ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY, EPIDEMIOLOGY AND PUBLIC HEALTH

SEROPREVALENCE AND ISOLATION OF *TOXOPLASMA GONDII* FROM SHEEP AND GOATS IN CENTRAL ETHIOPIA

BY
MUKARIM ABDURAHAMAN

JUNE, 2012
DEBRE ZEIT, ETHIOPIA

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A Thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Masters of Veterinary Science in Tropical Veterinary Public Health

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LIST OF ABBREVIATIONS

μl	Micro Litre
AARDO	Ambo Agricultural and Rural Development Office
AIDS	Acquired immune Deficiency Syndrome
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESZARDO	East Shoa Zone Agricultural and Rural Development Office
g	Gram
HIV	Human Immuno Deficiency Virus
i.p	Intraperitoneal
IFA	Indirect Fluorescent Antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRR	Incidence Risk Ration
Masl	Meters above sea level
MAT	Modified Agglutination Test
MDAT	Modified direct Agglutination Test
$NaCl_2$	Sodium Chloride
^{o}c	Degree Celsius
OR	Odds Ratio
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PI	Post Inoculation
RFLP	Restricted Fragment Length Polymorphism
SFT	Sabin Feldman Test
UK	United Kingdom
USA	United State of America

ABSTRACT

A cross-sectional study was conducted in Central Ethiopia from September, 2011 to May, 2012 to estimate the seroprevalence and risk factors of toxoplasmosis in sheep and goats of Central Ethiopia. Additionally, follow-up experimental study on mice was conducted during the same period in order to: isolate viable *T. gondii*, enumerate bradyzoites cysts and compare the diagnosis of *T. gondii* infection by Modified Agglutination Test (MAT) and bioassay. Blood and heart samples (n=357) from sheep and goats originating from Ambo, Ada'a-Liben and Fentale districts were collected from HELEMEX export abattoir, Debre Zeit and sera were examined for *T. gondii* antibodies by MAT. Hearts of those seropositive sheep and goats (n=54) were bioassayed in mice. Sera and brain of mice were examined for *T. gondii IgG* antibodies and tissue cysts, respectively. To this effect, the overall seroprevalence of anti-*Toxoplasma gondii* antibodies in sheep and goats was 20.5% (n=73/357). The present study revealed that seroprevalence was higher in small ruminants of Ambo (Odds ratio [OR] = 2.7, 95% confidence interval [CI]: 1.07, 6.80; 0.036) than Ada'a-Liben district, in sheep (OR=1.7, 95% CI: 1.02, 2.90; P=0.041) than goats and from samples of March (OR=5.4, 95% CI: 1.70, 17.07; P=0.004) than from samples of April. Among the 54 bioassays performed, viable *T. gondii* was isolated from hearts of 8 (34.78%) of 23 sheep and 13 (41.9%) of 31 goats. Quantification of cysts per brain of mice showed an average of 34.73 tissue cysts with a minimum and maximum of 0 and 554 respectively. Most appreciably, 2 of 21 *T. gondii* isolates from sheep and goats were mouse-virulent. The final Zero-inflated Poisson regression model of potential predictors of cyst count showed that cyst count was higher from heart samples of Ambo (Incidence risk ratio [IRR]= 1.8, 95% CI: 1.15, 2.69; P=0.009) and Ada'a-Liben (IRR = 3.2, 95% CI: 2.22, 4.76; P=0.000) districts than Fentale district, from hearts of female animals (IRR = 2.1, 95% CI: 1.36, 3.22; P=0.001) than male animals, from hearts of adult sheep and goats (IRR = 1.5, 95% CI: 1.11, 2.08; P=0.009) than young sheep and goats, from hearts of goats (IRR = 1.5, 95% CI: 1.19, 1.78; P=0.000) than sheep hearts and from MAT positive mice (IRR = 1.9, 95% CI: 1.32, 2.82; P=0.001) than MAT negative mice.

There was a significant agreement (Kappa=0.735, P=0.000) between MAT and microscope examination of cysts for diagnosis of *T. gondii* infection in mice. In conclusion, the present study confirms the widespread presence of *T. gondii* antibodies and tissue cysts in sheep and goats in Central Ethiopia. Results of the present study indicate that asymptomatic sheep and goats can harbor mouse-virulent *T. gondii* which might be hazardous for people who consume raw or undercooked meat. Emphasis should be made on the hazards associated with the consumption of raw meat as ingestion of raw meat could be source of human infection in Ethiopia. This is the first report on isolation of viable *T. gondii* from sheep and goats in Ethiopia. A further study to characterize the genotypes of isolated strains is in progress.

Keywords: *Bioassay*, *MAT, Tissue cysts, Sheep, Goat, Toxoplasma gondii*

1. INTRODUCTION

Toxoplasmosis is one of the most prevalent parasitic infections of medical and veterinary importance due to its negative impacts on health and production. It is caused by an obligate intracellular parasite called *Toxoplasma gondii* (Jithendran, 2004; Ghazaei, 2006; Dubey, 2010b). *Toxoplasma gondii* is one of the most successful protozoan parasites due to its ability to manipulate the host immune system and establish a chronic infection (Dubey, 2010b).

In 2008, the centennial of the discovery of *T. gondii* was celebrated. However, toxoplasmosis is still a neglected and underreported disease, despite having adverse health effects similar to, for example, those of salmonellosis and campylobacteriosis. Human vaccines are not available and the results of the usage of the current antiparasitic therapies are quite disappointing (Kijlstra and Jongert, 2008).

Toxoplasmosis is found worldwide in nearly one-third of the human population (Tenter *et al*., 2000; Dubey, 2010b). The occurrence of toxplasmosis has been significantly increasing due to the opportunistic infection of immunocompromised patients, such as acquired immune deficiency syndrome (AIDS). In these people deaths usually result from rupture of cysts that lead to continued multiplication of tachyzoites (Roberts and Janovy, 2000). Hence, encephalitis is reported to be the predominant clinical manifestation of toxoplasmosis in these AIDS patients and is believed to be due to reactivation of latent infection (Jithendran, 2004). *Toxoplasma gondii* causes severe encephalitis in up to 40 % of AIDS patients, and with 10 - 30% case fatalities (Tenter *et al.,* 2000). Furthermore, in ocular toxoplasmosis 25% cases develop blindness (Kijlstra and Jongert, 2008). Most ocular cases are now attributed to acquired toxoplasmosis, thus preventive strategies should be directed not only to pregnant women but also in the general population (Kijlstra and Jongert, 2008).

Among livestock, sheep and goats are more widely infected with *T. gondii* than cattle and chicken. This parasite causes abortion and neonatal death with significant economic losses to sheep, goat and pig farmers (Tenter *et al.,* 2000; Ghazaei, 2006). This is more serious especially when primary infection occurs during pregnancy (Radostits *et al*., 2006). The infection does not usually cause clinical symptoms in cattle (Ghazaei, 2006). Toxoplasmosis causes abortion, stillbirths and neonatal mortality in sheep and it is manifested by encephalitis and pneumonia (Radostits *et al*., 2006).

Diagnosis of toxoplasmosis is performed by isolation of the parasite from patients and more commonly by serological tests such as indirect fluorescent antibody (IFA) and enzyme linked Immunosorbent Assay (ELISA) (Yang *et al.,* 2000). Adequately, serodiagnos tic techniques have proved useful in diagnosing Toxoplama infection in both humans and animals, for example modified agglutination test (MAT) (Dubey, 1997a) and ELISA (Hashemi-Fesharki, 1996). The MAT is the major recommended test for diagnosis of *T. gondii* in several animals and humans (Dubey, 2010a). Dubey (1997a) found that MAT has highest sensitivity among all serological tests and this was confirmed by the results obtained by Shaapan *et al*. (2008), who demonstrated the benefits of using more sensitive and specific MAT for the detection of *T. gondii* antibodies in sheep sera which is cheaper, easier than the other tests and does not need special sophisticated equipment.

There have been a large number of serological surveys conducted in many countries examining the prevalence of toxoplasmosis in farm animals and humans from North and South America (Dubey *et al.,* 2005; Ragozo *et al.,* 2008; Carneiro *et al*., 2009; Alvarado-Esquivel *et al.,* 2009; Lopes *et al*., 2010), Europe (Pereira-Bueno *et al*., 2004; Acici *et al.*, 2008; Gilot- Fromont *et al*., 2009).

In Africa different reports indicate widespread occurrence of toxoplasmosis. Sharma *et al.* (2003) reported *Toxoplasma* infection rate of 30% in goats in Botswana. So far limited studies have been undertaken to investigate the magnitude of *T. gondii* infection in animals

and humans in Ethiopia. A preliminary serological study made by Negash *et al. (*2004) in sheep and goat population around Nazareth showed an overall seroprevalence of 54.7% in sheep and 26.7 % in goats using ELISA and MDAT. In another seroprevalence study of human toxoplasmosis of workers at Addis Ababa abattoir, Yimer *et al*. (2005) reported a prevalence of 96.8% Using an indirect haemagglutination assay, Bekele and Kasali (1989) also reported 22.9 %, 11.6 % and 6.6 % prevalence in sheep, goats and cattle in central Ethiopia, respectively.

Generally, there is paucity of data on sero-epidemiology of *T. gondii* in animals and humans in Ethiopia though numerous literatures associate human toxoplasmosis with consumption of raw or undercooked meat products of animal origin. The exact sero-epidemiological distribution and risk factors for the infection of food animals and humans in Ethiopia are unknown but, such studies are important. This is because consumption of raw meat is common and a popular tradition in Ethiopia. In addition, significant segments of citizens in the country are highly susceptible and suffer serious consequences of the disease due to immuno-compromisation. Thus, human toxoplasmosis in Ethiopia might have strong linkage with seroprevalence of the infection in food animals. Little is known about isolation of *T. gondii* from animals in African countries and no published information is available in Ethiopia. Therefore, this study was intended with the following objectives:

OBJECTIVES

Main objective

The main objective of this study was to diagnose *T. gondii* infection in small ruminants by laboratory isolation (bioassay in mice) from three districts of Central Ethiopia.

Specific Objectives

The specific objectives of this study were:

- To determine seroprevalence and risk factors of toxoplasmosis in sheep and goats in the study area
- To isolate *T. gondii* from seropositive sheep and goats heart on mice.
- To quantify the number of *T. gondii* bradyzoites cysts per brain of mice.
- To compare the diagnosis of *T. gondii* infection using serology and bioassay

2. LITERATURE REVIEW

2.1. *Toxoplasma gondii*

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan organism with large numbe of in termediate hosts, including all warm blooded animals and humans that belongs to the Kingd om Animalia, Phylum Apicomplexa, Class Protozoa, Subclass Coccidian, Order Eucoccidia, Family Sarcocystidae and Genus *Toxoplasma* (Dubey, 2010b). The parasite *T. gondii* was discovered by scientists Charles Nicolle in North Africa and Alfonso Splendore in Brazil around 100 years ago (Innes, 2010). Felids, particularly the domestic cat, are its definitive hosts and the only animal species in which oocyst develops (Dubey, 2004). *T. gondii* is regarded as one of the most successful parasite on the earth because of its broad host range, its high infection rates and its benign co-existence with the host (Tenter *et al.,* 2000; Dubey, 2010b). *Toxoplasma gondii* has nucleus most clearly demonstrated with Giemsa stain, located near one pole of the cell. Its cytoplasm contains mitochondria, microtubules, endoplasmic reticulum, ribosomes, Golgi apparatus and rhoptries which are useful in differentiating various *Sarcocystidae* (Jones *et al.,* 2001).

