paramount (Aguirre et al., 2019). Collaboration between veterinary authorities, public health agencies, and environmental experts is crucial for a comprehensive and effective control strategy. The One Health approach emphasizes integrating human, animal, and ecosystem health to address complex diseases like toxoplasmosis (Aguirre et al., 2019). To effectively manage and control this disease, interdisciplinary collaboration is essential, fostering new and innovative approaches. A dashboard system combining health and ecological indicators can aid in identifying prevention and management needs quickly. Transdisciplinary research, capacity building, and participatory methodologies are crucial in implementing One Health interventions for toxoplasmosis (Etougbétché et al., 2022). Local socioeconomic and cultural factors and regional and international policies must be considered to develop practical and sustainable solutions. By applying expertise through One Health, simultaneous benefits for humans, animals, and the environment can be achieved (Aguirre et al., 2019).

In this study, *T. gondii* isolates in the Khomas region of Namibia were successfully diagnosed and genetically characterised (Chapter 6). The limited number of sequenced isolates resulted in only one genotype being detected (*T. gondii* type III), which is of intermediate virulence (Dardé, 2004). However, more genotypes could probably be present in the country. Therefore, further research is required to determine the full extent of the number of genotypes present in the country.

Accurate diagnosis and genetic characterization of *T. gondii* infection are crucial for effective surveillance, prevention, and control of toxoplasmosis (Liu et al., 2015). Thus, investing in further research and surveillance is important to understand better the prevalence, risk factors, and transmission dynamics of toxoplasmosis in Namibia. This knowledge will aid in formulating evidence-based control strategies by furnishing essential data to policymakers.

Detecting *T. gondii* DNA in apparently healthy slaughtered animals in this study (Chapter 6) carries significant food safety and public health implications. Studies have shown that a considerable portion of human toxoplasmosis cases can be attributed to the consumption of raw or undercooked infected meat or cross-contamination from such meat (de Barros et al., 2022; Jones et al., 2001). It is, therefore, advisable to ensure the thorough cooking of sheep and goat meat to reduce the risk of transmission to humans. As a result, it becomes imperative to educate the public about the risks associated with consuming inadequately cooked meat to prevent toxoplasmosis. Raising awareness about safe food handling practices and the importance of thoroughly cooking meat can be crucial in safeguarding public health (Jones and Dubey, 2012). By disseminating this vital information, individuals can be empowered to make informed decisions and protect themselves from the potential health hazards linked to this parasitic infection. Additionally, routine vaccination of sheep and goats should be considered to reduce the risk of abortions and human exposure to this pathogen.

Namibia must also explore international best practices that have successfully managed similar diseases in livestock industries. By studying how other countries have effectively tackled similar challenges, valuable insights can be gained to inform disease control efforts within the country. Learning from the experiences and strategies implemented elsewhere can help identify innovative approaches that may be adapted to the unique circumstances of Namibia's livestock sector. Such an approach allows for integrating proven methods and avoiding potential pitfalls, ultimately strengthening the overall disease control measures. By fostering an open and collaborative exchange with other nations, Namibia can tap into a wealth of knowledge and expertise to enhance its disease management initiatives, ultimately promoting a healthier and more resilient livestock industry.

The *N. caninum* DNA could not be detected in bovine brain, heart or blood samples (Chapter 6), although serological evidence of its presence was found (Samkange et al., 2023b). One potential explanation for this disparity is the low prevalence of neosporosis in the country, as demonstrated in a previous serosurvey where only 5.7% (n=736) of the samples were positive (Samkange et al., 2023b). The small proportion of positive cases in the serosurvey might have contributed to the inability to detect *N. caninum* DNA in bovine tissues during the molecular study (Chapter 6). A

larger sample size might be necessary to enhance the sensitivity of future molecular investigations. Increasing the number of samples could significantly improve the probability of detecting *N. caninum* DNA (Pfeiffer, 2002). Alternatively, targeting aborted foetuses in future studies will increase the probability of detecting positive cases, as these could serve as potential sources with higher levels of *N. caninum* DNA. Overall, the discrepancy between the serological evidence and molecular findings underscores the importance of selecting appropriate study populations and methodologies to understand the prevalence of neosporosis comprehensively.

