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TOXOPLASMA GONDII IN SELECTED SITES OF CENTRAL ETHIOPIA: SEROPREVALENCE, RISK FACTORS AND BIOASSAY IN PIGS

MSc Thesis

By

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Bishoftu, Ethiopia

TOXOPLASMA GONDII IN SELECTED SITES OF CENTRAL ETHIOPIA: SEROPREVALENCE, RISK FACTORS AND BIOASSAY IN PIGS

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Tropical Veterinary Parasitology

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June, 2014

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
HCL	Hydrochloric acid
hrs	Hours
i.p	Intraperitoneum
IFAT	Indirect immunofluorescent antibody test
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MAT	Modified agglutination test
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PAS	Periodic acid-Schiff
RPM	Revolution perminute

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ABSTRACT

The objectives of this study were to estimate the sero-prevalence of *Toxoplasma gondii* infection, assess potential risk factors and isolate viable *T. gondii* tissue cyst in mice from hearts of seropositive pigs. A cross-sectional study was used to collect blood samples from a total of 557 pigs in Central Ethiopia. A systematic random sampling technique was used for blood sample collection. For bioassay 5 mice were used per pig sample. Modified Agglutination Test (MAT) was used to test sera of pigs and mice. A questionnaire survey was made to assess potential risk factors and knowledge of farm attendants about toxoplasmosis. For investigation of risk factors questionnaire and sera samples from 402 pigs were considered. Results of the questionnaire survey indicated that most of the farm attendants had no knowledge of health risk of cats either to human or animals. Absence of rodent control, neonatal mortality and history of abortion was found among herds of the study farms. The overall estimated seroprevalence was (32.7%; 95% confidence interval (CI): 0.288-0.366). Multivariable logistic regression analysis showed that extensively managed pigs are nearly twice (adjusted odds ratio [aOR]:=1.91, 95% CI: 1.01, 3.63) at higher risk of acquiring toxoplasmosis than intensively managed pigs and pigs supplied with feed containing additional animal byproduct had four times (OR=3.84, 95% CI: 2.01, 7.36) higher risk of acquiring *T. gondii* infection. Viable *T. gondii* was isolated from 48% (24/50) of the seropositive pigs. Most isolates (91.7%, 22/24) are avirulent for mice. One isolate from Bishoftu which killed four mice on day 28 and 29 postinoculation suggesting intermediate virulence. A perfect agreement was observed between MAT and microscopic cyst detection (Kappa =0.85) used as diagnostic method on mice.This is the first report of detection of *T. gondii* antibodies and isolation of viable *T. gondii* from pigs in Ethiopia. The high seroprevalence and isolation rate indicate that *T. gondii* parasite is widespread in the study areas and pigs could serve as an important source of *T. gondii* infection for pork people. Further epidemiological studies are essential for designing appropriate prevention and control strategies in pigs.

Keywords: *T. gondii*, sero-epidemiology, bioassay, pig, mice, Central Ethiopia

1. INTRODUCTION

Swine production forms an integral part of farmer's economy in many parts of the world. Many countries practice different kinds of production approaches. Swine production is increasing from time to time in many parts of tropical countries. The driving forces for the rapid increment in the demand of pork in the international market are due to the increased number of pork consumers and the profitability of the sector (Serres, 2001).

In Ethiopia, although the population of pigs has shown increment from19, 000 in 1980 (FAO, 2004) to 29,000 in (FAO, 2005), the industry is still at its infancy. In the past years adequate emphasis was not given for the sector. Unlike other livestock distribution, swine farms are predominantly found in the central part of the country particularly, in Addis Ababa and its surroundings. Currently large numbers pigs are kept around Holata (Western Shewa, Welmera district of Oromia Regional State) mixed with other livestock. The major feeds sources available for pig in Ethiopia are wheat bran, oil seed cakes and human food remain (leftover food). Some pigs also feed on pasture, crop residue and garbage (Abdu and Gashaw, 2010). The sector is left aside by development partners and researchers. In Ethiopia Christian Orthodox and Muslim religion followers which account 43.5% and 33.9% of the total population, respectively, do not consume pork (CSA, 2007).There is limited information on the constraints limiting pig production in Ethiopia. On the other hand, many of the advantages of pig production such as high fecundity rate, high feed conversion efficiency, early maturity, short generation interval, relatively small space requirement and ability to produce maximally under varied management systems (Lekule and Kyvsgaard, 2003) have not been appreciated and exploited in Ethiopia. Thus, promotion of public intervention in pig production, marketing and consumption is imperative (MoARD, 2007). Few studies reported constraints of pig production (Abdu and Gashaw, 2010; Tekle *et al*., 2013). However, studies on the status of *T. gondii* infection, isolation of the parasite from pig and the role of pigs in transmitting toxoplasmosis to humans have never been undertaken in Ethiopia.

Toxoplasma gondii is one of the most widely prevalent cyst forming Apicomplexan parasites and have been recorded worldwide. The parasite has significant impact on animal production particularly in sheep, goats and pigs. It is also causes major disease in the public health sector (Sudan *et al.*, 2013). One-third of the human population is infected by this parasite worldwide (Tenter *et al.,* 2000; Dubey, 2010). The rate of infection in humans and other animal hosts has been reported differently in various parts of the world. *Toxoplasma* infection in humans, especially in people with defective immune system, pregnant women, HIV/AIDS patients, children and those with underlying disease could entail serious damage (Rashidi *et al.,* 2013).

Toxoplasmosis ranks high on the list of diseases that lead to the death of patients with AIDS (Dubey, 2010). However, following the widespread usage of highly active anti-retroviral therapy (HAART) opportunistic infections including *T. gondii* in AIDS patients have been declining significantly (Huruy *et al.,* 2010). Infection of pregnant women may result either in abortion or congenital infection of the fetus. The congenital infection of fetuses results in hydrocephalus, intracranial calcification and retinochoroiditis (Radostits *et al*., 2006).

Major routes of transmission are different in human populations and depend on social culture, eating habits, and/or environmental factors (Demar *et al.,* 2007). Human infection commonly occurs through the ingestion of food and water contaminated with infectious oocysts shed from cats or by ingesting undercooked meat containing the viable tissue cysts. Elsewhere in the world, among meat animals, pigs are considered to be one of the most important sources of human infection and the potential risk to acquire *T. gondii* by ingesting raw or improperly cooked pork products represents a potentially serious problem and a critical food safety issue (Dubey, 2009). Moreover, increasing popular animal friendly production systems which allows outdoor accesses for pig let the suspicion by increasing risk exposure of pig to *T. gondii* of an increased risk on pork meat consumers (Kijlstra *et al.,* 2004a).

Diagnosis of toxoplasmosis is performed by isolation of the parasite from patients and more commonly by serological tests. Techniques such as histology, immunohistochemistry and Polymerase Chain Reaction (PCR) are also used detect *T. gondii* organisms in tissue samples. Serological techniques such as the Sabin-Feldman dye test, Modified Agglutination Test (MAT), Enzyme-Linked Immunosorbent Assay (ELISA), indirect fluorescent antibody test (IFAT) and immunoblotting are widely used to detect *T. gondii* specific antibodies (Gamble *et al*., 2005; Dubey, 2010).

Even though, currently studies on *Toxoplasma gondii* seroprevalence and associated risk factors have been documented in different species of livestock in Ethiopia, there are no studies conducted on sero-epidemiology and isolation of *T. gondii* parasite from pigs. Therefore the zoonotic importance of pigs in the overall epidemiology of toxoplasmosis in humans also remained unknown. On the other hand, prior research on *T. gondii* is important for subsequent prevention and control of its health impact so as to improve public hazards associated with pigs.

Therefore, the objectives of the present study were to:-

- Estimate the seroprevalence of *Toxoplasma gondii* infection in pigs in selected sites of Central Ethiopia using MAT.
- Identify the potential risk factors to *T. gondii* infection in pigs.
- Isolate *T. gondii* parasite from hearts of seropositive pigs using mouse bioassay.