Toxoplasma gondii can take several different forms: the oocyst; the tachyzoites; and the cyst. Oocysts in freshly passed feces are unsporulated (non-infective) and sub-spherical to spherical in shape and 10-12 mm in diameter (Tenter *et al.,* 2000; Acha and Szyfres, 2003). *Toxoplasma gondii* has three infective stages, tachyzoites (endozoites) individual and in-group, bradyzoites (xystoxites) in tissue and sporozoites in sporulated oocysts. Sporulated oocysts contain two ellipsoidal sporocysts which are 2mm x 6-8mm in size. Tachyzoites are often crescent (banana) shaped and 2mm x 6mm in tissue. Tissue cysts vary in size from 5mm to 7mm and contain few to several hundred bradyzoites (Dubey *et al.,* 1998; Acha and Szyfres, 2003). Bradyzoites differ only slightly from tachyzoites in having a nucleus situated towards the posterior end whereas the nucleus in tachyzoites is more central. Besides,

bradyzoites are more slender than tachyzoites and less susceptible to destruction by proteolytic enzymes.

There are three colonial lineages of *T. gondii,* designated as Type I, Type II and III. Type I is highly virulent for mice and can kill in less than 7 days, where as those of type II induce chronic pathology in susceptible strains of mice and type III is least virulent. In humans, type I and II are more frequently associated with congenital infection whereas type II is common in patients in whom disease has reactivated like in AIDS patients (Alexander *et al.,* 2000; Dubey, 2010b). *T. gondii* strain frequently isolated from animals is genotype III (Howe and Sibley, 1995).

2.2. Structure and Life Cycle of *Toxoplasma gondii*

There are three infectious stages of *T. gondii*: the tachyzoites, the bradyzoites, and oocysts. These stages are linked in a complex life cycle (Dubey, 2010b). The tachyzoite consists of various organelles and inclusion bodies including a pellicle (outer covering), cytoskeleton (inner membrane complex, subpellicular microtubules, apical rings, polar rings, a conoid), secretory (rhoptries, micronemes, dense granule), micropore, a mitochondrion, endoplasmic reticulum, a Golgi complex, ribosomes, rough and smooth endoplasmic reticula, micropore, nucleus, amylopectin granules, and an apicoplast (Figure 1) (Dubey *et al.,*1998).

Bradyzoites differ structurally only slightly from tachyzoites. They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located (Figure 1). The contents of rhoptries in bradyzoites are usually electron dense, whereas those in tachyzoites are labyrinthine. However, the contents of rhoptries in bradyzoites vary with the age of the tissue cyst. Bradyzoites in younger tissue cysts may have labyrinthine rhoptries, whereas those in older tissue cysts are electron dense. Also, most bradyzoites have one to three rhoptries that are looped back on them (Dubey, 2010b).

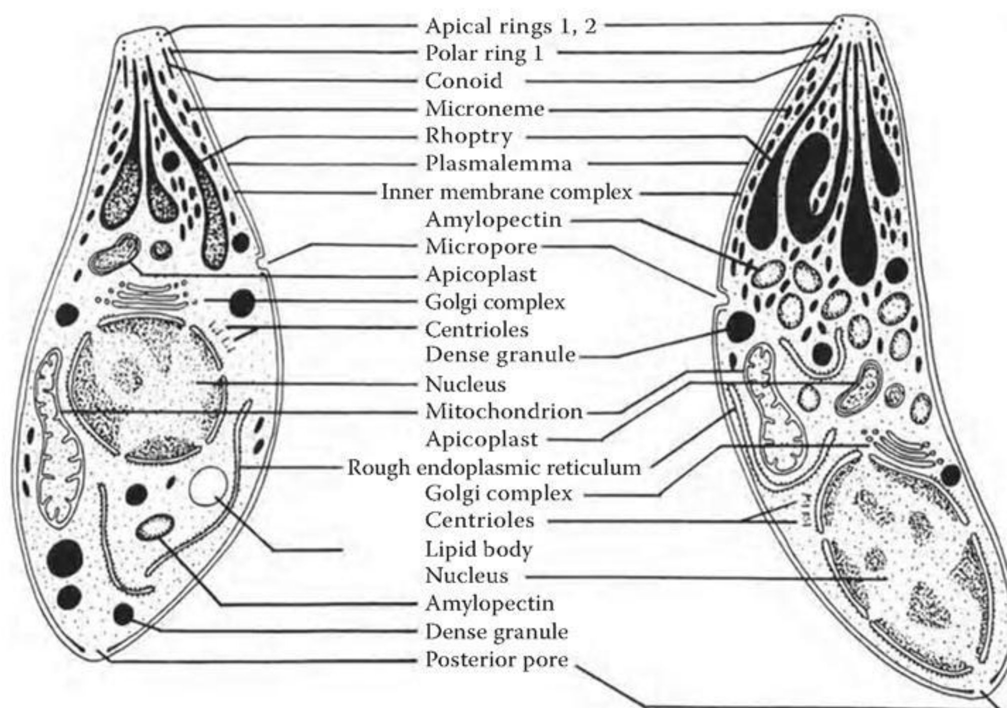

Figure 1: Schematic drawings of a tachyzoite and a bradyzoite of *T. gondii*.

Source: (Dubey *et al.,* 1998*).*

The life cycle of *T. gondii* involves two phases. The sexual phase takes part in the definitive host (*Felidae* family), and the asexual phase in any warm-blooded animal including humans and in birds (Dubey, 2010b). The sexual phase or entero-epithelial cycle occurs in the cat's intestine after ingestion of any infective form. Members of the cat family can become infected by any of the three infective stages through ingesting of food or water contaminated with oocysts, by consuming infected tissues containing tachyzoitesor bradyzoites, or transpl acentally. Zoites penetrate the epithelial cells of the small intestine and initiate a sequence of numerous generations (termed Types A to E) where, after a variable number of schizogonies , microgamonts and macrogamonts are formed. The microgamonts fertilize the macrogamon ts and oocysts are formed. The oocysts are excreted unsporulated and uninfective when pass

ed in the feces (Tenter *et al.,* 2000; Dubey, 2010b). Sporulation occurs in the environment after 1-21 days, dependent on temperature and moisture. Sporulation may take one day at 24-25ºC, five days at 15ºC and twenty one days at 11ºC) (Dubey, 2010b).

Bradyzoite-induced cycle is known and the most efficient; nearly all cats fed tissue cysts can shed the oocyst, whereas less than 30% of cats fed tachyzoites or oocysts shed the oocysts (Speer and Dubey, 2005). In oocyst and tachyzoites-induced cycles, it is hypothesized that after oocyst ingestion *T. gondii* invades many cat tissues. Bradyzoites produced in extra-intestinal tissues return to the intestine to initiate the bradyzoite-induced coccidian cycle. However, tissue cyst rupture is considered infrequent, and thus the complete coccidian cycle occurs only in few cats after ingestion of oocysts (Tenter *et al.,* 2000; Dubey, 1998). Millions of oocysts are produced because of profuse multiplication of *T. gondii* in the feline intestine, usually without clinical signs**.** *Felidae* excrete *T. gondii* oocysts in the feces 3 to 10 days after ingesting bradyzoites, after 13 days of ingesting tachyzoites, and after 18 days of ingesting sporulated oocysts (Speer and Dubey, 2005).

The asexual phase or extra-intestinal cycle occurs in any warm blooded animal after infection by any infectious stage (Dubey, 2010b) (Figure 2). After ingestion, sporozoites are released and invade the macrophages of the intestine. The sporozoites are differentiated into motile tachyzoites. Tachyzoites multiply rapidly by asexual process (endodyogeny) in a variety of cells and eventually encyst in several tissues, particularly in the brain tissues. They are also responsible for congenital infections during pregnancy. When host immunity develops, the process slow down leading to chronic infection, and the tachyzoites enter the bradyzoite (or cystozoites) stage, resulting in tissue cysts. Tissue cysts persist for a long time perhaps for the life of the host (Dubey, 1998; Tenter *et al.,* 2000; Jones *et al.,* 2003). It has been hypothesized that tissue cysts rupture occasionally and the released bradyzoites are killed in immunocompetent hosts. However, in immunosuppressed hosts such as AIDS patients, bradyzoites released from tissue cysts may multiply locally and spread to other organs. Encephalitis is the predominant clinical manifestation of toxoplasmosis in AIDS

patients and is believed to be due to reactivation of latent infections (Lift and Remington, 1992). The location and number of tissue cysts in animals differed with hosts and the strain of *T. gondii*. In mice and rats, more tissue cysts were found in the brain than in visceral tissues, irrespective of the strain given. However, in higher mammals more tissue cysts were present in muscular tissues than in the brain (Dubey, 1997b).

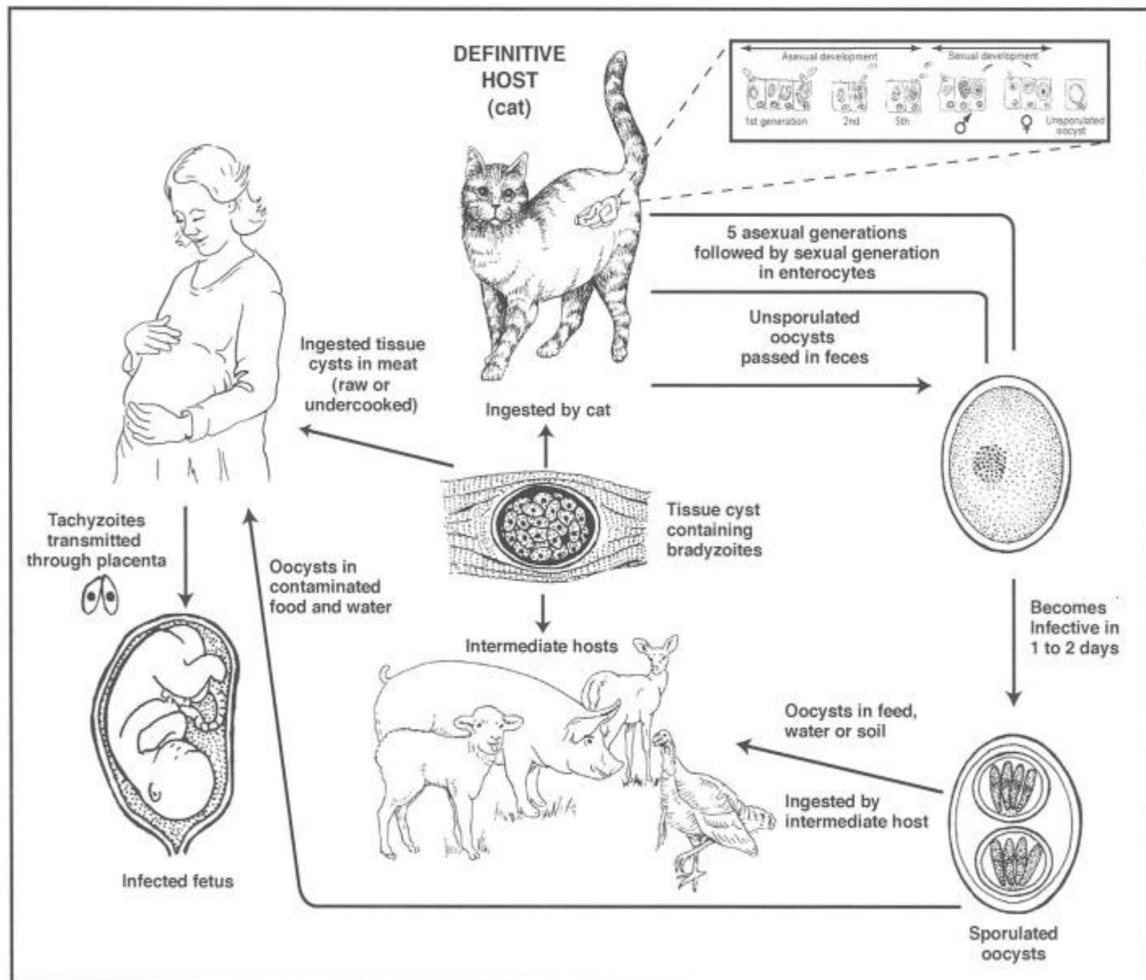

Figure 2: Life cycle of *Toxoplasma gondii* Source: (Dubey, 2010b)