Comparing *T. gondii* serosurvey results in sheep reveals a similar discordance between serological and molecular findings. The serosurvey in sheep indicated a very low prevalence of 3.68% (n = 299) (Samkange et al., 2023b); however, the molecular survey conducted on abattoir samples (hearts and brains) yielded higher results (Chapter 6). This discrepancy highlights the complexities involved in studying parasitic infections and the importance of considering multiple factors that may influence the detection of pathogens. Additionally, the parasite's tissue-specific distribution might contribute to the variation between the two studies (Hughes et al., 2008, 2006). This emphasizes the necessity for comprehensive approaches incorporating both serological and molecular methods while carefully considering the selection of target tissues and sample sizes. Researchers can obtain a more accurate understanding of the prevalence and distribution of parasitic infections in animal populations through a well-rounded and complementary analysis. Further investigations employing a combination of techniques are crucial to shedding light on the complexities of these parasitic diseases and ensuring effective control measures are developed to safeguard human and animal health.

However, it is essential to note that this research was limited to the Khomas region of Namibia, and therefore, the findings cannot be extrapolated to the rest of the country. Hence, further research is necessary to obtain a comprehensive picture of the situation in other regions of the country.

7.2. Recommendations

In light of the findings of this research and the above discussions, the following is a summary list of recommendations:

1. Initiate targeted public awareness campaigns to educate livestock producers about neosporosis and toxoplasmosis, including their potential impacts and preventive measures. Utilize workshops, educational materials, farmers' magazines, and online platforms to reach a wider audience.
2. Facilitate regular communication between animal health practitioners (AHPs) and livestock producers to share information about neosporosis and toxoplasmosis. Provide training to AHPs in effective communication strategies to promote open dialogues and address concerns.
3. Foster community involvement and ownership in disease control programmes by engaging local communities, farmers, and livestock producers. Nurture a sense of ownership and responsibility among participants to enhance adherence to disease prevention measures.
4. Provide specialized training and continuous education for AHPs to keep them up-to-date with the latest advancements in neosporosis and toxoplasmosis research, diagnosis, and control strategies.
5. Establish efficient reporting mechanisms to encourage AHPs to regularly request testing for *N. caninum* and *T. gondii* in cases of abortions. This will aid in monitoring disease prevalence and the potential implementation of suitable control measures.
6. Foster collaboration between government veterinary services, research institutions, and private veterinary practitioners to develop comprehensive disease control programs and guidelines.
7. Adopt a One Health approach for toxoplasmosis by promoting interdisciplinary collaboration between veterinary authorities, public health agencies, and environmental experts to address the public health risks associated with the disease.
8. Invest in further research and surveillance to better understand the prevalence, risk factors, and transmission dynamics of neosporosis and toxoplasmosis in Namibia. Use this data to inform evidence-based control strategies.

9. Educate the public about the food safety risks of consuming inadequately cooked meat to prevent toxoplasmosis and other foodborne/zoonotic diseases.
10. Learn from international best practices in managing similar diseases in livestock industries to inform disease control efforts within Namibia.
11. Conduct further studies in different regions of Namibia to understand the epidemiology of neosporosis and toxoplasmosis throughout the country.

By implementing these recommendations, Namibia can work towards a more holistic approach, leading to more effective control measures for neosporosis and toxoplasmosis in the livestock industry.

7.3. Conclusions

In conclusion, the findings presented in this study shed light on the prevailing knowledge gaps and communication challenges surrounding neosporosis and toxoplasmosis among livestock producers and animal health practitioners in the Khomas region of Namibia. The results of the KAP study revealed a significant lack of awareness about these diseases among livestock producers, while animal health practitioners demonstrated a general but insufficient level of knowledge. Furthermore, the absence of regular testing requests for *N. caninum* and *T. gondii* in cases of abortions and limited discussions about these diseases with farmers further emphasize the need for improved communication between AHPs and livestock producers.

Identifying serological and molecular evidence of neosporosis and toxoplasmosis in Namibian livestock underscores the urgency of addressing these parasitic infections for the sake of animal health and public health. The low prevalence of neosporosis found in the country highlights the need for larger sample sizes in molecular studies to improve detection rates. Additionally, targeting aborted fetuses in future research could enhance the probability of identifying *N. caninum* DNA.