2. LITERATURE REVIEW

2.1. Biology of *Toxoplasma gondii*

Toxoplasma belongs to the phylum *Apicomplexa*, which consists of intracellular parasites that have a characteristically polarized cell structure and a complex cytoskeletal and organellar arrangement at their apical end (Dubey *et al.,* 1998). Many other protozoan parasites of medical and veterinary or economical importance exist within the phylum *Apicomplexa*, with varying degrees of biological similarity to *T. gondii*. In the taxonomy of *Apicomplexa*, *Toxoplasma gondii* is further classified into class Coccidia, order Eimeriida, and family Sarcocystidae. Other members of this phylum include human pathogens (*Plasmodium*: the cause of malaria, Cryptosporidium: an animal parasite and an opportunistic pathogen of humans, *Babesia*, *Cyclospora*, *Isospora*), and animal pathogens (*Eimeria*: the causative agents of chicken coccidiosis, *Theileria*: tick-borne parasites of cattle in Africa, *Neospora*: major cause of bovine abortion (Dubey and Lindsay, 1996) and *Sarcocystis*) (Black and Boothroyd, 2000; Saleh, 2006).

Taxonomy of *Toxoplasma gondii* (from:http://www.ncbi.nlm.nih.gov/Taxonomy/)

Domian:	Eukaryota
Kingdom:	Alveolata
Phylum:	Apicomplexa
Class:	Coccidia
Subclass:	Eucoccidiorida
Order:	Eimeriorina
Family:	Sarcocystidae
Genus:	*Toxoplasma*
Species:	*Toxoplasma gondii*

2.1.1. Tachyzoites

The tachyzoite is often crescent-shaped and is approximately 2μm x 6μm, the size of a red blood cell. The anterior end of the tachyzoite is pointed and the posterior end is round (Hill *et al.,* 2005). It has a pellicle, subpellicular microtubules, a polar ring, a conoid, rhoptries, micronemes, mitochondria, endoplasmatic reticulum, golgi apparatus, ribosomes, rough surface endoplasmatic reticulum, micropores and a centrally located nucleus (Figure 1). The conoid, the rhoptries and the micronemes are characteristic a structure of this parasitic form.The pellicle consists of three membranes. The inner membrane is discontinuous in three areas, at the polar ring (anterior), at the micropore (lateral) and the posterior end. The polar ring is an osmiophilic thickening of the inner membrane at the anterior end of the tachyzoite. The conoid is located at the polar end. It is a cylindrical cone which consists of six to eight fibrillar elements arranged like a compressed spring.This structure is probably associated with the penetration of the tachyzoite through the membrane of the host cell. Terminating within the conoid are the rhoptries. These are four to ten gland like structures with an anterior narrow neck and posterior-sac-like end reaching as far as the nucleus. Each compartment has its own complement of proteins whose function is consistent with the timing of their release: micronemes release their contents early during the attachment-invasion process and then the rhoptries are released as invasion proceeds. Finally the dense granules discharge their contents during and after the formation of the PV, modifying the PV environment for intracellular survival and replication of the parasite and this happens when invasion is essentially complete. When the parasite has attached to the host cell, their contents are discharged through the conoid. The micronemes are tube like structures at the anterior end of the organism. They are usually fewer than 100 situated at the conoidal end of the parasite and are also involved in invasion of the host cell. The conoid can rotate, extend and retract and is important when the parasite searches for an attachment site at the host cell. The micropores are sites specialized for the uptake of nutrients through endocytosis. After entry into the host cell, the parasite is surrounded by a parasitophorous vacuole membrane (PVM) (Black and Boothroyd, 2000, Saleh, 2006). The PV vacuole provides a safe environment for the tachyzoites to multiply, because it is resistant to acidification and lysosomal fusion. Generation time of tachyzoites is 6 to 8 h (in vitro) and the parasites exit the cell, usually after 64 to 128 parasites have accumulated per cell (Radke and White, 1998).

This stage has a high rate of multiplication and requires an intracellular habitat to survive and multiply. Tachyzoites enter the cells by direct penetration or by phagocytosis and multiply by endodyogeny* within the host cell. The tachyzoites are associated to the acute phase of infection, during which they invade host cells. After invasion, the tachyzoite continue to divide until the host cell is filled with parasites and form rosettes. Cell cytoplasm becomes filled with parasites leading to cell disruption, upon which the released tachyzoites invade contiguous cells by active invasion of the host cell membrane or by phagocytosis (Hill *et al.,* 2005; De Sousa, 2009).

Endodyogeny* is a process in which two daughter cells form internally within the mother cell (Morrissette and Sibley 2002).

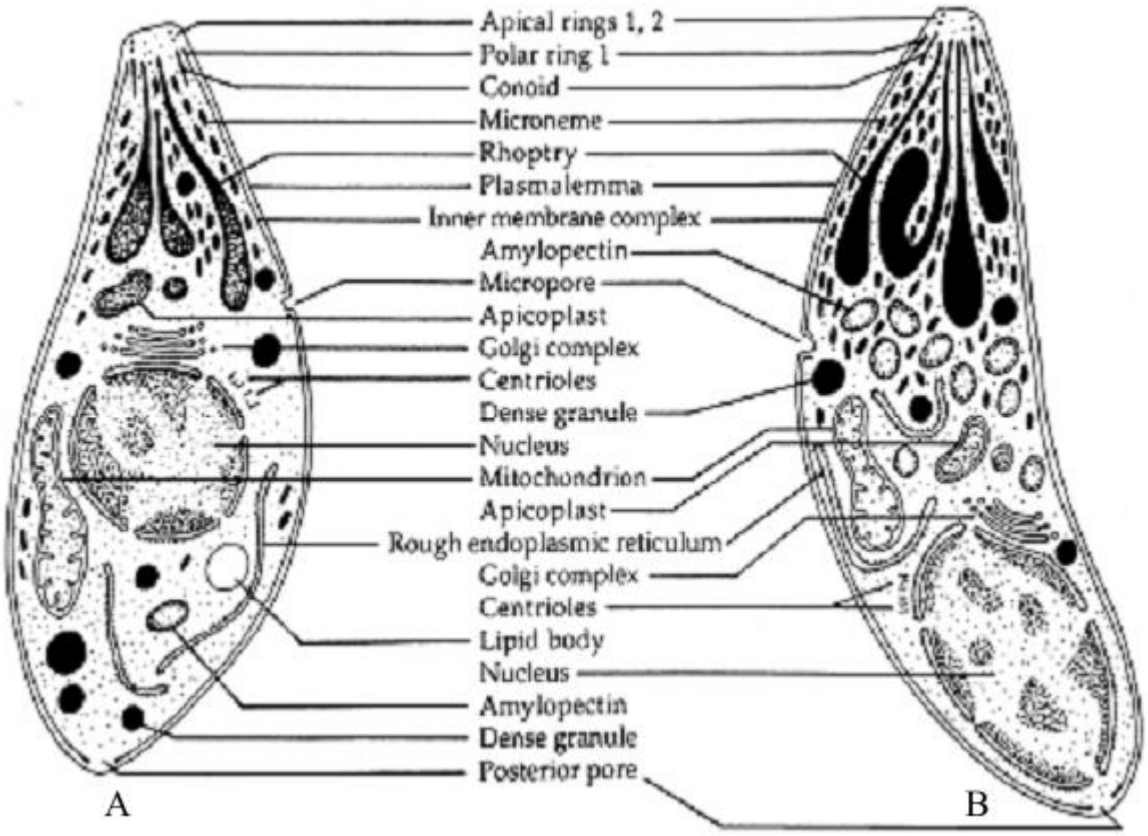

Figure 1. Morphology of tachyzoites (A) and bradyzoite (B) of *T. gondii* Source: Dubey *et al.,* 1998