2.3. Molecular biology

The *Toxoplasma gondii* nucleus is haploid except during the sexual division in the intestine of the cat (Pfefferkorn, 1990). The total haploid genome contains 14 chromosomes and 7793 genes, with a total genome size of 63,495,144 base pairs (Khan *et al.,* 2007). It is an unusual parasite because of its broad host range and with only one species in the genus. Prior to the development of genetic markers, *T. gondii* isolates were grouped by their virulence to outbred mice (Dubey *et al.,* 2010a). During the 1980s and 1990s methods were developed to recognize genetic differences among *T. gondii* isolates from humans and animals (Dubey, 2010b). The isolated *T. gondii* strains have been classified into three types (I, II, III) (Howe *et al*., 1997; Howe and Sibley, 1995), based on restriction fragment length polymorphism (RFLP). They proposed that type I isolates were 100% lethal to mice, irrespective of the dose, and that types II and III generally were avirulent for mice (Howe *et al.,* 1996). New clonal types have already been proposed, for example, type IV, which is associated with ocular toxoplasmosis (Grigg *et al*., 2001), type X, from sea otters (Miller *et al*., 2004) and an identical mixed I/III genotype which has been described in humans from Africa (Ajzenberg *et al*., 2004).

Lehmann *et al*. (2006) made the first in-depth study of genetic variability among more than 275 *T. gondii* isolates obtained worldwide from one host (free-range chicken) and in one laboratory (Dubey *et al*., 2002) and found geographic differences, with some isolates confin ed to Brazil whereas others were worldwide in distribution. Phenotypically, *T. gondii* isolates from asymptomatic chickens from Brazil were mouse virulent (Dubey *et al*., 2002). Recent studies have indicated a higher genetic diversity among *T. gondii* isolates in livestock and wildlife in the Americas (Dubey, 2009). Population structure of *T. gondii* and genetic variability among isolates are now under extensive Investigation (Dubey, 2010b).

2.4. Epidemiology of *Toxoplasma gondii*

2.4.1. Host Range and Distribution

Toxoplasmosis is worldwide in distribution and cats have a major influence in the epidemiology of the disease. Islands with geographical isolation and absence of cats have been found to be free of toxoplasmosis (Acha and Szyfres, 2003; Dubey, 2010b). This fact is explained by the preying habits of this group and their diet that includes wild birds, rodents and *Toxoplasma* infected placentas and stillborn fetuses in some cases (Steven *et al.,* 2000). Historically, cats have been associated with domestic animals as an aid to rodent control (Radostits *et al.,* 2006).

Toxoplasma gondii infection in man and animals is widespread throughout the world, but varies in different geographical areas of a country. Causes for these variations are not yet known. Environmental conditions may determine the degree of natural spread of *T. gondii* infection. Infection is more prevalent in warm climates and in low-lying areas than in cold climates and mountain regions and in humid areas than in dry areas. This is probably related to conditions favoring sporulation and survival of oocysts in the environment (Dubey, 2010b).

There have been a large number of serological surveys conducted in many countries examining the prevalence of toxoplasmosis in farm animals and humans from North and South America (Dubey *et al.,* 2005; Ragozo *et al.,* 2008; Carneiro *et al*., 2009; Alvarado-Esquivel *et al.,* 2009; Lopes *et al*., 2010), Europe (Pereira-Bueno *et al*., 2004; Acici *et al.*, 2008; Gilot- Fromont *et al*., 2009), Africa (Bekele and Kasali, 1989; Guebre-Xabier *et al.,* 1993; Achu-Kwi and Ekue, 1994; Sharma *et al*., 2003; Negash *et al*., 2004; Yimer *et al.,* 2005), Asia (Yang *et al*., 2000; Sharma *et al.,* 2008; Huang *et al*., 2010). According to ACIAR (2007) *T. gondii* is widespread, with seroprevalence rates of 2-70% in humans, 35-73% in cats, 75% in dogs, 11-36% in pigs, 11-61% in goats, and less than 10% in cows.

The extent of *T. gondii* infection in cats depends on the availability of infected birds and small mammals (Dubey, 2010b).Toxoplasmosis has been confirmed in some 200 species of vertebrates, including primates, ruminants, swine, equine, carnivores, rodents, marsupials, insectivores and numerous avian species (Tenter *et al.,* 2000; Pal, 2007; Dubey, 2010a). In general among domestic animals, high reactor rates have been found in cats, sheep, goats and swine; lower levels in horse and dogs; and low level in cattle (Tenter *et al.,* 2000; Acha and Szyfres, 2003; Sukthana, 2006, Dubey, 2010b). *Toxoplasma gondii* infection is also prevalent in game animals. Among wild game, *T. gondii* infection is most prevalent in black bears and in white-tailed deer. Approximately, 80% of black bears are infected in the United States and about 60% of raccoons have antibodies to *T. gondii*. Because raccoons and bears scavenge for their food, infection in these animals is a good indicator of the prevalence of *T. gondii* in the environment (Acha and Szyfres, 2003).

2.4.2. Toxoplasmosis in Sheep and Goats

Toxoplasma gondii causes abortion and neonatal mortality in sheep worldwide. *Toxoplasma gondii* has been recognized as one of the main causes of infective ovine abortion in New Zealand, Australia, the United Kingdom, Norway, and the United States (Dubey, 2010b). Actual losses in lambs due to toxoplasmosis are difficult to estimate because (1) the disease is usually sporadic, (2) only a small number of aborted lambs are submitted for diagnosis, (3) those submitted may be inadequately examined, (4) unsuitable material may be sent for diagnosis, (5) the serologic test may not be specific, and (6) toxoplasmosis does not produce clinical disease in the ewe, so this disease does not alarm the farmer as much as other bacterial and viral infections (Dubey, 2010b). Congenitally infected lambs that survive the first week after birth usually grow normally and can be a source of infection for humans.
The worldwide prevalence of *T. gondii* in sheep is high and varies from 3% to 95.7% (Dubey, 2009). In Ethiopia, it ranges from 11.9 to 56 % and 22.9 % to 82 % in sheep and goat, respectively (Table-1) (Bekele and Kasali, 1989; Negash *et al.* 2004).

Although abortion and neonatal mortality are the main clinical signs, adult goats can develop clinical toxoplasmosis involving liver, kidneys and brain (Dubey, 2010b). Presumptive clinical caprine toxoplasmosis was reported from Bangladesh, West Indies, and People's Republic of China, Norway, and the Czech Republic. *T. gondii* was identified as a cause of abortion in California by Moeller (2001) from 3% of 211 aborted tissues, in Italy by Masala *et al.* (2007) from 6.4% of 362 fetuses. Borde *et al*. (2006) found association between *T. gondii* seropositivity and abortion on a goat farm in Tobago, West Indies. Generally, *T. gondii* infection in sheep and goats is responsible for heavy economic losses due to early embryonic death and resorption, fetal death and mummification, abortion, still-birth and neonatal death (Naoi and Yano, 2002; Acha and Szyfres, 2003; Dubey, 2010b). The global seroprevalence reports of caprine toxoplasmosis vary from 2.4 up to 90.9% (Dubey, 2010b).

Table 1: Serological prevalence of *Toxoplasma gondii* in Ethiopia

Study Area	Prevalence (%)				Test	References
	Cattle	Sheep	Goat	Humans		
Central Ethiopia	6.6	11.9	22.9	-	MDAT	Kassali and Teklye (1989)
Various parts of-Ethiopia	-	-	-	74.4	ELISA	Guebre-Xabier *et al.* (1993)
Addis Ababa				80.0	SFT	Tilahun *et al.,*(1998)
Debre Birhan	-	35	34	-	MDAT	Tilaye and Getachew (2002)
Nazareth	-	52.6	24	-	MDAT	Negash *et al.* (2004)
Nazareth	-	56	25.9	-	ELISA	Negash *et al.* (2004)
Addis Ababa	-	-	-	96.77	ELISA	Yimer *et al.* (2005)
South Omo	-	-	82	-	MAT	Teshale *et al.* (2007)
North Omo	-	-	79.5	-	MAT	Teshale *et al.* (2007)
East Shewa zone	-	-	62.2	-	MAT	Teshale *et al.* (2007)
Nazareth	-	-	-	60	MDAT	Negash *et al.* (2008)
South Wollo	-	45.4	37.2	76.5	MAT	Yibeltal (2008)
Central Ethiopia	-	31.6	19.8	-	ELISA	Zewdu *et al*., 2011a & 2011b

2.5. Public Health Importance of *Toxoplasma gondii*

2.5.1. Worldwide studies on human cases of toxoplasmosis

Toxoplasmosis is one of the most common parasitic zoonoses world-wide. Human toxoplasmosis can result from a congenital or an acquired infection (Dubey, 2010b). *In utero,* the fetus may be infected transplacentally, if a sero-negative woman is primarily infected during pregnancy. After birth, humans are usually infected with *T. gondii* by ingestion of oocysts in food or water that have been contaminated with cat feces, or by ingestion of tissue cysts. Therefore, ingestion of raw or lightly cooked meat containing live *T. gondii* tissue cysts, ingestion of raw or lightly cooked vegetables contaminated with oocysts or exposure to oocysts derived from cat feces, while gardening or playing (for children) are the most likely source of infections (Acha and Szyfres, 2003; Pal, 2007; Jones, 2003; Dubey, 2010b).

An epidemiological study on the seroprevalence of acquired toxoplasmosis in HIV infected patients in Nigeria showed that there was a higher seroprevalence of *T. gondii* antibodies among individuals aged 31 - 40 years (36.5%). Patients with concomitant toxoplasmosis and HIV infection manifested fever (63.5%), headache (44.7%), rashes (41.2%) and anorexia (34.1%) (Uneke *et al*., 2005). The most common infections were brain toxoplasmo sis (32.2%), meningeal cryptococcosis (21.5%) and tuberculosis (8.7%). Further studies done in Mexico showed that neurological manifestations in AIDS patients in the state of Yucatan are due to toxoplasmosis in 47% of the cases (Castro-Sansores *et al*., 2004). Lift and Remington (1992) also consider toxoplasmatic encephalitis as one of the most common and most treatable causes of AIDS-associated Central Nervous System (CNS) pathologies. Among patients with AIDS, more than 95% of toxoplasmatic encephalitis is due to the reactivation of a chronic/latent infection caused by the loss of cellular immune surveillance. A study done in Thailand showed that among HIV positive and *T. gondii* antibody positive

groups, 43.2% had symptoms and signs of acute toxoplasmosis involving eye and/or the central nervous system (Sukthana *et al.,* 2006).