The comprehensive recommendations outlined in this study aim to address the current challenges and pave the way for effective disease control and prevention strategies. Initiatives such as targeted public awareness campaigns and improved communication between AHPs and livestock producers are crucial to closing the knowledge gap and fostering a more proactive approach towards disease prevention. Namibia can effectively create a unified and sustainable strategy to combat

neosporosis and toxoplasmosis by engaging local communities and stakeholders and implementing a One Health approach.

Continued investment in research and surveillance will provide essential data to policymakers, enabling evidence-based control strategies for these diseases. Furthermore, emphasizing food safety education and learning from successful practices in other countries can further enhance Namibia's disease management initiatives.

Overall, with collaborative efforts and a commitment to knowledge dissemination and communication, Namibia can work towards safeguarding the health and well-being of its livestock, farmers, and the public at large. By taking proactive measures and adopting a holistic approach, the country can pave the way for a healthier and more resilient livestock industry, benefiting both animals and humans.

7.4. References

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8. Scientific publications connected with this dissertation

1. Samkange, A., Chitanga, S., Neves, L., & Matjila, T. (2023). Evaluation of knowledge, attitudes and practices regarding neosporosis and toxoplasmosis among farmers and animal health practitioners in Namibia. *Tropical Animal Health and Production*, 55(1), 28. <https://doi.org/10.1007/s11250-022-03441-y>
2. Samkange, A., Chitanga, S., Tjipura-Zaire, G., Mutjavikua, V. G., Smith, J. W., Neves, L., & Matjila, T. (2023a). Seroprevalence and associated risk factors of bovine neosporosis in the Khomas region of Namibia. *Onderstepoort Journal of Veterinary Research*, 90(1), a2077. <https://doi.org/https://doi.org/10.4102/ojvr.v90i1.2077>
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Appendices

Appendix 1 KAP study questionnaire: farmers

Target respondents: Farm owners or farm workers who spend a lot of time with the animals

Purpose: Most Namibian farmers have experienced abortions and other reproductive problems in their cattle and small stock (sheep & goats). *Neospora caninum* and *Toxoplasma gondii* are some of the possible causes in cattle and small stock, respectively. However, these two parasites have never been studied in Namibia. The purpose of this questionnaire is to solicit information on the knowledge, attitudes and perceptions of livestock farmers in the Khomas region with respect to *Neospora caninum* and *Toxoplasma*.

Confidentiality: We assure you that all the information you will provide will be treated in the strictest confidence.

DECLARATION OF INFORMED CONSENT

I _____ do hereby confirm that the contents of this questionnaire have been fully explained to me and therefore give my consent for this interview. I further give permission for the results of this interview to be published for research purposes as long as my personal details are kept anonymous.

Signature _____ Date _____

PART A: PERSONAL INFORMATION

NAME (optional): _____ Age: 25-39 ☐ 40-50 ☐ 51-60 ☐ >60 ☐

SEX: Male ☐ Female ☐

Farm/village name: _____ Establishment size _____

Designation: *Owner* *worker* *Leasing* *Other* _____

Type of establishment: *Commercial* *Resettlement* *Communal*

Contact number: _____ Email: _____

PART B: KNOWLEDGE

1. What are your educational qualifications?
 - A. Degree/diploma
 - B. Secondary school
 - C. Primary school
 - D. None
2. How long have you been farming? _____
3. What can cause abortions in cattle, sheep or goats? _____

4. If livestock abortions occur at your farm, how much would it cost you for each of the following:
 - A. Calf aborted _____
 - B. Lamb aborted _____
 - C. Kid aborted _____
 - D. Don't know _____
5. Have you ever experienced abortions at your farm? _____
6. If yes, what do you think were the causes of those abortions? _____

7. Do you know of any abortion-causing livestock diseases, which can affect people? ____

8. Do you know how animals get infected with abortion-causing diseases?

9. What can you do to prevent abortions in your animals? _____

10. Have you ever heard of a disease called neosporosis or toxoplasmosis? _____

11. Do you know which animals are affected by a disease called neosporosis?

12. 11. Do you know which animals are affected by a disease called toxoplasmosis?