2.1.2. Bradyzoites and tissue cyst

Bradyzoites result from the conversion of tachyzoites into a slow-dividing stage and form tissue cysts (Fig. 2). These cysts are more or less spheroid in brain cells or elongated in muscular cells. They vary in size from 10 μm for the younger cysts, containing only two bradyzoites, to up to 100 μm for the older ones, containing hundreds or thousands of densely packed bradyzoites (Robert-Gangneux and Dardé, 2012). Although tissue cysts may develop in visceral organs, including the lungs, liver and kidneys, they are more prevalent in the neural and muscular tissues, including the brain, eyes, and skeletal and cardiac muscles. Intact tissue cysts probably do not cause any harm and can persist for the life of the host without causing a host inflammatory response (Dubey *et al.,* 1998). Bradyzoites appear structurally similar to tachyzoites by light microscopy, but ultrastructurally by electron microscopy bradyzoites have a more posterior nucleus, more honeycombed rhoptries than tachyzoites, and contain amylopectin granules (Weiss and Kim, 2011) (Figure 1). A distinctive morphology of bradyzoites is that their

parasitophorous vacuole becomes thickened forming the tissue cyst wall. The cyst wall is rich in carbohydrate and stains with various lectins (Zhang *et al.,* 2001). They are more slender and less susceptible to destruction by proteolytic enzymes than tachyzoites. Bradyzoites have a latent metabolism, well adapted to long-term survival. The death of the host cell may trigger the disruption of the cyst wall and the consequent liberation of bradyzoites. The resistance of bradyzoites to the acid pepsin (1- to 2 hrs survivals into pepsin-HCl) allows their transmission through ingestion. Intact tissue cysts probably do not cause any harm and cysts remain intracellular throughout their life span of the host (Dubey *et al.,* 1998; Hill *et al.,* 2005; Robert-Gangneux and Dardé, 2012). Bradyzoites can be released from tissue cysts to form tachyzoites again, causing a reactivated infection in immunocompromised hosts (Montoya and Liesenfeld, 2004). Modifications of the environmental pH, shifting the temperature, IFN-γ treatment, or the inhibition of the mitochondrial respiratory chain induces transition from the tachyzoite to the bradyzoite stage (Tomavo, 2001). Oocyst ingestion by host can also result in bradyzoites formation and to a small extent with tachyzoites or contaminated meat with tissue cysts. It is believed that tissue cysts can periodically rupture thus releasing parasites that re-invade host cells and establish new tissue cysts (Reiter-Owona *et al.,* 2000).

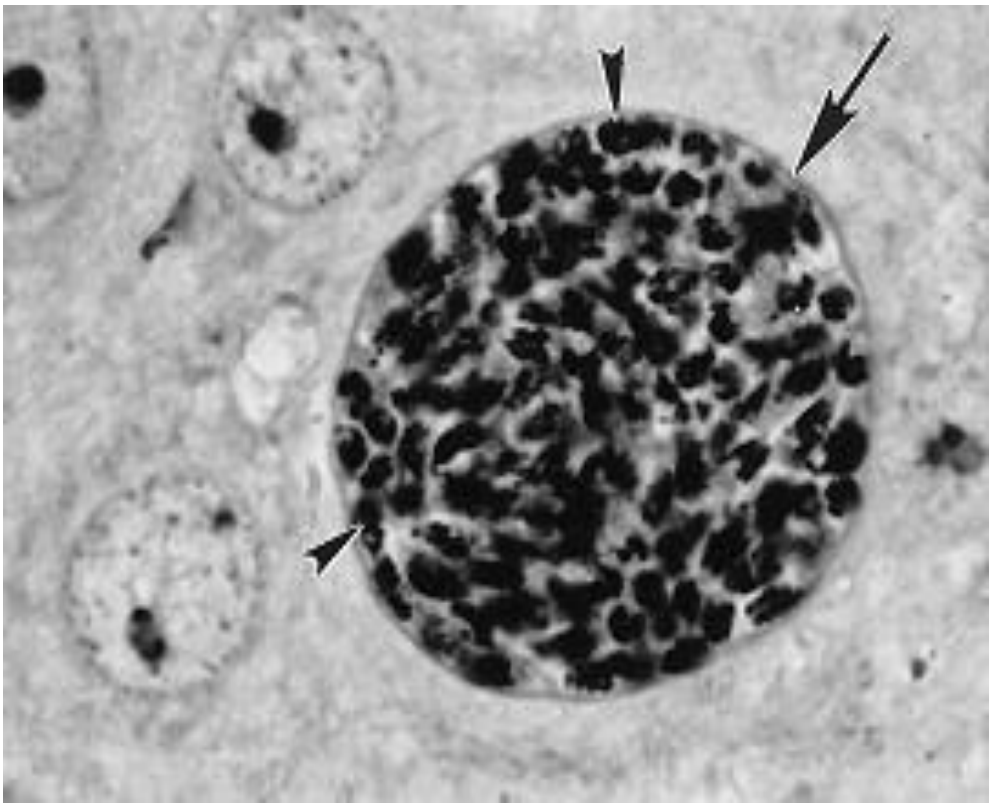

Figure 2. Tissue cyst with numerous periodic acid-Schiff (PAS) positive bradyzoites (arrowheads) enclosed in a (PAS) negative cyst wall (arrow). Source: Dubey *et al.,* 1998.

2.1.3. Oocyst

Unsporulated oocysts are subspherical to spherical with 10x12μm in diameter (Figure 3). Sporulated oocysts are subspherical to ellipsoid with 11x13μm in diameter. Each sporulated oocyst contains two sporocysts measuring 6x8μm. Each sporocyst divides into 4 sporozoites, measuring 2 x 6 to 8μm. Thus, the sporulated oocysts contain eight sporozoites. Oocyst wall of sporulated oocysts consist of three layers. One electron-dense outer layer, an electron-lucent middle layer and a moderately electron-dense inner layer (Dubey *et al*., 1998). Oocysts sporulation depends on the temperature and oxygen and may take 1 to 21 days. Sporulation takes place in 2 to 3 days at 24ºC, 5 to 8 days at 15ºC and 14 to 21 days at 11ºC. Sporulation does not occur below 4ºC or above 37ºC. Sporulated oocysts of *T. gondii* are very resistant to environmental conditions. They survive short periods of cold and dehydration and remain infectious in moist soil or sand for up to 18 months. They are highly impermeable and, therefore, are also very resistant to disinfectants (Frenkel, 2000). Ultraviolet (UV) treatment can be an effective

disinfection method to inactivate *T. gondii* oocysts in drinking water (Dumètre *et al.*, 2008).