Ocular toxoplasmosis used to be attributed to congenital infection, but Gilbert and Stanford (2000) recently compared prenatal and postnatal toxoplasmosis. They concluded that at least two thirds of ocular toxoplasmosis is caused by postnatal infection, which has major public health implications. An outbreak of acquired *T. gondii* infection occurred in the greater area of British Columbia in 1994-1995. In total, 100 patients with acquired and 12 patients with congenital toxoplasmosis were identified.

Among meat producing animals pigs, sheep and goats often harbor *T. gondii* in edible tissue and therefore, raw or under cooked meat from these animals constitute a major risk of the infection for humans. Virtually all edible portions of an animal can harbor viable *T. gondii*. In Europe, parasitism rates in excess of 50% have been found in the meat of sheep and swine slaughtered in abattoirs (Acha and Szyfres, 2003). In Europe and in the USA, tissue cysts have been found in commercial cut of pork, thus pork has been generally considered to be a major source of *T. gondii* infection in human (Tenter *et al.,* 2000). In one study in Iowa (Xiao *et al.,* 2010), viable *T. gondii* was isolated from 17% of 1,000 adult pigs (sows) from a slaughter plant. On the other hand, as cattle appears to be naturally resistant and capable of clearing the infection, beef is generally regarded as not important source of human infection. Less frequently, the edible tissue in infected poultry, rabbits, dogs and horses can harbor *T. gondii* (Quinn *et al.,* 1994; Dubey, 2010b).

2.5.2. Toxoplasmosis in Immunocompromised Patients

In immunocompetent individuals, most infections of *T. gondii* go unnoticed. After infection, the parasite multiplies in various tissues until the body mounts immunity, and then the parasite hides in tissue as cysts and enters the latent phase. During acute toxoplasmosis, symptoms are often influenza-like: swollen lymph nodes, or muscle aches and pains that last

for a month or more. Rarely, a patient with a fully functioning immune system may develop eye damage or nasal lesions from toxoplasmosis. But in those who are immounocompromise d, it can be life-threatening. In these individuals, toxoplasmosis is almost always happens as a result of reactivation of chronic infection (Montoya and Liesenfeld, 2004).

Reactivation of the infection has greatest impact in late AIDS, where up to 25% of patients will develop toxoplasmic encephalitis characterized by unrestricted replication of the tachyzoites stage of the parasite. This is mainly associated with the loss of T-cell function (Alexander *et al.,* 2000). *Toxoplasma gondii* infection is a common opportunistic infection ranging from 20 - 80% in patients with AIDS in the USA and Europe (Tenter *et al.,* 2000). According to Yibeltal (2008), 89.6% of the HIV positive individuals in South Wollo, Ethiopia are positive for anti-*T. gondii* IgG. Similarly, higher MDAT titers were encountered in pregnant and immunocompromised individuals of Nazaret town residents (Negash *et al.,* 2008).

2.6. Diagnosis

Diagnosis of toxoplasmosis is carried out by isolation of the parasite from patients and directly by polymerase chain reaction (PCR), hybridization, histology and more commonly by serological tests such as indirect fluorescent antibody (IFA) and enzyme-linked Immunosorbent Assay (ELISA) (Yang *et al.,* 2000). Adequately, serodiagnostic techniques have proved useful in diagnosing *Toxoplasma* infection in both humans and animals, for example modified agglutination test (MAT) (Dubey, 1997a) and ELISA (Hashemi-Fesharki, 1996). The MAT is the major recommended test for diagnosis of *T. gondii* in several animals and humans (Dubey, 2010b).

2.7. Treatment

The drugs which are used commonly are sulfadiazine (15-25 mg/kg) and pyrimethamine (0.44 mg/kg); they act synergistically. Though they cannot eradicate infection, the drugs are beneficial if given in the acute stage of the disease when there is active multiplication of the parasite.

These drugs are believed to have little effect on the bradyzoite stage. Sulfonamides, trimetoprim, pyrimethamine and clindamycin have been used to treat cats with clinical toxop lasmosis (Elmore *et al.,* 2010). Combining clindamycin with pyrimethamine may offer more effective therapy (Dubey, 2010b). The most typically used and successful treatment in immu nosupressed patients is the combination of pyrimethamine/sulphadiazine and folic acid (Jone s *et al.,* 2003).

2.8. Prevention and Control

Because *T. gondii* is transmitted by multiple modes and sources, it is difficult to establish the definite modes of transmission on an individual basis. But cats are keys to the transmission of *T. gondii* as they are the only definitive hosts that shed the oocysts. Therefore, prevention and control methods should target on *Felidae*. Cats should be provided with adequately cooked meat and should also be prevented from hunting birds and rodents (Urquhart *et al.,* 1996).

The seroprevalence of *T. gondii* in food animals (pig farm) had been greatly reduced by vaccinating cats orally with a strain of *T. gondii* that does not produce oocysts in cats but immunizes them against shedding of oocysts (Mateus-Pinilla *et al.,* 1999). Sulfadiazine-pyrimethamine or monensin can be used as chemoprophylaxis to prevent oocyst shedding.

At present, there is no non-viable, effective vaccine to prevent *T. gondii* infection in animals and humans, with none on the horizon. The only available vaccine is a commercially

produced live preparation for sheep. It consists of tissue culture grown S48 *T. gondii* tachyzoites attenuated by over 3000 passages in mice (Buxton and Innes, 1995). The vaccine stimulates effective protective immunity for at least 18 months following a single subcutaneous injection, and showed a significant reduction in reproduction wastage. The vaccine has a short shelf life and is a potential risk to humans (Dubey, 2010b).

Further options for the prevention and control of toxoplasmosis in sheep and goats include; avoiding their exposure to the oocyst by keeping feeds in a closed container and by proper disposal of cat feces or chemoprophylaxis by adding the anticoccidial drug such as monensin to the feed is effective in reducing lamb losses (Smith and Sherman, 1994).

Practicing good hygienic measures appears to be the best option to minimize transmission of *T. gondii* to humans. This can be done by increasing the public awareness through health education; especially for women of childbearing age, immunocompromised groups and children. The lesson should include avoidance of consumption of raw or under cooked meat or unpasteurized goat's milk products and raw vegetables; wearing of gloves when cleaning cats litter, and washing hands thoroughly with soap after handling suspected materials; washing kitchen materials with hot soapy water after contact with raw meat, poultry, or seafood, or with unwashed fruits or vegetables (Jones *et al.,* 2003; Sukthana, 2006; Pal, 2007).

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in three purposively selected districts of East Shoa (Fentale and Ada'a-Liben districts) and West Shoa (Ambo district) Zones of Oromia Reginal State.

Ada'a-Liben district is located in East Shoa Zone and found at a distance of 45 km from Addis Ababa. It is situated at a longitude of 38^0 58' E to 39^o22'E and latitude of 08^0 22'N to 8° 56' N. The altitude of the district ranges from 1500 to over 2000 meters above sea level (masl). The area is characterized by a tepid to cool submoist agro- ecology. The average rainfall is about 839 mm, while the mean minimum and maximum temperatures recorded for 27 years ranged from 7.`9ºC to 28ºC with an overall average of 18.5ºC (IPMS, 2004). Farming system is mixed crop and livestock production.

Fentale district is located in East Shoa Zone 190 Kms East of Addis Ababa and situated between longitude of 39.93^0E to $39^0$56'0"E and latitude of 8.975^o N to 8.58'30" N. It lies at an altitude range of 955 masl (at Metehara Plain) to 2007 masl (at Mount Fentale); with annual average rainfall of 553 mm ranging from 560 - 630 mm. Average temperature ranges from 29 to 38^0C. With its arid and semi-arid climate, pastoral and agro-pastoral production system predominates in the area (ESZARDO, 2010).

Ambo district is found in West Shoa Zone of Oromia Regional State. The area is found at a longitude of 37^0 32' to 38^0 3' E, and latitude of 8^0 47' to 9^0 20' N and the altitude within the district ranges from 1400 to 3045 masl. The climatic condition of the area is 23% highland, 60% mid altitude, and 17% lowland with an annual rainfall and annual temperature ranging from 800 – 1000 mm and 15°C – 29^oC, respectively. The mean temperature is 18.6 °C. The rainfall is bi-modal with the short rainy season from February to May and long rainy season (over 58.8% of the total annual rainfall) from June to September. Agriculture, of mixed type, is the main occupation of the human population in the area. Major livestock reared include

cattle, sheep, goats, poultry, and pack animals (mules, horses, and donkeys) (AARDO, 2010).

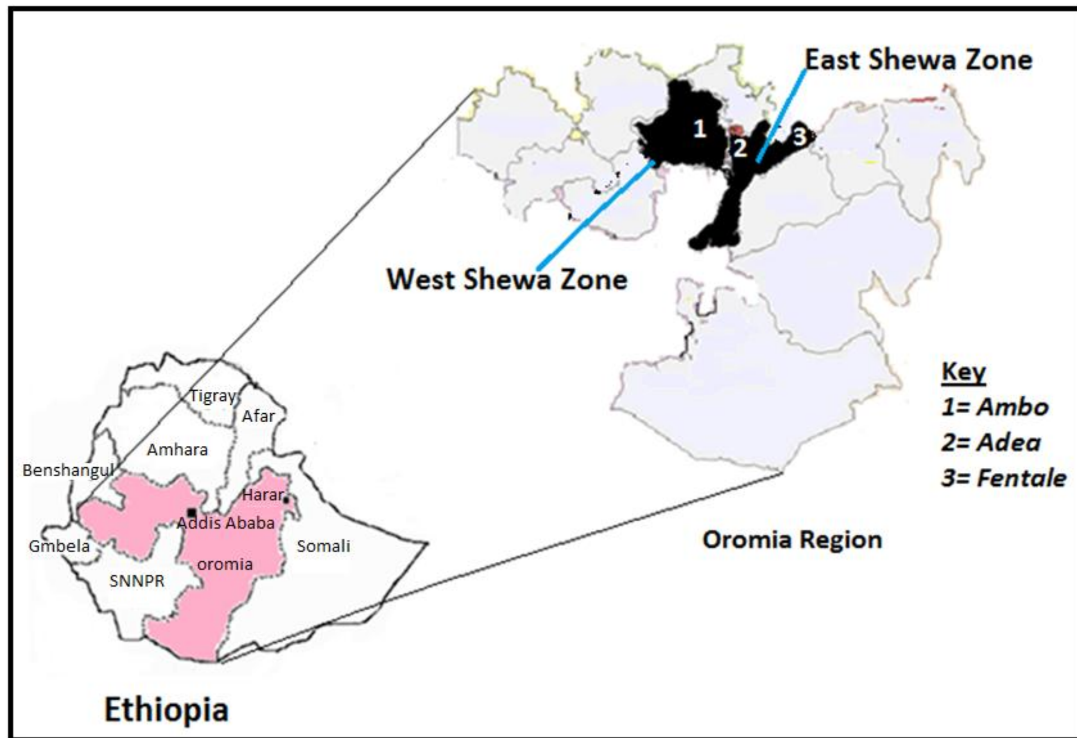

Figure 3**:** Study areas (districts) in east Shoa and west Shoa zones, Central Ethiopia

The livestock and human populations of the study areas are summarized in Table 2. The statistics are based on the agricultural sample survey report on livestock and livestock characteristics (CSA, 2008b), and summary and statistical report of the 2007 population and housing census results (CSA, 2008a), respectively.