13. What vaccines do you know which can prevent abortions? _____

14. Does keeping dogs at your farm pose any risk to your animals? If yes, what? _____

15. Does keeping cats at your farm pose any risk to your animals? If yes, what? _____

PART C: ATTITUDES

1. When abortions occur in your animals why would it worry you? _____

2. If abortions occurred at your farm what would you do? _____

3. If your animals tested positive for neosporosis and/or toxoplasmosis, will you do anything to control these diseases?

4. If yes, what would you do? _____

5. If you knew that an animal which you intent to buy had aborted before, would you still buy it? Why?

-
6. In terms of causes of livestock losses, how would you rank them in order of importance? (e.g. predation, abortions, diseases, injuries etc.)

7. Is it a good practice to eat undercooked meat, and why? _____

8. Do you think that other farmers which you regularly interact with know or have heard about neosporosis _____ and/or _____ toxoplasmosis?

PART D: PRACTICES

1. How much does livestock production contribute to your livelihood?
- A. 100%
 - B. 75%
 - C. 50%
 - D. 25%
 - E. Less than 20%
2. On an average year, how many animals do you sell?
- A. Cattle _____
 - B. Sheep _____
 - C. Goats _____
3. How many animals do you slaughter for household consumption per year?
- A. Cattle _____
 - B. Sheep _____
 - C. Goats _____
 - D. Other (specify) _____
4. What vaccinations do you give to your animals and how often for each?

-
5. Do you give anything to your animals to prevent abortions in cattle? If yes, what?
-
6. Do you give anything to your animals to prevent abortions in sheep & goats? If yes, what?
-
7. What do you do with animals that have aborted:
- A. Once _____
 - B. Twice _____
 - C. Thrice _____
8. Where do you source your breeding replacement stock? _____
9. Do you first get any information regarding the farm from which you buy your replacement stock?
-
10. How do you want your meat grilled and why?
- A. Well-done _____
 - B. Medium done _____
 - C. Rare _____
 - D. Other _____
11. What records do you keep for your animals? _____
-
-
-

Appendix 2: KAP study questionnaire: animal health practitioners

Target respondents: Animal health practitioners in Namibia (veterinarians & animal health technicians)

Purpose: Most Namibian farmers have experienced abortions and other reproductive problems in their cattle and small stock (sheep & goats). *Neospora caninum* and *Toxoplasma gondii* are some of the possible causes in cattle and small stock, respectively. However, these two parasites have never been studied in Namibia. The purpose of this questionnaire is to solicit information on the knowledge, attitudes and perceptions of the animal health practitioners in the country with respect to *Neospora caninum* and *Toxoplasma*.

Confidentiality: We assure you that all the information you will provide will be treated in the strictest confidence.

DECLARATION OF INFORMED CONSENT

I _____ do hereby confirm that the contents of this questionnaire have been fully explained to me and therefore give my consent for this interview. I further give permission for the results of this interview to be published for research purposes as long as my personal details are kept anonymous.

Signature _____ Date _____

PART A: PERSONAL INFORMATION

Age group: 25-39 ☐ 40-50 ☐ 51-60 ☐ >60 ☐

SEX: Male ☐ Female ☐

Town/village: _____

Type of Practice: Private ☐ State ☐ Other _____

Qualification: Veterinarian ☐ Para-veterinary professional ☐

Type of animals the practice attends to: Companion animals ☐ Production animals ☐ Mixed ☐

PLEASE USE YOUR WALK-AROUND KNOWLEDGE TO COMPLETE THIS QUESTIONNAIRE; YOU DON'T NEED TO LOOK THE INFORMATION UP

PART B: KNOWLEDGE

1. What are the common causes of abortions do you know in the animals below:
 - a. Cattle: _____

 - b. Sheep & goats: _____

2. What is the level of your knowledge of *Neospora caninum*?
 - a. Knowledgeable
 - b. Average
 - c. Below average
 - d. No knowledge
3. How is *N. caninum* transmitted? _____
4. What are the final hosts of *Neospora caninum*? _____
5. Does *N. caninum* cause any clinical signs in the final host? Yes No
6. If yes to above, what are the main clinical signs? _____

7. Which animals are affected by *N. caninum*? _____
8. What is the main problem caused by *N. caninum* in cattle? _____

9. Is *N. caninum* a zoonosis? *Yes* *No*
10. Is there an effective vaccine against neosporosis? *Yes* *No*
11. What is the level of your knowledge of *Toxoplasma gondii*?
- a. Knowledgeable
 - b. Average
 - c. Below average
 - d. No knowledge
12. How is *T gondii* transmitted? _____
13. What are the final hosts of *T gondii*? _____
14. Does *T gondii* cause any clinical signs in the final host? *Yes* *No*
15. If yes to above, what are the main clinical signs? _____