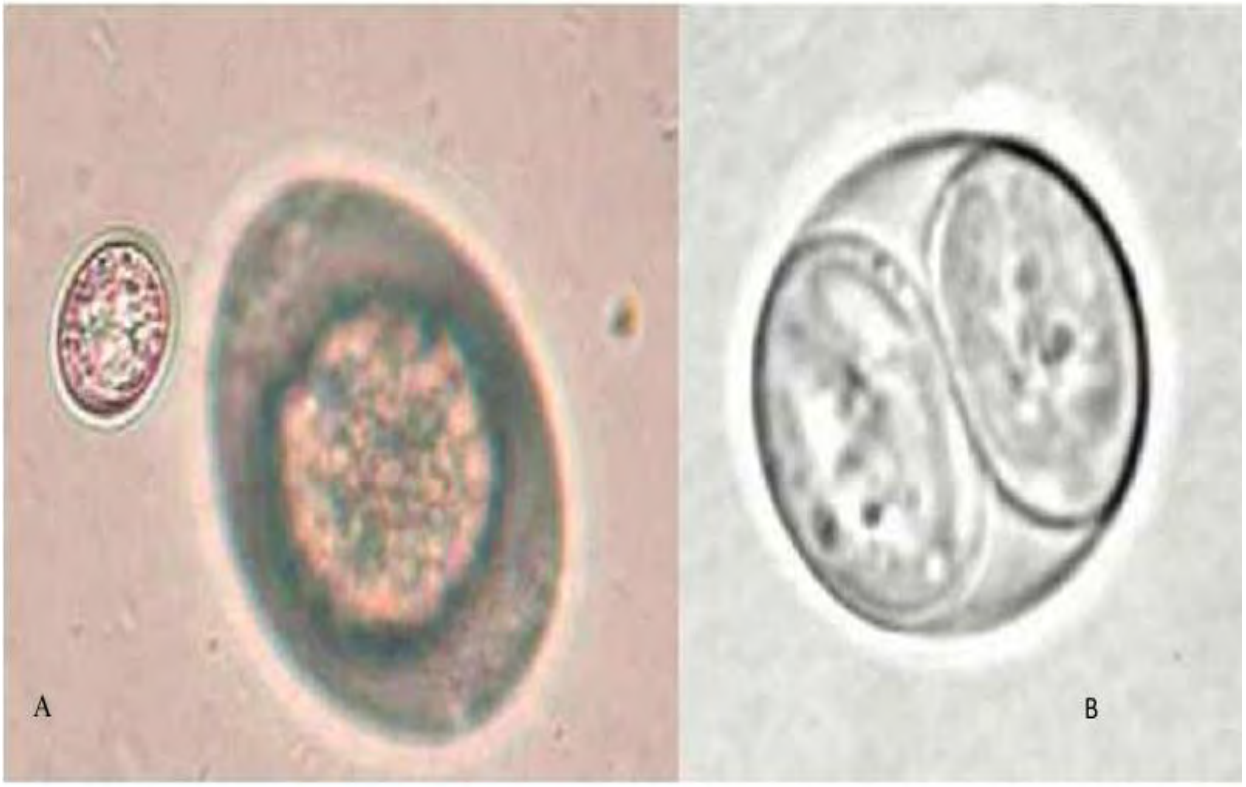

Figure 3. *Toxoplasma gondii* oocyst. Unsporulateed oocyst (A) (Blagburn, 2010) and Sporulated oocyst (B) (CDC, 2006).

2.2. Life cycle of *Toxoplasma gondii*

Toxoplasma gondii is transmitted in diverse ways and its life cycle is often described as 'complex'. However, completing the life cycle is not necessary for its existence. *T. gondii* reproduces both asexually and sexually, lies dormant in the hosts under the control of the hosts' immune responses, and survives in the environment (Jokelainen, 2013). Life cycle of *T. gondii* includes definitive and intermediate hosts. The sexual and asexual cycle of the parasite can take place in intestinal epithelial cell of the cat (definitive host), but in the intermediate host only asexual cycle takes place (Dubey, 2008).

2.2. 1. Life cycle in the intermediate hosts

Toxoplasma gondii has a heteroxenous biological life cycle and can virtually infect all species of warm-blooded animals (mammals and birds), including humans as intermediate hosts and felines as final hosts (Dubey *et al.,* 2004b; Dubey *et al.,* 2007a).When intermediate host ingests oocysts sporozoites are released into the gut lumen and pass through the gut epithelium to enter cells in the lamina propria. In case an intermediate host ingests tissue cysts the released bradyzoites behave similarly to sporozoites in which upon ingestion both sporozoites and bradyzoites invade the intestinal epithelium, differentiate into the rapidly growing tachyzoite form and disseminate throughout the body. Both sporozoites and bradyzoites transform into tachyzoites that enter a host cell where they divide rapidly until the cell bursts (Weiss and Kim, 2011; Opsteegh, 2011). Tachyzoites can infect virtually any nucleated cell type, although a tropism for certain cell types (for instance retinal vascular endothelial cells) has been reported (Smith *et al.,* 2004). After invading a cell, the tachyzoites can rapidly divide and after death of the host cell they will invade adjacent cells or after traveling through the blood stream attach to cells elsewhere in the body. The tachyzoite stage can transform into a slowly dividing bradyzoite (Klaren and Kijlstra, 2002) because as immunity develops, replication of tachyzoites decreases and tissue cysts develop which do not normally produce a host reaction (Saavedra, 2003). Ingesting tissues of an intermediate host containing tissue cysts infects carnivorous and omnivorous animals (Figure 2). This is also the route whereby cats and other Felidae become infected, thereby completing the life cycle of the parasite (Kijlstra *et al.,* 2004a).

2.2.2. Life cycle of Toxoplasma gondii in definitive hosts

Cats and wild felids are the only definitive hosts that may pass oocysts with their faeces and play an important role in the epidemiology of toxoplamosis (Bayarri *et al.,* 2012). Although domestic cats play the most important role in the dissemination of *T. gondii* oocysts, reports have also indicated that oocysts of *T. gondii* are present in feces of naturally infected jaguar (*Panthera onco*), ocelots (*Felis pardalis*), cheetah (*Acinonyx jubatus*), bobcats (*Llynx rufus*) and Canadian lynx (*Lynx conadensis*) (Coetzer and Tustin, 2004). Cats acquire the infection in one of two main ways: via consumption of cysts

present in the organs/tissues of a chronically infected intermediate host prey (such as mice) or via ingestion of oocysts within their food or water (Figure 4). After ingestion of tissue cysts the cyst wall is digested by gastric acid, bile and lytic enzymes of the upper digestive tract, which results in releasing bradyzoites and the released bradyzoites, invade the intestinal epithelium. Besides systemic dissemination after conversion to the invasive tachyzoite stage, some organisms inside the epithelium of definitive host undergo five different developmental stages that reproduce asexually by endodyogeny, where two daughter cells are created inside one and by schizogeny to differentiate into micro and macro gametocytes within 2 days of infection and involving the formation of multiple merozoite cells around a previously divided nucleus. The gametes fuse to form a zygote, which subsequently secretes a cyst wall to develop into oocysts. Oocysts rupture the intestinal epithelial cells to disseminate into the lumen and hundreds of millions are excreted in feces for days or weeks. Oocysts undergo sporulation outside of the body to become infective to other hosts (Dubey *et al.,* 1998; Dabritz and Conrad, 2010). The persistence of oocysts in the environment increases the probability of transmission to humans or animals. Cats with toxoplasmosis typically show no signs of the disease (Elmore *et al.,* 2010). The shortest prepatent period, i.e. the time from infection until the shedding of oocysts is 3 to 10 days after ingestion of tissue cysts. The prepatent period is 13 days or more after consuming tachyzoites and 18 days or more after oocysts ingestion (Dubey, 2008). Almost all cats shed oocysts after tissue cysts ingestion whereas less than 30-50% of cats shed oocysts after ingestion of tachyzoites or oocysts (Dubey, 2009). The prevalence of *T. gondii* infection in cat populations not only depend upon the availability and contact with infected prey species, where infection levels depend upon access to and ingestion of oocysts and infected tissue but also transmission of the infection via a congenital route. After a primary infection cats become immune, and do not usually excrete oocysts again if re-infected. This immunity can remain for up to 6 years and some cats can shed oocysts again if re-infected. However, only in smaller amounts than after the primary infection (Dubey, 1995).