Table 2**:** Human and livestock population in the study area

Study Areas	Cattle	Sheep	Goat	Camel	Poultry	Human
West Shoa Zone						
Zone Total	2,072,485	1,035,326	1,037,159	-	1,823,471	2,072,485
Ambo District	110,796	52,714	43,339	-	NA	110,796
East Shoa Zone						
Zone Total	1,357,522	696,891	660,631	1,017,255	527,412	1,357,522
Adaa District	131,273	55,305	55,491	NA	68,892	131,273
Fentale District	82,225	69,482	89,717	131,273	33,639	82,225

NA= Data Not Available Source: (CSA, 2008a; CSA, 2008b)

3.2. Study animals

Sheep and goats of the study districts slaughtered for human consumption at HELEMEX export abattoir, Debre Zeit were traced and identified using information supplied by attendant and the abattoir manager. The sera of the animals were serologically tested for the presence of IgG antibodies against *T. gondii.* At slaughter, the whole heart and blood in the chambers of the heart were collected and transported in ice box packed with ice to the microbiology laboratory of the Department of Microbiology, Immunology, Epidemiology and Public Health (MIEP) of the Collage Veterinary Medicine and Agriculture of the Addis Ababa University for serological testing and bioassay. In this study, small ruminants of male and female sexes were sampled. However, few females were sampled because of females normally kept for breeding purpose and those arriving at the abattoir are culls and infertile animals. Sheep and goats aged over six months were included in this study.

Small ruminant production in the study areas is mainly characterized by traditional and extensive type of management system, which includes sedentary and pastoral husbandry systems. Sedentary farming is a feature of the highlands and midland areas while transhumance pastoralism prevails in lowland areas of Fentale district. Moreover, semi-intensive farming is practiced in the urban and peri-urban areas of Ada'a-Liben and Ambo districts. Afar, Arsi-Bale and Western highland breeds of goat predominate in Central Ethiopia. Similarly sheep breeds common in the area are Afar, Black Head Somali, Arsi-Bale and Horro. These entire breed types are kept for mutton production. However, pastoralists in Fentale district use goats and rarely sheep for dairy purpose.

3.3. Study designs

The study designs were both cross-sectional and follow up experimental. It was undertaken from September, 2011 to May, 2012. Serological investigation and isolation of *T. gondii* were carried out from sheep and goats of study districts slaughtered for human consumption.

Samples collected at the slaughter line were immediately transported to the microbiology laboratory of the Department of MIEP in cold chain. In the laboratory, sera were separated and examined for *T. gondii* antibodies. Hearts of those seropositive samples were bioassayed in *Toxoplasma* negative Swiss Albino mice (National Veterinary Institute, Debre-Zeit). After two months of follow-up these mice were humanely killed (using di-ethyl ether) and their sera and brain were subjected for serology (modified agglutination test [MAT]) and *T. gondii* tissue cysts enumeration, respectively.

3.4. Sampling methods

The number of study animals were determined based on an expected prevalence of 31.6% for sheep, 19.8 % for goats from a recent study by Zewdu *et al*. (2011a and 2011b), and 5% absolute precision using the formula described by Thrusfield (2007).

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

where, n = required sample size

P_{exp}= expected prevalence

d= desired absolute precision

Therefore, based on this formula and aforementioned figures the calculated sample sizes were 333 and 244 for sheep and goats, respectively. But out of the total anticipated sample size (577), 134 sheep and 223 goats were sampled, due to interruption in incoming of the animals to the abattoir and delay in arrival of serological kit and bioassay chemicals.

3.5. Laboratory investigation

3.5.1. Serological tests

Toxoplasma gondii-specific IgG antibodies (in sheep, goat and mouse sera) were detected by the modified agglutination test (MAT, Toxo screen DA, biomerieux® SA mercy-I' Etoile/France) following the procedure described by Dubey and Desmonts (1987). Briefly, the serum samples were diluted 1:40 and 1:4000 using phosphate buffer saline (PBS, PH=7.2) and 25µl of diluted sera were placed in U- bottom well of microtitre plate. Sera were treated with 0.2 M 2-mercaptoethanol to remove non-specific IgM or IgM-like substances. Sedimentation of antigen at the bottom of the well and clear agglutination above half of well were recorded as negative and positive results respectively. The detailed procedure is annexed (**Annex 1)**.

3.5.2. Bioassay of sheep and goat tissues for *T. gondii* in mice

Heart tissue samples of sheep and goats, weighing at least 50 gram (g), were taken for bioassay in mice after digestion in pepsin (Merck KG.A, Darmstadt, Germany) as described previously by Dubey (1998). Briefly, after removing fat, auricles and blood, 50 g of the myocardium of each sheep and goats were chopped and gently ground in a blender without any fluid. Aqueous saline solution (125 ml, 0.85% NaCl) was then poured into the blender and homogenized for 30 seconds at top speed. This homogenate was incubated with an acid

pepsin solution for 1 hour at 37 ^{0}C, centrifuged, the sediment neutralized and suspended in 5-10ml antibiotic solution (Dubey, 2010b). The homogenate was inoculated (1 ml/mouse) intraperitonealy (i.p.) into female Swiss albino mice (five mice for each study animal tissues) as described by Dubey (2010b). The inoculated mice were observed daily for illness for 60 days post inoculation and the information on number of survivors, dead, day of death, symptoms, and weight (initial and final) were recorded. The mice were fed with pelleted feed and municipal chlorinated water ad libitum. Surviving mice were bled on day 60 after i.p inoculation. Serum samples were tested for antibodies of *T. gondii* using MAT and brains of all mice were examined for tissue cysts as described by Dubey, (2010b). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues or mice sera reacted positively for MAT (**Annex 2).** Non-infected mice (5) were kept separately as negative control.

3.5.3. Quantification of cysts from the mouse brain

Briefly, two months after i.p challenge, the whole brains from those mice which survived were removed by sagital dissection after anesthetizing with di-ethyl ether. The brain was homogenized in 1 ml PBS by using a mortar and pestle. The number of cysts in three aliquots of each 10 µl were counted under microscope with a 10X objective and summed. The total number of cysts in the brain of each mouse was determined by multiplying the number of cysts in the 30 µl sample examined with the dilution factor (Goodwin *et al*., 2008; Fritz *et al*., 2012).

3.6. Data analysis

Data generated from laboratory investigation were recorded and coded using Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). The seroprevalence was calculated as the number of serologically positive samples divided by the total number of samples tested. The association of the potential factors (district, species, age, sex, breed and sampling months with *T. gondii* IgG seropositivity was analyzed by univariable logistic regression. Non-collinear variables with P-value ≤ 0.25 in univariable analysis were offered to the final multivariable model. Zero-inflated Poisson regression was used to model brain tissue cysts counts in mice. The agreement between bioassay and MAT used for the diagnosis of *T. gondii* infection was done using kappa test. The clustering nature of the data was considered in the analysis. The 95% confidence interval and a significance level of $\alpha = 0.05$ were used.

4. RESULTS

4.1. Overall seroprevalence of Toxoplasmosis in sheep and goat

The overall seroprevalence of anti-*Toxoplasmagondii* antibodies in sheep and goats was 20.5%. Serum samples from sheep showed significantly high proportions of positive reactions compared to goats ($P < 0.05$). The overall prevalence in small ruminants was not statistically significant between study districts ($P \geq 0.05$). The highest seropositive percent for sheep and goats was recorded from Ambo and Fentale districts, respectively (Table 3).

Table 3**:** Overall IgG seroprevalence of *T. gondii* infection in sheep and goats of study districts

Study Districts	Sheep		Goats		Total	
	No. tested	No. positive (% prevalence)	No. tested	No. positive (% prevalence)	No. Tested	No. positive (% prevalence)
Ada'a	6	1 (16.7)	76	5 (10.4)	54	6 (11.1)
Fentale	57	9 (15.8)	99	21 (21.2)	156	30 (19.2)
Ambo	71	25 (35.2)	76	12 (15.8)	147	37 (15.2)
Total	134	35 (26.1)	223	38 (17.0)	357	73 (20.5)

Sheep: Pearson chi2 (2) = 6.4711; P = 0.039; Goat:Pearson chi2 (2) = 2.7926; P = 0.248; Shoat:Pearson chi2 (2) = 5.0511; P = 0.080.

The MAT test done demonstrated antibody titers ranging from 1: ≤ 60 to 1: ≥162,000. The distributions of most prevalent positive titers in sheep were: 8 with titer ≤ 60, 7 with titer 54,000 and 7 with titer ≥162,000. Similarly, the distributions of most prevalent positive titers in goats were: 12 with titer ≤ 60, 7 with titer ≥1620 and 4 with titer 54,000 (Table 4).

Table 4: MAT titer of seropositive sheep and goats samples

Species	No. tested	No. positive	Reciprocal MAT titers							
			Positive at 1:40				Positive at 1:4000			
			≤60	180	540	≥1620	≤6000	18000	540000	≥162000
Sheep	134	35	8	1	2	4	3	3	7	7
Goat	223	38	12	5	3	7	2	3	4	2
Total	357	73	20	6	5	11	5	6	11	8

The results of univariable logistic regression analysis of the potential risks revealed districts, species and study months as significantly associated variables with *T. gondii* IgG seropositive (Table 5).

Table 5: Chi-square and univariable logistic regression of potential risk factors associated with *T. gondii* seropositivity in sheep and goats of study districts

Variable	No. tested	No. positive (%)	X^2 (p-value)	Univariable OR (95% CI)	P-value
District					
Ada'a-Liben	54	6 (11.1)	5.0511 (0.080)	1.0	-
Fentale	156	30 (19.2)		1.9 (0.75, 4.86)	0.178
Ambo	147	37 (25.2)		2.7 (1.07, 6.80)	0.036
Species					
Goat	223	38 (17.0)	4.2415 (0.039)	1.0	-
Sheep	134	35 (26.1)		1.7 (1.02, 2.90)	0.041
Age					
Young (≤1 yr)	232	46 (19.8)	0.1569 (0.692)	1.0	-
Adult (> 1yr)	125	27 (21.6)		1.1 (0.65, 1.90)	0.692
Sex					
Male	348	70 (20.1)	0.9423 (0.332)	1.0	-
Female	9	3 (33.3)		2.0 (0.48, 8.14)	0.340
Breed					
Arsi-Bale (s)	10	1 (10.0)	19.2810 (0.004)	1.0	-
Arsi-Bale (g)	84	9 (10.7)		1.1 (0.12, 9.54)	0.945
Western highland(g)	75	12 (16.0)		1.7 (0.20, 14.81)	0.624
Horro (s)	71	25 (35.2)		4.9 (0.59, 40.86)	0.143
Afar (s)	46	9 (19.6)		2.2 (0.24, 19.57)	0.483
Afar (g)	64	17 (26.6)		3.3 (0.38, 27.65)	0.280
Blackhead Ogaden(s)	7	0 (0.0)			
Months					
April	45	4 (8.9)	16.1684 (0.003)	1.0	-
January	68	7 (10.3)		1.2 (0.32, 4.28)	0.805
February	97	20 (20.6)		2.7 (0.85, 8.31)	0.092
December	86	21 (24.4)		3.3 (1.06, 10.34)	0.039
March	61	21 (34.4)		5.4 (1.70, 17.070	0.004

4.2. Bioassay and serology on mice

Bioassays of seropositive sheep (n=23) hearts was done on mice. *Toxoplasma gondii* was isolated from 8 (34.78%) of 23 bioassayed sheep (Table 6). Number of *T. gondii* isolates was higher from sheep with higher MAT titers (Figure 4). *Toxoplasma gondii* bradyzoites cysts were isolated from 1 of 6 sheep with titer of 1: ≤ 60, 0 of 1 sheep with titer of 1:180, 1 of 4 sheep with titer of 1: ≥ 1620, 3 of 7 sheep with titer of 1:54000 and 3 of 4 with titer of 1: ≥ 162000.