16. Which animals are affected by *T gondii*? _____
17. What is the main problem caused by *T gondii* in sheep and goats? _____

18. Is *T gondii* a zoonosis? _____
19. If yes, how do humans get infected? _____

20. Is there an effective vaccine against *T gondii*? *Yes* *No*
21. If yes, what's its name? _____
22. Does veterinary inspected meat pose any dangers to the public? Why? _____

PART C: ATTITUDES

1. Do you think *N caninum* is present in Namibia? _____
2. Is *N caninum* important for Namibia? _____
3. Should cattle farmers in Namibia be worried about *N caninum*? Why?

4. If *N caninum* vaccine was readily available would you recommend it to farmers for routine vaccination? Why? _____

5. Do you think *N caninum* and/or *T gondii* should be part of the routine abortion screening panel at the
Central _____ Veterinary _____ Laboratory?

6. If *T gondii* vaccine was readily available would you recommend it to farmers for routine vaccination?
Why? _____
7. Do you think that the veterinary fraternity in Namibia is very informed about neosporosis?

8. Do you think that the veterinary fraternity in Namibia is very informed about toxoplasmosis?

9. Do you think it's important to regularly discuss toxoplasmosis and/or neosporosis with livestock farmers? _____

PART D: PRACTICES

1. For cattle abortion cases, which diseases do you normally request lab tests for?

2. For sheep & goat abortion cases, which diseases do you normally request lab tests for?

3. Have you ever requested tests for *N caninum* and/or *T gondii*? YES NO

4. How do you normally communicate/interact with livestock owners? _____

5. Do you routinely hold farmers' meetings? YES NO

6. What topics do you normally cover during farmers' meeting? _____

7. Have you ever talked to farmers about *N caninum* and/or *T gondii* on any gathering? _____

8. In the past 12 months did you talk to any cat owner about toxoplasmosis?

Appendix 3: *Neospora caninum* risk factors questionnaire: farmers

Target respondents: Cattle owners/workers who are familiar with the animals and husbandry practices on the farm

Purpose: Neosporosis, caused by *Neospora caninum*, is one of the most common causes of abortions in cattle worldwide. In Namibia, the status of this disease in cattle is unknown. As part of a doctoral dissertation, this questionnaire has been designed to solicit information about any factors on this farm that could increase the likelihood of neosporosis being present in your animals.

Confidentiality: Any information provided will be treated as strictly confidential.

DECLARATION OF INFORMED CONSENT

I _____ do hereby confirm that the contents of this questionnaire have been fully explained to me and therefore give my consent for this interview. I further give permission for the results of this interview to be published for research purposes as long as my personal details are kept anonymous.

Signature _____ Date _____

PART A: Farm information

Farm name: _____ Farm size _____

Region: _____

Farm owner/designate: _____

Contact no. _____ Email: _____

Person filling in the questionnaire (occupation): _____

Production type: (tick appropriate box)

Beef production

Stud farm

Dairy production

Mixed (specify)

Other animals
(specify)

PART B: Animal information

Number of heifers: _____

Number of breeding cows: _____

Number of bulls: _____

Number of calves born last season _____

Other animals on the farm and their numbers:

PART C: Other questions

1. How do you feed your animals? _____

2. Do you supplement your animals? If yes, what is used and what time of the year? ____

3. Do you herd your animals? If yes, who/what herds them? (e.g. hired herders, dogs...)

4. What vaccinations do you use? _____

5. What type service do you use for your cows & heifer?
- a. Artificial insemination
 - b. Natural service
 - c. Both A and B
6. Where do your animals drink water?
- a. Water troughs
 - b. Open ponds in the field
 - c. Water troughs and ponds in the field
 - d. Other (specify) _____
7. How often do you clean water troughs?
- a. Weekly
 - b. Monthly
 - c. Yearly
 - d. Other (specify) _____
8. Do you have any dogs on the farm? YES ☐ NO ☐
9. If yes, how many dogs? _____ breed(s) _____
10. If YES, are the dogs kept indoors or they move around freely on the farm?
- A. Indoors
 - B. Move freely
 - C. Others _____
11. Did you see any stray dogs on the farm over past 12mo _____
12. If yes, when did you last see them & how many? _____