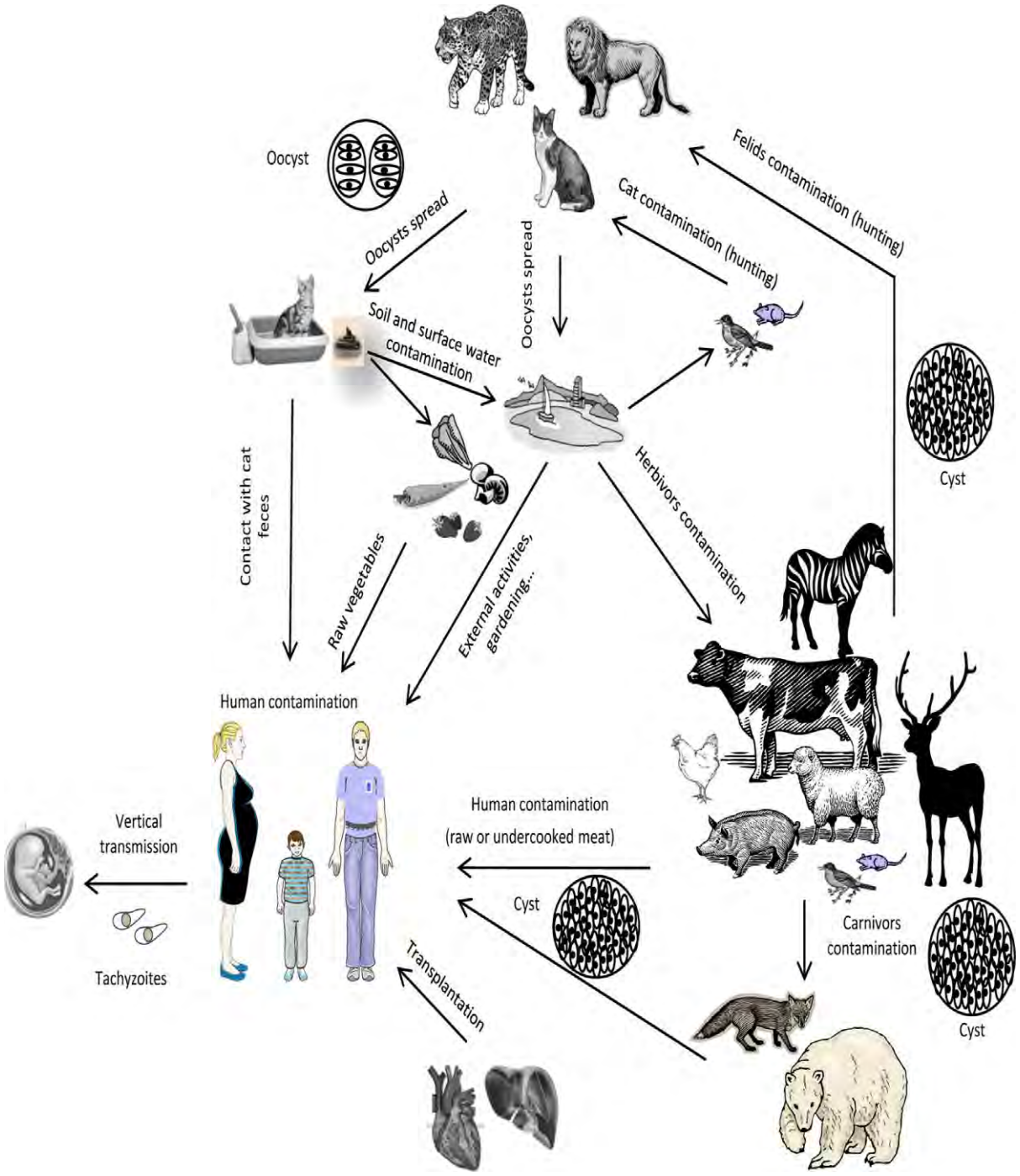

Figure 4. Life cycle of *Toxoplasma gondii*. (Source: Robert-Gangneux, 2014)

2.3. Epidemiology

2.3.1. Transmission routes

Widespread natural infection of the environment is possible since a cat may excrete millions of oocysts after ingesting as few as 1 bradyzoite or 1 tissue cyst, and many tissue cysts may be present in one infected mouse (Dubey, 2001). Ingesting oocysts in water, soil or feed is probably the most common route for *T. gondii* infection in non-carnivorous mammals and birds (Tenter *et al.,* 2000). The risk of *T. gondii* infection is higher in animals raised in extensive management than in intensive management. Sheep and goats on pasture show high sero-prevalence in many countries. Most *T. gondii* infections in pigs are due to oocysts via consumption of contaminated feed and soil (Dubey *et al.,* 1995c). Transmission to swine may also occur by the consumption of tissues of animals such as rodents and birds infected with *T. gondii* tissue cysts and by cannibalism. Studies have shown that *T. gondii* infection in pregnant sows can give rise to transplacental transmission, resulting in mummified fetuses, stillbirths and congenital infections, which have devastated the swine industry, causing significant economic losses (Huang *et al.,* 2010*).*

Carnivorous animals get infected by feeding on chronically infected meat, prey and carrion (Montoya and Liesenfeld, 2004). *T. gondii* may survive in food animals for years in tissue cysts (Hill and Dubey, 2002). Infection in felids and intermediate hosts, including humans, is acquired by ingesting uncooked or raw meat or carcasses contaminated with *T. gondii* cysts (McAllister, 2005). Although *T. gondii* is unable to reproduce sexually in non felids, asexual stages in infected tissues of intermediate hosts serve as a source of parasite transmission to other animals by carnivorism, which helps maintain enzootic *T. gondii* in the food chain (Dubey *et al.,* 1999).

Congenital transmission is also a unique method that *T. gondii* has developed to maintain reservoir infection in certain intermediate hosts. During pregnancy the tachyzoites can cross the placenta and infect the developing offspring. Congenital infection can occur in cats, and congenitally infected kittens can excrete oocysts, providing another source of oocysts for contamination (Hill and Dubey, 2002). For sheep and rodents, evidence has been reported indicating that congenital transmission may occur during successive pregnancies. Although congenital transmission has been reported in pigs, it is not known

whether transmission can occur during multiple pregnancies. In rodents, the congenitally infected offspring may transfer the disease to its offspring leading to a long-lasting reservoir of *T. gondii* even in the absence of *Felidae* as definitive host (Webster, 1994).

2.3.2. Risk factors for toxoplasmosis

Toxoplasma gondii has been found in almost every country of the world in many species of carnivores, insectivores, rodents, pigs, herbivores, primates and other mammals as well as in birds. Seroprevalence of *T. gondii* is highly variable among different geographic regions. Even for the same continent, deep variations can be found for the different countries. Several reasons may explain this fact such as: diet, preparation of food, hygiene, environmental conditions, definitive host population (wild and domestic *Felidae*) and different laboratory techniques used for serodiagnosis (De Sousa, 2009).The variation in climate has a marked influence on the habitat of *T. gondii*; for example, an elevation in ambient temperature and precipitation can modify the soil humidity, so that the sporulated oocysts persist for a long time viable in the moist environment (Meerburg and Kijlstra, 2009). Risk factors can also be associated to host factors (age, sex), and farm management (Pinheiro, *et al.,* 2009). Small flock size and access of cats to drinking water are potential risk factors for *T. gondii* infection in sheep. Prevalence in small ruminants is generally very high due to the continuous contamination of pastures by *T. gondii* oocysts (Cenci-Goga *et al.,* 2013).

Seropositivity is distinctly lower and more varying in horses, rabbits and poultry. This may reflect epidemiological factors such as different types of confinement, hygiene of stables and different types of feed. By contrast, seropositivity is usually high in dogs, indicating their continuous exposure to a natural environment and the cumulative effect of age. All of these animals may harbor a considerable number of tissue cysts in their organs, including skeletal muscles, and thus have importance in food-borne transmission to humans who consume their meat (Tenter *et al.*, 2000).

2.3.3. Prevalence of T. gondii in different species

Seroprevalence studies have been conducted in different animal species in different parts of the world (Bisson *et al.,* 2000; Spisak *et al.,* 2010; Berger-Schoch *et al.,* 2011a). Pig, cat, sheep and goat are domestic animal species most seriously affected by the *T. gondii*.

2.3.3.1. Prevalence in pigs

Seroprevalence of *T. gondii* in pigs has been reported in many different countries of the world, which showed that pigs are commonly affected animal with *T. gondii* (Suaréz – Aranda *et al.,* 2000; Saavedra and Ortega, 2004; Damriyasa *et al.,* 2004; Klun *et al.*, 2006; Tsai *et al.,* 2007; van der Giessen *et al.,* 2007; Correa *et al.,* 2008; Dubey and Jones 2008; Poljak *et al.*, 2008, Villari *et al.,* 2009; Veronesi *et al.,* 2010; Wu *et al.,* 2012; Deksne, and Kirjušina, 2013). A serological survey conducted in USA and other countries indicated a high prevalence of *T. gondii* in pigs, as 23% of 11229 pigs slaughtered in USA from 1983 to 1984 were found to be seropositive (Dubey, 1996). The seroprevalence in domestic swine on large commercial farms in developed countries has declined over the year, probably due to improvement in management practices. However, the current animal friendly pig management under non-confined condition has led to increased seroprevalence in Europe and America (van der Giessen *et al.,* 2007, Dubey, 2009). Serological testing of domestic pigs in Zimbabwe has showed widespread *T. gondii* infection and also indicated that seroprevalence was directly related to the hygienic conditions under which these animals were kept. In addition, seroprevalence was lowest in fattening pigs from large commercial farms (19.75%, n=238) and the highest in backyard scavenging pigs (35.71%, n= 70) (Hove *et al.,* 2005).