Serological test (MAT) of bioassayed mice (n=253) was done and 53 (20.95%) mice showed positive reaction. Fourteen mice (14) with cyst positive bioassays tested positive in MAT, with only one and three mice seropositive in each. In contrast, serology was positive in two bioassays in which cysts were not observed; notably, two of these originated from sheep with the higher specific antibody levels detected (1:≥1620 and 1:54000) (Table 6). Four of the eight (8) *T. gondii* isolates were from sheep of Fentale and Ambo district. One isolate (Id No.110) killed one mouse at 44 days post inoculation (Table 6).

Table 6: Isolation of *T. gondii* from sheep of Ada'a-liben, Ambo and Fentale districts, Central Ethiopia

District	Animal Id	Reciprocal MAT titer of seropositive Sheep	Bioassay in mice			
			Mice with cysts/mice examined (n)	Seropositive mice/mice examined (n)	Mice died/infected	Days of mice death PI (mice no.)
Ada'a-Liben	A1	≤60	0/2	0/2	3/5	2 (3)
Ambo	68	54000	1/5	0/5	0/5	Survived
	72	≥162000	0/5	0/5	0/5	Survived
	94	≥162000	3/3	3/3	2/5	2 (2)
	108	54000	1/3	3/4	1/5	2 (1)
	110	≥162000	1/1	1/1	3/5	44 (1), 2 (2)
Fentale	81	54000	0/5	0/5	0/5	Survived
	112	≥162000	0/2	0/2	3/5	2 (3)
	167	≤60	0/5	0/5	0/5	Survived
	172	≤60	0/1	0/1	0/1	Survived
	173	≤60	0/5	0/5	0/5	Survived
	175	≤60	0/5	0/5	0/5	Survived
	198	≥1620	0/5	1/5	0/5	Survived
	202	≥1620	0/5	0/5	0/5	Survived
	203	≤60	1/5	1/5	0/5	Survived
	204	≥162000	5/5	5/5	0/5	Survived
	251	180	0/5	0/5	0/5	Survived
	256	54000	0/5	0/5	0/5	Survived
	257	54000	3/5	3/5	0/5	Survived
	258	≥1620	0/5	2/5	0/5	Survived
	259	54000	0/5	0/5	0/5	Survived
	260	≥1620	¼	0/4	1/5	2 (1)
	261	54000	0/5	5/5	0/5	Survived

Among goats, *T. gondii* was isolated from 13 of 31 (41.9%) bioassayed samples. Out of the 13 goat samples that gave positive result for cyst count, one sample (Id No. 176) killed one mouse at 48 day post inoculation and all the rest survived the infection**.** Ten goats samples out of 31 (32.3%) gave positive MAT results on mice. Out of 147 survived mice inoculated with goat heart tissue homogenates, 29 (29/147, 19.7%) mice reacted positive for MAT (Table 7 and 8).

Table 7: Isolation of *T. gondii* from goats of Ada'a-liben, Ambo and Fentale districts, Central Ethiopia

District	Animal Id	Reciprocal MAT titer of seropositive Goats	Bioassay in mice			
			Mice with cysts/mice examined (n)	Seropositive mice/mice examined (n)	Mice died/infected	Days of mice death PI (mice no.)
Ada'a-Liben	A2	≥6000	5/5	5/5	0/5	Survived
	A3	≤60	0/5	0/5	0/5	Survived
	A6	≥1620	0/5	0/5	0/5	Survived
	A14	≤60	1/5	0/5	0/5	Survived
	A32	540	0/5	1/5	0/5	Survived
Ambo	142	≥1620	4/5	4/5	0/5	Survived
	176	54000	5/5	5/5	1/6	48 (1)
	182	≤60	1/5	1/5	0/5	Survived
	186	≤60	0/5	1/5	3/5	2 (3)
	190	180	0/5	0/5	0/5	Survived
	191	540	0/5	0/5	0/5	Survived
	192	≥162000	5/5	5/5	0/5	Survived
Fentale	91	54000	0/5	0/5	0/5	Survived
	128	≤60	1/5	0/5	0/5	Survived
	132	≥1620	0/5	0/5	0/5	Survived
	133	≤60	¼	0/4	1/5	2 (1)
	218	≤60	1/5	0/5	0/5	Survived
	220	540	0/5	1/5	0/5	Survived
	221	180	0/4	0/4	1/5	2 (1)
	222	≥1620	0/5	0/5	0/5	Survived
	223	≥1620	0/4	0/4	1/5	2 (1)
	224	≥162000	5/5	5/5	0/5	Survived
	225	180	0/3	0/3	2/5	2(1),3(1)
	230	54000	0/5	0/5	0/5	Survived
	231	18000	0/5	0/5	0/5	Survived
	234	180	0/5	0/5	0/5	survived
	236	≥1620	0/3	0/3	2/5	2(2)
	237	18000	0/5	1/5	0/5	Survived
	239	≥1620	2/4	0/4	1/5	2(1)
	240	180	1/5	0/5	0/5	Survived
	250	540	2/5	0/5	0/5	Survived

The success of *T. gondii* isolation in mice generally increased with antibody titers in sheep and goats (Figure 4).

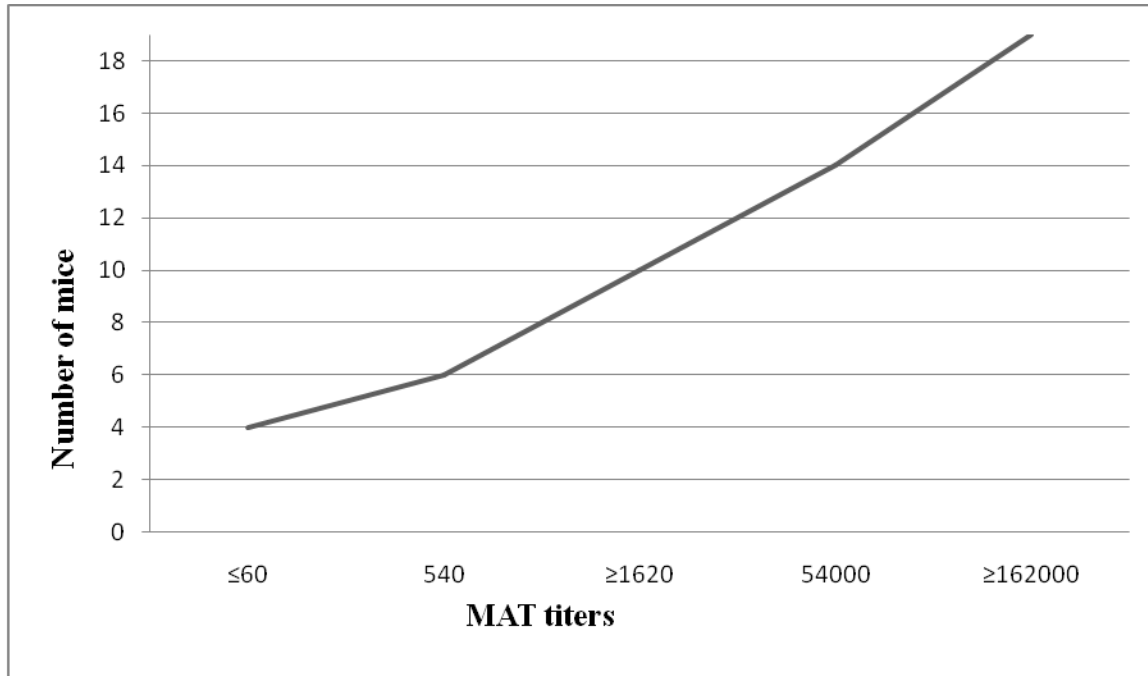

Figure 4: Relationship between sheep and goats MAT titers and number of *T. gondii* cyst isolations in mice

4.3. Quantification of cysts from the mouse brain

During the follow-up study, majority of mice were asymptomatic. Enumeration of the brain cysts showed an average of 34.73 brain tissue cysts within cyst positive samples with a minimum and maximum of 0 and 554 respectively (Table 8).

Table 8: Summary of serology and cyst count in artificially infected mice

No. of bio-assayed		No. of seropositive mice/tested (%)	Cyst Positive			Mean cyst count	Variances	Range
Species	No		MAT+	MAT-	Total			
Sheep	23	23/106(21.7)	14	2	16	22.46	3980.91	0-392
Goat	31	30/147(20.4)	29	9	37	43.6	12114.16	0-554
Total	54	53/253(20.95)	42	11	53	34.73	8786.283	0-554

The mean cyst count from sheep samples was lower than goats' samples. It is evident from the descriptive statistics that the variance of overall cyst count is much higher relative to the mean indicating an over-dispersion. Hence, we opted to use zero-inflated Poisson regression to model predictors of mice brain tissue cyst count. The results of univariable analysis of the potential variables revealed that districts, sex, breed, reciprocal MAT titers of sheep and goats and MAT status of mice as significantly associated variables with bradyzoite cyst count in mice ($P<0.05$). In the final multivariable zero-inflated Poisson regression model, study district, sex, age, species and MAT status of mice were found to be independent predictors' of bradyzoite cyst count. Positive weight difference of mice (i.e., final weight minus initial weight) was found to be protective for cyst count. Breed was excluded from the final model due to collinearity with district (Table 9).