13. What is the type of fencing around the farm?

- a. Jackal-proof/diamond mesh with no access to stray dogs
- b. Strands of steel wire
- c. Other (specify) _____

14. Are the following animals present on the farm:

- a. Jackals _____ Sightings: few/moderate/many
- b. Other canids _____ Sightings: few/moderate/many

15. Did you experience any abortions on your farm in the last 3 years? YES ☐ NO ☐

16. Which animals aborted?

- a. Heifers
- b. Adult cows
- c. Both

17. How many abortions occurred?

- a. >10%
- b. <10%

18. When did the abortions occur? _____

19. What time of the year did the abortions occur? _____

20. In the last 3 years, did you have cases of retained placentas or uterine infections? How many?

21. Did you observe dogs eating aborted materials or placentas on the farm?

a. Yes

b. No

22. What do you do with cows that abort?

a. Keep on the farm

b. Sell or slaughter

23. Where do you get your replacement heifers from?

A. Reared at the owner's farm _____

B. From other farms _____

C. From auctions _____

D. Other (specify) _____

24. When was the last time you bought in animals? How many?

25. What routine vaccinations are done on the farm? _____

26. What is the average annual rainfall on this farm? _____

27. Rainfall patterns over the past 3 seasons (*circle the appropriate answer*):

a. Current season: *Normal* *Above average* *Below average*

b. Last season: *Normal* *Above average* *Below average*

c. Season before last: *Normal* *Above average* *Below average*

28. What do you supplement your animals in dry season?

a. Buy or harvest hay

b. Licks

c. Both A and B

NUMBER OF BLOOD SAMPLES COLLECTED

Cattle _____

Completed by _____

Signature _____

Date _____

Appendix 4: *Toxoplasma gondii* risk factors questionnaire: farmers

Target respondents: Sheep & goats owners/workers who are familiar with the animals and husbandry practices on the farms

Purpose: Toxoplasmosis, caused by *Toxoplasma gondii*, is a zoonotic disease that is one of the most common causes of abortions in sheep and goats worldwide. In Namibia, the status of this disease in sheep & goats is unknown. As part of a doctoral dissertation, this questionnaire has been designed to solicit information about any factors on this farm that could increase the likelihood of toxoplasmosis being present on this farm/settlement.

Confidentiality: Any information provided will be treated as strictly confidential.

DECLARATION OF INFORMED CONSENT

I _____ do hereby confirm that the contents of this questionnaire have been fully explained to me and therefore give my consent for this interview. I further give permission for the results of this interview to be published for research purposes as long as my personal details are kept anonymous.

Signature _____ Date _____

PART A: Farm information

Farm name: _____ Farm size _____

Geo-coordinates/location: _____

Farm owner: _____

Contact details: _____

Production type: (tick appropriate box)

Beef

Stud farm

Dairy

Mixed (specify)

Other animals
(specify)

PART B: Animal information

- Number of cattle: _____
- Number of ewes: _____ Breeds _____
- Number of lambs _____
- Number of rams _____ Breeds _____
- Number of does (she-goats): _____ Breeds _____
- Number of bucks (he-goats) _____ Breeds _____
- Number of kids _____
- Lambs born in the last 2 years: Last year _____ Year before last _____
- Kids born in the last 2 years: Last year _____ Year before last _____

- Other animals on the farm and their numbers:

PART C: Other questions

-
1. How do you feed your animals (sheep and/or goats)? _____

 2. Do you supplement your animals? If yes, what is used and what time of the year? ____

 3. Do you herd your animals? If yes, who/what herds them? (e.g. hired herders, dogs...) _____

 4. What vaccinations, if any, are used and at what time of the year? _____

 5. Do you deworm your animals? If yes, what do you use and how often? _____

 6. Do you have problems with external parasites? If yes, which ones and how do you control them?

 7. Do you have cats on this farm? YES ☐ NO ☐
 8. If yes, how many cats? _____
 9. How do you dispose of cat litter? _____
 10. Did you see any stray cats on the farm over past 12mo _____
 11. If yes, when did you last see them & how many? _____
 12. Where do your animals drink water?
 - a. Water troughs
 - b. Open ponds after rain
 - c. Both