2.3.3.2. Prevalence in sheep and goats

Seroprevalence of *T. gondii* in sheep was reported as 32.9% (Pinheiro *et al.,* 2009) to 80% in Brazil (Rossi *et al.,* 2011), 84.5% in Serbia (Klun *et al.,* 2006), 52.2% in Saudi Arabia (Sanad and Al Ghabban, 2007), 29.1% in Iran (Bonyadian *et al.,* 2007) and 5.6% in South Africa (Samra *et al.,* 2007). In goats prevalence varied from 67.2 % in Zimbabwe (Hove *et al.,* 2005) to 4% in Senegal (Deconinck *et al.,* 1996). Only limited epidemiological information is available on the seroprevalence of *Toxoplasma gondii* in domestic livestock

in some African countries (Table 1). In Uganda sero-prevalence of *Toxoplasma gondii* is 31% in goats (Bisson *et al.,* 2000). The limited studies undertaken to investigate the magnitude of *T. gondii* infection in animals in Ethiopia indicated high sero-prevalence ranging from 22.9% to 56% in sheep and 11.6% to 82% in goats (Bekele and Kasali, 1989; Deconinck *et al*., 1996; Negash *et al.,* 2004; Teshale *et al.,* 2007; Yibeltal, 2008; Gebremedhin *et al.,* 2013; Zewdu *et al*., 2013; Gebremedhin and Gizaw, 2014). Food producing animals are important sources of *T. gondii* infection in humans. This has necessitated epidemiological investigation of the parasite in food animals in order to provide effective prevention and control of toxoplasmosis in animals and humans.

2.3.3.3. Prevalence in dog and cats

Toxoplasma gondii prevalence in cats have been recorded, 17.98% in China (Zhang *et al.,* 2009), 8.1-16.1% in Korea (Kim *et al.,* 2008), 40.3% in Ankara (Ozkan *et al.,* 2008), 44.1% in Czech Republic (Sedlak and Bartova, 2006), 91.8% in Mexico (Castillo-Morales *et al.,* 2012), 25.5-51.9% in Spain (Miro *et al.,* 2004), 70.2% in Belgium (Dorny *et al.,* 2002), in Iran 24.75% (Mosallanejad *et al.,* 2011) and 8.3% in Thailand (Arunvipas *et al.,* 2013). Other studies conducted in different countries showed the prevalence of *T. Gondii* infection in dogs 6.1 % in Thailand (Arunvipas *et al.,* 2013) and 26.9% in Brazil (Langoni *et al.,* 2013). High prevalence (25%) of *T. gondii* infection in dogs was also found in Maiduguri, Nigerian (Kamani *et al.,* 2010). In Egypt 50% prevalence of *T. gondii* was found in cat faeces (Awadallah, 2010) whereas in Ethiopia a preliminary study, antibodies to *T. gondii* were found in 85.4% of stray cats from Addis Ababa (Dubey *et al.,* 2013a; Tiao *et al.,* 2013).

2.3.3.4. Prevalence in chickens

Prevalence in poultry also varies markedly according to production systems. *Toxoplasma* infection in industrialized poultry farms is practically low or absent, while the seroprevalence in free-range or backyards chickens is usually high, up to 100%. Due to their habit of feeding close to the ground, free-range chickens are indeed considered a good indicator of environmental contamination by *Toxoplasma* oocysts (Dubey, 2010). In the developing world seroprevalence may be up to 65% in free-range chickens and tissue cysts

have been found in 81% of seropositive birds (Kijlstra and Jongert, 2008). However, in developed countries broiler chickens tend to be intensively reared and have a short life span, but no recent data are available on seroprevalence in these birds (Food Standards Agency, 2012). In free range chickens the prevalence of 65.2% was reported from Brazil (da Silva *et al.,* 2003) and 38.4% Addis Ababa, Ethiopia (Tilahun *et al.,* 2013).

2.3.3.5. Prevalence in other domestic animals

Tissue cysts are found only rarely in beef or buffalo meat, although antibodies have been demonstrated up to 92% of cattle (Avezza *et al.,* 1993) and up to 20% of buffaloes as evidence of past exposure to the parasite (El Ridi *et al.,* 1990). Although, horses are resistant to *T. gondii* (Dubey and Jones, 2008) the surveys conducted in different countries indicated seropositivity of 38.1% in Egypt (Ghazy *et al.,* 2007) and 1% in Sweden (Uggla *et al.,* 1990). Toxoplasmosis has also been reported in dairy cattle 13.3% in Sudan (Elfahal *et al.,* 2013), 5.7% in Southern China (Zhou *et al.,* 2012) and (71.3%) in South East Iran (Sanati *et al.,* 2012). *T. gondii* antibodies in camel sera were re-ported from Saudi Arabia (Al-Mohammed, 2012), Sudan (Khalil and Elrayah, 2011) and Egypt (Hilali *et al.,* 1998).

Table 1. *Toxoplasma gondii* prevalence in some domestic animals in some African countries

Geographic origin	Host	Test type	Sample size	Prevalence %	References
S. Africa	Sheep	IFAT	600	5.6	Samra *et al.,* 2007
Egypt	Chicken	MAT	121	40.3	Dubey *et al.,* 2003
	Goats	IHAT	306	59.4	Barakat *et al.,* 2009
	Sheep	ELISA	320	44	Barakat *et al.,* 2009
Morocco	Sheep	ELISA	261	27.6	Sawadago *et al.,* 2005
Ethiopia	Chicken	MAT	601	30.5	Gebremedhin *et al.,* 2014
	Sheep	ELISA	1130	31.6	Gebremedhin *et al.,* 2013
	Goats	MAT	641	74.8	Teshale *et al.,* 2007
	Cattle	IHAT	785	6.6	Bekele and Kasali, 1989
Nigeria	Dog	LAT	168	25	Kamani *et al.,* 2010
	Cat	LAT	105	36.2	Kamani *et al.,* 2010
	Pig	ELISA	302	29.14	Onyiche *et al.,* 2013
Uganda	Goats	ELISA	784	31	Bisson *et al.,* 2000
Ghana	Goats	ELISA	526	26.8	van der Puije *et al.,* 2000
	Pig	ELISA	641	39	Arko-Mensah *et al.,* 2000
	Sheep	ELISA	732	33.2	van der Puije *et al.,* 2000
Zimbabwe	Pig	IFAT	474	26.5	Hove *et al.,* 2005

2.4. Strains of *Toxoplasma gondii*

Recombinant and clonal isolates of *T. gondii* exist because of its replication both sexually and asexually (Sibley *et al.,* 2009). The population biology of *T. gondii* indicates that, despite the existence of a well-described sexual cycle in cats, the parasite appears to reproduce in nature largely clonally, with sexual recombination occurring only extremely rarely which in turn this can contribute to genetic variation of the parasite (Grigg *et al.,* 2001). Furthermore, recent studies from remote geographical regions revealed that there is genetic variability than previously reported of *T. gondii* isolates obtained either from animals or humans (Ajzenberg *et al.,* 2004; Lehmann *et al.,* 2004). Generally it was believed that *Toxoplasma gondii* had a clonal population structure with three predominant lineages, namely types I, II and III which was originated from a variety of human and animal sources in North America and Europe (Su *et al.,* 2006a). Moreover, another fourth clonal lineage (Type 12) close to type II strains has recently been described and is the most common type in wildlife in North America (Dubey *et al.,* 2011; Rajendran *et al.,* 2012). Type I strains are highly virulent, whereas type II and type III are significantly less virulent (Boothroyd and Grigg, 2002). Type I strains *T. gondii* infection in human showed an increased frequency in AIDS patients as well as ocular toxoplasmosis (Khan *et al.,* 2005; Vallochi *et al.,* 2005) while type II strains are the most prevalent cause of human toxoplasmosis both in congenital infection and in AIDS patients in North America and Europe (Boothroyd and Grigg, 2002; Nowakowska *et al.,* 2006; McLeod *et al.,* 2012). Type I strains do not readily produce tissue cysts or participate in the sexual cycle, whereas Type II and III strains maintain the ability to complete the entire life cycle (Sibley and Boothroyd, 1992). Variations within the strain types are very rare, which suggests that type I, II, and III have undergone clonal expansion (Su *et al.,* 2003b). Less than 1% of the isolates contains highly polymorphic DNA sequences, and is defined as "exotic" or "atypical" (Ajzenberg *et al.,* 2004*).* Interestingly, atypical and exotic strains have alleles that are found in type I and III strains, but rarely from type II (Boothroyd and Grigg, 2002).