Table 9: Potential predictors of mice brain cyst count using Zero-inflated Poisson Regression Model

Variable	No. of mice	No. of Cyst positive mice (%)	Univariable		Multi-variable	
			IRR(95%.CI)	P-value	IRR(95%.CI)	P-value
District						
Fentale	128	20(15.6)	-	-	-	
Ada'a-Liben	27	6(22.2)	4.6 (3.17, 6.56)	0.000	3.2(2.22,4.76)	0.000
Ambo	98	27(27.6)	3.4(2.27, 5.01)	0.000	1.8(1.15, 2.69)	0.009
Age						
≤1year	166	27(16.3)	-	-	-	
>1years	87	26(29.9)	1.3(0.66, 2.49)	0.461	1.5(1.11, 2.08)	0.009
Sex						
Male	241	48(19.9)	-	-	-	-
Female	12	5(41.7)	1.6(1.11, 2.36)	0.013	2.1(1.36, 3.22)	0.001
Species						
Sheep	106	19(17.9)	-		-	-
Goats	147	34(23.1)	1.5(0.75,3.02)	0.251	1.5(1.19, 1.78)	0.000
Breed						
Afar	128	20(15.6)	-	-		
Horro	64	12(18.8)	2.6(1.46, 4.68)	0.001		
Arsi-bale	27	6(22.2)	4.6(3.17, 6.56)	0.000		
W/high land	34	15(44.1)	4.0 (2.69, 5.92)	0.000		
MAT reciprocal titers of shoats						
≤540	102	6(11.3)	-	-	-	-
≥1620	143	47(88.6)	1.9 (1.15, 3.27)	0.013	1.02(0.75, 1.40)	0.896
MAT status of mice						
Negative	200	11(5.5)	-	-	-	-
Positive	53	42(79.3)	3.4 (2.19,5.26)	0.000	1.9 (1.32, 2.82)	0.001
W2-W1			0.9 (0.88, 0.99)	0.026	0.9 (0.89, 0.97)	0.001

W2-W1= final weight minus initial weight of mice

4.4. Test agreement between mice serology MAT and cyst count

All sera and brain tissues (each n= 253) were examined by MAT and microscope for *T. gondii* antibodies and bradyzoite cysts, respectively. Analysis of MAT status (positive/negative) versus brain cyst (positive/negative) showed that there was a significant agreement (kappa = 0.735, P= 0.000) (Table 10).

Table 10: Comparison of MAT and microscopic cyst examination to detect *T. gondii* infection

	Cyst status		
MAT	Positive	Negative	Total
Positive	42	11	53
Negative	11	189	200
Total	53	200	253

Sensitivity = 79.24%   Kappa = 0.735, P = 0.000

A tissue cyst is a collection of bradyzoites surrounded by a well-defined host cell membrane which is thin wall and in this study; it is isolated from mice brain (Figure 5)

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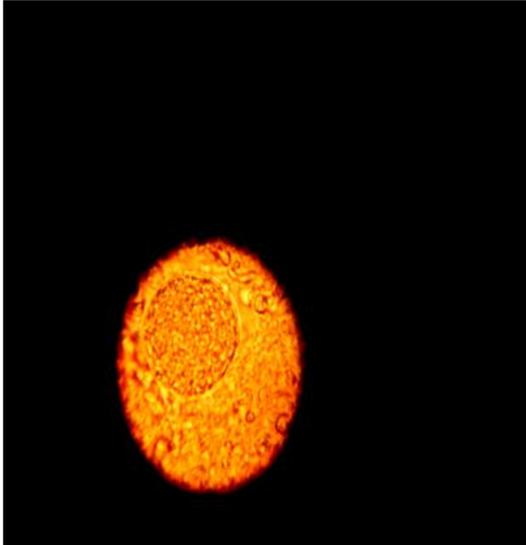
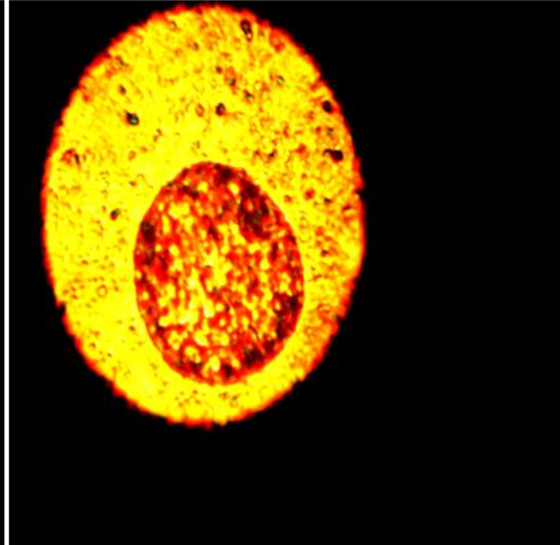
a) b)





a) Light Microscope at 40X magnification (Ambo)
b) Light Microscope at 4X magnification, (large cyst at 10X but for camera purpose, it is magnified at 4X) (Fentale)

Figure 5: Tissue cyst of *Toxoplasma gondii* isolated from mouse brain containing hundreds of bradyzoites which was developed after inoculation of *T. gondii* seropositive heart tissue homogenate

5. DISCUSSION

Sheep and goats are important for meat and milk production, and they are economically important in many countries. As they can be chronically infected with *T. gondii,* these animals are potential sources of *T. gondii* transmission to humans.

In the present study serology and bioassay were used for the diagnosis of *T. gondii* infection. Different studies prevalence from 0-100 % was recorded in different areas of the world (Olivier *et al*., 2007) depending up on cat density, weather conditions, age of the animals and husbandry practice (Tenter *et al*., 2000; Dubey, 2004; Ghazaei, 2006). The 26.1% toxoplasmosis prevalence in sheep observed in this study is in close agreement to the prevalence estimated in previous studies in Ethiopia (Bekele and Kassali, 1989; Zewdu *et al.,* 2011a), Morocco (Sawadogo *et al*., 2005), Turkey (Oncel and Vural, 2006), Pakistan (Lashari and Tasawar, 2010) and Iran (Bonyadian *et al.,* 2007). Lower prevalence values of 3.8, 4.3, 11.2 and 12.1% were recorded by Sharma *et al.* (2008) in India, Samra *et al.* (2007) in South Africa, Ramzan *et al.* (2009) in Pakistan and Dubey and Foreyt (2000) in the North America, respectively. Much higher prevalence were reported by Negash *et al.* (2004) in central Ethiopia, Hove *et al.* (2005) in Zimbabwe, Romanelli *et al*. (2007) in Brazil, Sanad and Al-Ghabban (2007) in Saudi Arabia, Klun *et al*. (2006) in Serbia and Mason *et al*. (2010) in United Kingdom.

The observed *T. gondii* seroprevalence among goats (17 %) in Central Ethiopia was in consonant with the earlier reports from Ethiopia (19.8% -24%), (Bekele and Kassali, 1989; Negash *et al.,* 2004; Zewdu *et al*., 2011b) but lower than the reports of Teshale *et al*. (2007) (62 -84%). It is also lower than the 28.9%, 27. 9% and 25.4% reported from Brazil (Bisson *et al*., 2000), Thailand (Jittapalapong *et al*., 2005) and Pakistan (Ramzan *et al*., 2009), respectively.

On the other hand the prevalence recorded in the present study is lower than the 67.9% and 59.4% reported from Zimbabwe (Hove *et al*., 2005) and Egypt (Barakat *et al*., 2009), respectively.
The differences in the overall prevalence observed in the current study and the above studies could be due to differences in the relative cat densities, the access of sheep and goats to contaminated feed and water, the climatic variation, sample size and the diagnostic techniques used (Dubey, 2004; Innes *et al*., 2009).

With regards to risk factors, our study showed that seroprevalence was higher in small ruminants of Ambo (Odds ratio [OR] = 2.7, 95% confidence interval [CI]: 1.07, 6.80; 0.036) than Ada'a-Liben district, and sheep (OR=1.7, 95% CI: 1.02, 2.90; P=0.041) than goats. This variation can be defined by the difference in environmental temperatures and moistures in these areas. The influence of the environment on the epidemiology of toxoplasmosis has been well documented (Dubey, 2004; Tenter *et al*., 2000).

The lowest prevalence in sheep (15.8%) was recorded in Fentale district, which is characterized by a hot and arid climate compared to the moist agro climate in Ambo. Humidity is favorable to a higher chance of oocyst survival in the environment and infectivity to sheep, thereby contributing to the higher seroprevalence. It is well known that a dry climate has an adverse effect on the persistence and dissemination of oocysts of *T. gondii* (Jones *et al.,* 2001; Dubey, 2010b).

In the current study, the prevalence *T. gondii* between the study months were significant (P<0.05) ranging from 8.9% in April to 34.4% in March (Table 5). It has been suggested that the incidence of toxoplasma infection is likely to decline during warmer and drier seasons because of a reduction in the number of viable oocysts in the environment (Dubey, 2010b).

Bioassay and serology on mice

Most importantly, for the first time viable *T. gondii* was isolated by bioassay in mice from tissues of 8 (34.78%) of 23 and 13 of 31 (41.9%) bioassayed of seropositive sheep and goat heart's in Ethiopia, respectively.

In this study, the level of isolation of *T. gondii* from bioassayed of seropositive sheep and goats (21/54, 38.8%) is higher compared with 16 (19.5%) of 82 seropositive sheep from Brazil (Ragozo *et al*., 2008) and 8 of 30 (26.6%) seropositive ewes from France (Dume`tre *et al*., 2006). It is slower compared to 16 of 17 (94%) of seropositive lambs from the United States (Dubey *et al*., 2008) and 12 of the 12 (100%) from goats from Brazil (Ragozo *et al.,* 2009). These differences may be due to the density of *T. gondii* in tissues of sheep and goat, and the type of tissues surveyed. *Toxoplasma gondii* localizes more often in muscle than the brain of sheep and goat (Dubey, 2010b).

In this study, bradyzoite cysts were observed in some MAT seronegative mice (Table 8). The reason behind the presence of bradyzoite cyst in the brain of MAT seronegative mice could be attributed to benign nature of inoculated bradyzoites with no or minimal ability to illicit antibody response (Waree *et al*., 2007).

Most significantly, 1 of the 8 *T. gondii* isolates from sheep (Table 6) and 1 of the 12 isolates from goats (Table 7) were mouse-virulent. This is lower compared to the report from Brazil where, 9 of the 16 *T. gondii* isolates from sheep (Ragozo *et al.,* 2008) and 12 of the 12 *T. gondii* from goats (Ragozo *et al.,* 2009) were mouse-virulent. The present mouse virulence detected was also contrast to *T. gondii* isolates from sheep from Europe and the United States where the 8 isolates from adult ewes from France (Dume`tre *et al*., 2006) and 52 of 53 isolates from lambs from United States (Dubey *et al*., 2008) were not virulent for mice.

Seropositive mice which died during the course of follow-up might be linked to the larger dose of bradyzoites received during i.p injection. On the other hand, the 14 mice died within 2 days following i.p inoculation (Table 6 and 7) possibly be due to septic peritonitis following unintended puncturing of intestine or other vital organs (Fritz *et al.,* 2012).

In the present study, the majority of mice were asymptomatic and quantification of the brain cysts showed an average of 34.73 tissue cysts with a minimum and maximum of 0 and 554 respectively (Table 8). Fritz *et al*., (2012) reported a mean bradyzoite cyst of 600 and 31,362.5 per mouse from subcutaneously and orally oocyst infected mice, respectively. The number of cysts seems to depend on at least three factors: 1) the immune system of the host, 2) host-derived genetic factors, and 3) parasitic factors (Waree *et al*., 2007).