13. How often do you clean water troughs?

- a. Weekly
- b. Monthly
- c. Yearly
- d. Other (specify) _____

14. Do cats have access to animal feeds? _____

15. Have you ever found cat faeces in your animal feed? _____

16. Did you experience any abortions on your farm in the last 5 years? YES ☐ NO ☐

If yes:

- Which animals? _____
- How many abortions? _____
- When did they occur? _____

17. What routine vaccinations are done on the farm & how often? _____

18. What is the average annual rainfall on this farm? _____

19. Rainfall patterns over the past 3 seasons (*circle the appropriate answer*):

- a. Current season: *Normal* *Above average* *Below average*
- b. Last season: *Normal* *Above average* *Below average*
- c. Season before last: *Normal* *Above average* *Below average*

NUMBER OF BLOOD SAMPLES COLLECTED

Goat _____

Sheep _____

Completed by _____

Signature _____

Date _____

Appendix 5: Ethical approval certificate (REC)



Faculty of Veterinary Science
Research Ethics Committee

22 February 2024

LETTER OF APPROVAL

Ethics Reference No: REC087-21
Protocol Title: Prevalence, associated risk factors and molecular analysis of *Neospora caninum* and *Toxoplasma gondii*; knowledge, attitudes and practices in Namibia
Principal Investigator: Dr A Samkange
Supervisors: Prof PT Matjila

Dear Dr A Samkange,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC087-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely



PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee



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University of Pretoria, Faculty of Veterinary Science
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Tel +27 (0)12 529 5260
Email mark.watson-ink@up.ac.za

Faculty of Veterinary Science
Fakulteit Veeartsenrykunde
Lefapha la Disaense tsa Bongakadirulwa

Appendix 6: Ethical approval (Humanities)



Faculty of Humanities
Fakulteit Geesteswetenskappe
Letwapha le Bumantho



20 June 2022

Dear Dr A. Samkange

Project Title: Prevalence, associated risk factors and molecular analysis of *Neospora caninum* and *Toxoplasma gondii*; knowledge, attitudes and practices in Namibia
Researcher: Dr A. Samkange
Supervisor(s): Prof PT Matjila
Department: Veterinary Tropical Diseases
Reference number: 26400422 (HUM005/0322)
Degree: Doctoral

Thank you for the application that was submitted for ethical consideration.

The Research Ethics Committee notes that this is a literature-based study and no human subjects are involved. The application has been approved on 20 June 2022 with the assumption that the document(s) are in the public domain. Data collection may therefore commence, along these guidelines.

Please note that this approval is based on the assumption that the research will be carried out along the lines laid out in the proposal. However, should the actual research depart significantly from the proposed research, a new research proposal and application for ethical clearance will have to be submitted for approval.

We wish you success with the project.

Sincerely,



Prof Karen Harris
Chair: Research Ethics Committee
Faculty of Humanities
UNIVERSITY OF PRETORIA
e-mail: tracey.andrew@up.ac.za

Research Ethics Committee Members: Prof Ms Harris (Chair); Mr A Sison; Dr A M de Bree; Dr A van der Linde; Dr F Gertse; Mr KT Gwender Andrew; Dr E Johnson; Dr D Krige; Prof D Maree; Mr A Mohamed; Dr J Neneke; Dr J Dicks; Dr C Potjaght; Prof D Poykari; Prof M Steyn; Prof E Talana; Ms D Mokoena

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Tel: +27 (0)12 420-9844; Fax: +27 (0)12 420-9845; email: ghwender@up.ac.za | www.up.ac.za/faculty-of-humanities

Appendix 7: Ethical clearance certificate (University of Namibia)


ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: NEC0007 **Date:** 26/04/2022

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

Title of Project: PREVALENCE, ASSOCIATED RISK FACTORS AND MOLECULAR ANALYSIS OF NEOSPORA CANINUM AND TOXOPLASMA GONDII; KNOWLEDGE, ATTITUDES AND PRACTICES IN NAMIBIA

Principal researchers: Dr. Alaster Samkango

Staff Number/ Student number: 102442

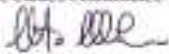
Remarks: the research proposal respects all the parameters to obtain the ethical clearance

Centre for Research Services

Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee
4. The ethics committee retains the right to:
 - i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
 - ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.



Dr. Umberto Molini (Chairperson Neudamen Decentralized Ethics Committee)



Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)