Type II is the most common lineage worldwide, followed by the type III strain. Type I strain, though widely distributed, has been infrequently isolated. Several new clonal genotypes have been identified from South America (Majumdar, 2010). Domestic animal infections in the United States and Europe are typically characterized as Types II and III strains (Dubey *et al.,* 2004b). In Australia, genotype II strains have been reported from a human isolate (Sibley and Boothroyd, 1992) and a dog isolate (Al-Qassab *et al.,* 2009) and atypical and type II-like strains have been isolated from native Australian wildlife (Parameswaran *et al.,* 2010). More recently, highly virulent atypical genotypes in French Guiana and Brazil have caused severe disease in immunocompromised individuals, fetuses and otherwise healthy individuals (Carme *et al.,* 2009; Dubey *et al.*, 2012). In Africa, a clonal population structure consisting of additional common clonal lineages known as the Africa 1 to 3 haplogroups, coexisting with type II and III lineages, has been described (Mercier *et al.,* 2010). In Ethiopia four ToxoDB (#1, #2, #3 and #20) genotypes of *T. gondii* was identified from feral cats (Dubey *et al.,* 2013b).

2.5. *Toxoplasma gondii* in pig

Among production animals, pigs are the most common animals that harbor *T. gondii* (Silva *et al.*, 2003). Most pigs acquire infection postnatally by ingestion of oocyst from contaminated environment or ingestion of infected tissue of animals (Dubey, 2009). *T. gondii* infections in pigs are usually subclinical. However, outbreak of toxoplasmosis characterized by high body temperature, gradually loss of appetite, high morbidity reaching 57% and mortality of approximately 2% has been reported (Li *et al.,* 2010). *T. gondii* is also responsible for pneumonia, myocarditis, encephalitis, and placental necrosis in pig (Hill *et al.,* 2005). In sows infected during pregnancy, abortion rates of up to 44% may occur. During transplacental infection birth of premature, or weak and dead piglet may occur over a very short period of time (Kim *et al.,* 2009). The mortality associated with toxoplasmosis in pigs is greater in young than in adult pigs. *T. gondii* infection in pigs can be confirmed by definitive histological examination or by bioassays that require isolation of organisms from fresh tissue. By this approach, the prevalence of *T. gondii* infection was found to range from as low as 4% to as high as 69% in pigs from the USA (Dubey, 1990).

In developing countries, such as Peru and Brazil, there is a high prevalence of specific antibodies (34% and 9%, respectively) to *T. gondii* in pigs (Saavedra, 2003). The prevalence of *T. gondii* varied considerably in pigs depending on such factors as herd management, housing, age group and herd location (Suaréz-Aranda *et al.,* 2000; Venturini *et al.,* 2004). Seropositive pigs might harbor tissue cysts in their meat after being naturally infected with *T. gondii*. The introduction of *T. gondii* into the pig farm increases the risk of infection in pregnant gilts and sows. Few pigs become infected prenatally by transplacental transmission of the parasite (Dubey, 2009). Rodents are considered as a reservoir host and play an important role in the transmission of the disease to pigs and humans (Kijlstra *et al.,* 2008).

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in three purposively selected sites located in Central Ethiopia, namely Addis Ababa, East Shewa (Bishoftu and Adama) and West Shewa (Ambo area) (Figure 5). The selection of those study areas were mainly based on availability of pig farms and ease of accessibility in terms of distance from Bishoftu College of Veterinary Mediicne and Agriculture.

Bishoftu is found in Adea district of East Shewa Zone of Oromia Regional State at a distance of 45 kms from Addis Ababa. It is situated at a longitude of 38°58’ E to 39°22’E and latitude of 08° 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 sub moist agro- ecology. The average rainfall is about 839 mm, while the mean minimum and maximum temperatures recorded for 27 years ranged from 7.9 ºC to 28 ºC with an overall average of 18.5 ºC (IPMS, 2004).

Ambo district is found in West Shewa 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 90 20' N and the altitude within the district ranges from 1400 to 3045 masl. The climatic condition of the area is 23% highland, 60% midland and 17% lowland with an annual rainfall and annual temperature ranging from 800–1000 mm and 15 °C – 29 °C, 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).

Addis Ababa is the capital city of Ethiopia. It covers about 540 km^2, of which 18.2 km^2 is rural. The city is located at 9°1’ 48 “N and 38° 44’- 24” E at an average altitude 2,200 of 3300 masl. The annual rainfall is about 800-1100 mm and a mean annual maximum and minimum temperature is about 21°C and 27°C respectively (Fikire *et al.,* 2012).

Adama is located 100 kms to the south east of Addis Ababa. It is situated 1600-1700 masl and in a latitude from 8^0-33.8N to 8^0-36N and a longitude from $39^0$11' 57E to $39^0$21'15E in the Rift valley of warm climate. Its average annual weather conditions are 21 ^{0}C with small amount of rainfall from June to September. In general, the town has sunny and windy weather conditions (Taye, 2011).

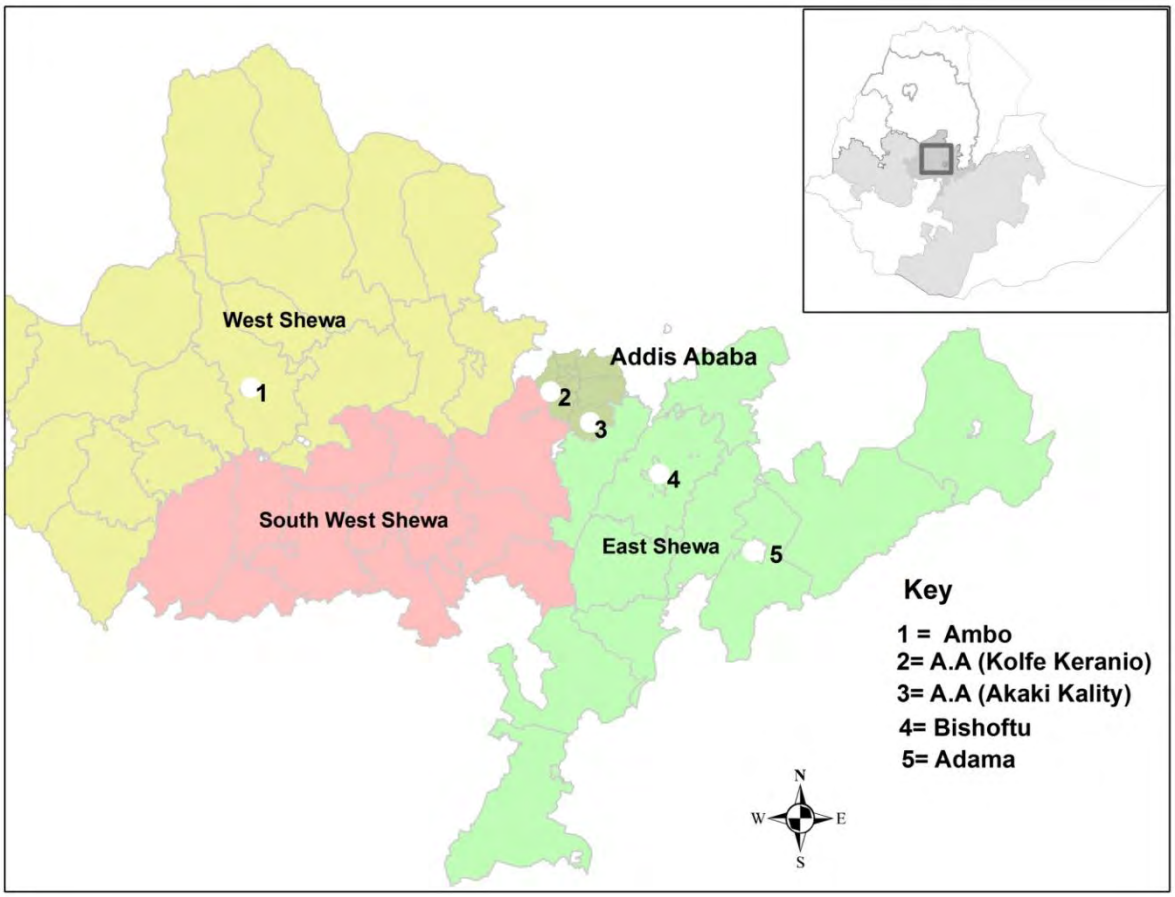

Figure 5. Study areas (East Shewa (Bishoftu, Adama), West Shewa (Ambo) and Addis Ababa (A.A), Central Ethiopia.