The results of analysis of multivariable Zero-inflated Poisson regression model indicated that district, sex, species, age of sheep and goats and MAT status have significant effect ($P < 0.05$) on the bradyzoite cyst count from brain mice. Cyst count was higher from heart samples of Ambo (Incidence risk ratio [IRR]= 1.8, 95% CI: 1.15, 2.69; P=0.009) and Ada'a-Liben district (IRR = 3.2, 95% CI: 2.22, 4.76; P=0.000) districts than Fentale district, from hearts of female animals (IRR = 2.1, 95% CI: 1.36, 3.22; P=0.001) than male animals, from hearts of adult sheep and goats (IRR = 1.5, 95% CI: 1.11, 2.08; P=0.009) than young sheep and goats, from hearts of goats (IRR = 1.5, 95% CI: 1.19, 1.78; P=0.000) than sheep hearts and from MAT positive mice (IRR = 1.9, 95% CI: 1.32, 2.82; P=0.001) than MAT negative mice. Positive weight change of mice during the two months follow-up period was found to be protective (IRR = 0.9, 95% CI: 0.89, 0.97; P=0.001) for cyst count (Table 9). All these variations could be accounted on the basis of host age, sex, environmental conditions, and cat densities (Tenter *et al*., 2000; Dubey, 2004; Ghazaei, 2006).

In this study, the comparison all sera from mice for which blood samples and brain tissues were tested by MAT and microscopic examination of cyst respectively. Comparison of results MAT (positive/negative) and cyst status (present/absent) revealed a significant agreement (Kappa=0.735; P=0.000) (Table 10). Thus, MAT can be considered as a satisfactory test to detect *T. gondii* infection.

6. CONCLUSION AND RECOMMENDATIONS

The results of present study confirm the widespread presence of *Toxoplasma gondii* antibodies and tissue cysts in sheep and goats in Central Ethiopia. Study months (which are reflection climatic condition), species and study districts were significantly associated with *T. gondii* seropositivity in small ruminants. District, species, age, sex of small ruminants and positive MAT test result in mice were independent predictors of bradyzoite cyst count in mice brain. Once more, isolation of mouse virulent *T. gondii* strains were observed in tissues of sheep and goats for the first time, and this has implications for human infection, as consumption of meat, milk and other animal products in raw or undercooked form is common in Ethiopia. Hence, sheep and goats could be a source of human infection in Ethiopia, especially for seronegative women of childbearing age and immunocompromised individuals.

Therefore, based on the above conclusion the following points are recommended:

- The role of the government and concerned professionals in the subject of toxoplasmosis should be underlined with due emphasis on enforcing hygienic measures, the education of people and provision of rendering facilities to kill the bradyzoites in meat.
- It would be useful to determine the prevalence of *T. gondii* antibodies and the actual parasites in milk and other body fluids of goats, as infected milk (with tachyzoites) could be of high risk to babies, expectant mothers and the immunocompromised people.
- Further epidemiological investigation of isolated *T. gondii* to characterize the genotypes is in progress and should be strengthened.

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8. ANNEXES

Annex 1. MAT Procedure (Dubey and Desmonts, 1987**)**

In the modified agglutination test (MAT, Toxo screen DA, biomerieux® SA mercy-I' Etoile/France), whole killed *T. gondii* tachyzoites were used and the serum samples were treated with 0.2 M 2-mercaptoethanol to remove non-specific IgM or IgM-like substances. The serum samples were diluted 1:40 and 1:4000 using phosphate buffer saline (PBS, Ph 7.2) and 25µl of diluted serum were placed in U- bottom well of microtitre plate and serial two to fourfold dilution were made in PBS. The stock antigen were then diluted 1:5 using the alkaline buffer containing the mercaptoethanol (sodium chloride,7.012g, boric acid (H_3BO_3) 3.092g 1.0 N sodium hydroxide 24 ml, bovine albumin 4 g, sodium azide 1g, 2-mercaptoethanol 14 ml, and distilled water to 1000 ml). The alkaline buffer were made freshly or not stored more than two weeks. Antigen (50µl) containing 2-mercaptoethanol was added to each serum well. Finally, the plate were sealed with cellophane and kept 5-18 hours at room temperature. The result was examined. Sedimentation of antigen at the bottom of the well means negative. A clear agglutination above half of bottom means positive.

MAT Instruction:

Screening:

1. In tubes, dilute the serum samples and control sera (R4 and R5):

- 1/20: 100µl of serum + 1.9 ml of PBS (R6)
- 1/2000: 25µl of the 1/20 dilution +2.5ml of PBS

2. Locate the patient sera on the report sheet then dispense 25µl of each serum dilution into the 2 wells provided.

3. Add 25µl of 0.2 mol/l 2-mercaptoethanol to each well, thus diluting the sera 1/40 and 1/4000.

4. Add 50µl R1 antigen suspension diluted 1/5 to each well.

Allow 1 well for **antigen control**:

- 2-mercaptoethanol(0.2mol/l) 25µl
- PBS 25µl
- Antigen 50µl

5. Homogenize using the vibrator or kline rotar.
6. Cover with a self- adhesive sheet. Leave for 5-18 hours at room temperature away from vibration and sources of drying.
7. Perform reading

Reading:

Antigen control: sedimentation of the toxoplasma in button or ring.

Positive control: agglutination of the toxoplasma in a mat covering **about half** of the well base. The mat may show slight shrinking around the edges (irregular shape).

Negative control: sedimentation of the toxoplasma in a button or ring.

Border line reaction: agglutination of the toxoplasma in a mat covering less than half of the well base**.**

Quantitative test (to determine the titer)

1. Depending on the result of screening test (serum positive at 1/40 or 1/4000) use either 1/20 or 1/2000 dilution in PBS to make serial threefold dilution

2. Dispense:
   - 50µl of 0.2 mol/l 2-mercaptopethanol in successive wells
   - 25µl of the chosen serum dilution (1/20 or 1/2000) in the 1st well.

3. Transfer 25µl from 1st well to the 2nd well. Repeat up to the 4th well

   ✓ A dilution series is obtained:
   - Either: 1/60,1/180,1/540, 1/1620
   - Or: 1/6000,1/18000.1/54000.1/162000.

4. Procedure and reading are performed as for the screening test.

<u>**Content of the kit (4X 96 wells)**</u>

Toxoplasma antigen 5x c 1x4ml	R1	Suspension of formalin-treated toxoplasma, RH Sabin strain grown in mice (14). **Store upright**. Shake vigorously before use to ensure homogeneous suspension. Dilute 1: 5 in BABS albumin buffer (R2). **The diluted suspension is stable 3 weeks** at 2 – 8 ^{o}C in a glass bottle
Diluent 1x16ml	R2	Colored BABS albumin buffer (red) pH 8.95 (sodium azide 1g/l). Ready to use. Avoid contamination
2-Mercaptoethanol(2ME) 1x 1 ml	R3	Solution (14.2 mol/l). Dilute in PBS (R6) to obtain a 0.2 mol/l solution. - 2ME---- 0.35 ml - PBS to ---25 ml **The dilution is stable**: **4 weeks at 2 -8 ^{o}C in a brown glass bottle**
Positive control serum 1x 1ml (lyoph.)	R4	Goat serum + sodium merthiolate 0.1 g/l. Reconstitute with 1 ml of sterile distilled water. **Stability**: - 4 wks at 2 – 8 ^{o}C, 6 months at -25-/+ 6 ^{o}C (in aliquots). This serum is calibrated against the WHO standard (3 rd IS). The titer (T) is indicated on the vial label as a reciprocal of the dilution
Negative control serum 1x1 ml (lyoph.)	R5	Goat serum + sodium merthiolate 0.1 g/l. Reconstitute with 1 ml of sterile distilled water. **Stability**: - 4 wks at 2 – 8 ^{o}C, 6 month at -25-/+ 6 ^{o}C (in aliquots).
PBS pH 7.2 for 1 liter(powder)	R6	Phosphate NaCl buffer (phosphate buffered saline) Make upto 1 liter with distilled water Stability after reconstitution: 2 months at 2-8 ^{o}C. Avoid contamination.

Annex 2. Bioassay of sheep and goat tissues for *T. gondii* in mice (Dubey (1998); Dubey, 2010b).

After removing fat, auricles and blood, 50 g of the myocardium from each heart were chopped and gently ground in a blender without any fluid. 125 ml of aqueous 0.85% NaCl solution (saline) were then poured into the blender and homogenised for 30 seconds at top speed. This homogenate was incubated with an acid pepsin solution(Merck KG.A, Darmstadt, Germany) for 1 hour at 37 ^{0}C, centrifuged, the sediment neutralized and suspended in 5–10 ml antibiotic solution (Dubey, 2010b).The homogenate was inoculated (1 ml/mouse) intraperitonealy (i.p.) into female Swiss albino mice (five mice for each study animal tissues) as described by Dubey (2010b). The inoculated mice were observed daily for illness for 60 days post inoculation and the information on number of survivors, dead, day of death, symptoms, and weight (initial and final weight) were recorded. Surviving mice were bled on day 55 after i.p inoculation. From each mouse serum sample were tested for antibodies of *T. gondii* using MAT. Mice were killed 55 days p.i. and brains of all mice were examined for tissue cysts as described (Dubey, 2010b). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Bioassay procedure

1. Trim connective tissue, fat from muscular tissues (50 gram heart) using nonporous, hard plastic cutting boards, scissors, or disposable razors. Cut muscle into small (1–2 cm) pieces and store in plastic bags or cups.

2. Grind muscle in a blender for 15 sec at low speed without saline. Then add 125 ml of saline and blend at top speed for about 30 sec. Rinse blender with 125 ml of saline

3. Pour the tissue homogenate into a 1,000-ml wide-mouth plastic jar with a disposable plastic liner.

4. To the pre warmed (37°C) homogenate add 250 ml of freshly prepared, pre warmed (37°C) acid pepsin solution (pepsin 5.2 g, NaCl 10.0 g, HCL 14 ml, and distilled water to make 1,000 ml, pH, 1.10–1.20). Incubate at 37°C in a shaking water bath for 60 min.

5. Filter the homogenate through two layers of gauze and centrifuge 250 ml of filtered homogenate in a 250-ml wide-mouth polypropylene centrifuge bottle (Nalgene) at 1,200 x *g* for 10 min.

6. Pour off the supernatant. Depending on the tissue, fatty scum may stick to the rim of the centrifuge bottle. To prevent this, suspend the sediment in 20 ml of phosphate buffered saline (PBS, pH 7.2) using disposable plastic pipettes. Transfer the homogenate in a 50-ml centrifuge tube with a conical bottom. Neutralize the homogenate with 12 to 15 ml of freshly prepared 1.2% sodium bicarbonate (pH 8.3) with phenol red as a pH indicator until the color changes to orange. After mixing, centrifuge at 1200 x *g* for 10 min.

7. Pour off the supernatant and add 5–10 ml of saline that contains 1,000 units penicillin and 100 µg of streptomycin per ml.

9. Inoculate 1 ml of tissue homogenate subcutaneously/intraperitonealy into each of 5 to 10 mice

10. Examine all inoculated mice for *T. gondii* infection.

Annex 3. Data collection formats

Data collection sheet for MAT tested artificially infected mice and shoat

SN	Date	Sampling place	Spp	Id no	Age in months	Sex	Breed	MAT status			
								Pos.	Titer	Neg.	Remark
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											

Annex 4. Data collection sheet to monitor IP infected mice

SN	Date	Mice id no	Days after IP injection	Wt of brain	Cyst counting					
					1 st	2nd	3rd	Total	Cyst/brain	Remark
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										

SIGNED STATEMENT OF DECLARATION

"I, the under signed, declare that the thesis is my original work and has not been presented for a degree in any University and that all sources of materials used for the thesis have been duly acknowledged"

Name: Mukarim Abdurahaman Kadir

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This thesis has been submitted for examination with our approval as University academic Advisors:

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