3.2. Study populations

The study was conducted in swine farms and pigs brought at Addis Ababa slaughterhouse from the four purposively selected sites in Central Ethiopia (Ambo, Addis Ababa, Bishoftu and Adama area). The origins of pigs considered for this study were predominantly from Bishoftu because many large and small scale commercial pig farms are concentrated around this area. In Bishoftu area pigs are mainly kept under intensive production systems. In Addis Ababa area both intensive and extensive management of pigs are common while in Ambo area pigs are kept under extensive management system. For pigs originated from Adama area data on management system was not available. All pigs of more than three months of age belonging to both sexes (male and female) were considered for the purpose of this study. Pig age was determined using tooth eruption patterns (Boitani and Mattei, 1992) (approximately at 1 year of age when permanent incisor 1 erupt (I1), at 16 - 20 months of age when permanent incisors 2 erupt (I2), at 8 - 10 months of age when permanent incisor 3 erupt (I3), at 9 - 10 months of age when canine teeth erupt and less than three months of age when temporary incisor 1 and 2 erupt)and pigs ≤1 year of age were categorized as young whereas pigs >1 year as adults.

3.3. Study designs and sample size

A cross-sectional study design was carried out in selected sites of Central Ethiopia from October, 2013 to May, 2014. The farms included in the study were purposively selected based on farm owners' willingness and accessibility. Accordingly a total of 557 individual pigs were sampled from purposely selected study areas. For blood collection from farms and Addis Ababa abattoir systematic random sampling technique was used.

Since there was no previous study on *T. gondii* infection in pigs in Ethiopia the sample size was calculated according to Thrusfield (2007) by considering 50% expected prevalence (*P*) and 95% confidence interval (Z= 1.96) with a 5% desired absolute precision (d) using the formula $N= (Z)^2 P(1-P)/d^2$. The calculated required sample size (N) was 384. However, total number of sampled animals was increased to 557 for better accuracy. For the purpose of *T. gondii* isolation 50 hearts from seropositive pigs were considered purposively and five mice were used per sample.

The abattoir was selected because slaughtered animals are received from all areas of the country, including from large commercial and small scale producers and the meat is sold locally by supermarket owners to consumers. The number of samples was determined by the number of animals that were slaughtered on the days of the visits and a total of 290 pigs were sampled. The majority of the pigs were ordered for slaughter by supermarket owners; therefore, information associated with the specific farm of origin was limited for some pigs particularly pigs from Adama area. Information recorded included sex, age and source when available. Out of total pigs sampled, blood samples of 186 pigs from Bishoftu, 57 pigs from Ambo and 24 pigs from Addis Ababa Akaki kaliti sub-city were sampled from live animals in the farm. Whereas blood and hearts of 290 pigs were sampled from Addis Ababa abattoir enterprise during slaughtering of pigs for human consumption. The latter pigs were originated from Adama, around Addis Ababa area and Bishoftu. For the isolation of *T. gondii,* hearts of sero-positive pigs (from 290 pigs sampled from abattoir) was taken for bioassay in *Toxoplasma* negative Swiss Albino mice.

3.5. Sample collection and transportation

Blood samples were aseptically collected from ear vein from pig on farms (n=267) by sterile vacuum tubes without anti-coagulant. From abattoir blood samples were collected from jugular vein (n=290) during slaughtering and corresponding heart were collected in sterile plastic bag with zipper and labeled with the same code of blood sample. These samples were immediately transported in ice box with ice packs to the Ethio-Belgium Laboratory of the College of Veterinary Medicine and Agriculture of the Addis Ababa University, Bishoftu for serological testing and bioassay of *T. gondii*. Sera were separated by centrifugation of the tubes at 3200 RPM for 10 minutes. The sera were transferred to eppendorff tubes and kept at –20 ^{0}C until serologically assayed. Separated sera were examined for anti- *T. gondii* antibodies (IgG) by MAT. In the laboratory the heart samples were kept in refrigerator at (+4 ^{0}C) for 48 hours until the result of MAT was obtained. The numbers of tissue samples for bioassay were selected from seropositive pigs obtained from slaughterhouse based on the number of tissues samples permitted on the days of the visits because of not all heart tissue samples from pigs slaughtered at Addis Ababa abattoir were allowed for sampling and organs were sent with their corresponding carcass to the supermarket. Finally 8-12 tissue samples were allowed at each day of slaughter visits of which tissues of seropositive pigs were used for bioassay until 50 samples attained.

3.6. Laboratory investigation

3.6.1. Modified Agglutination Test

Serum sample from pigs and mice were tested by MAT (Toxo screen DA, biomerieux® SA mercy-I' Etoile/France) to detect IgG antibodies against *T. gondii* following the protocol of the manufacturer. Sera were diluted at screening dilution of 1:40 and 1:4000 in order to avoid the false negative results that might occur at low dilutions when using sera with high antibody titers. Serum samples (100µl) were diluted using phosphate buffer saline (PBS, pH=7.2) and 25µl of diluted sera were placed in U- bottom well of microtitre plate. Sera samples were treated with 0.2 M 2-mercaptoethanol to remove non-specific IgM or IgM-like substances. Then, equal amount of antigen (50 µl) was added to each well. Sedimentation of antigen at the bottom of the well and clear agglutination above half of the well was recorded as negative and positive results, respectively. (See annex 1for the details of the procedure).

3.6.2. Bioassay of hearts of seropositive pigs for T. gondii

Female Swiss mice with 20-25 g weight were used in the bioassay. Isolation of *T. gondii* was made from heart tissues of sero-positive pigs. Approximately, weighting at least 50 g of heart tissue, was taken for bioassay after digestion in pepsin (Merck KG.A, Darmstadt, Germany) as described previously (Dubey, 2010) (Annex 2). All seropositive pigs were used for bioassay to mice.The mice used for bioassay were obtained from National Veterinary Institute (NVI), Ethiopia. Mice were daily checked for any signs of toxoplasmosis and recorded on data collection sheet for monitoring mice infected with heart tissue homogenate of seropositive pigs (Annex 3). The virulence of the parasite was classified according to Pena *et al.* (2008) who determined *T. gondii* virulence at isolation without knowledge of infecting dose based on mortality of mice within four weeks of infection and categorize isolates into three groups: virulent (100 % death of mice within four weeks), intermediate virulent (30 % to less than 100 % mortality), and non-virulent (< 30 % death within four weeks). After two months of follow-up the mice were humanely killed by anaesthetizing with di-ethyl ether. Blood samples from survived mice were collected for serological assay (MAT). Further the brain was subjected for *T. gondii* tissue

cysts detection and enumeration. The inoculated mice were considered positive with *T. gondii* infection when MAT positive and tissue cysts were found in the brains.

3.6.3. Quantification of cysts from the mouse brain

Brain from surviving mice was removed by sagittal dissection after anesthetizing with di-ethyl ether and then it was weighted and recorded. Then the whole brain was homogenized in 1ml PBS by using a mortar and pestle. The number of cysts in five aliquots of each 10µl was counted under 10X objective lens of a microscope and summed. The total number of cysts in the brain of each mouse was determined by converting the sum of cysts in 50 µl to the whole volume of the brain homogenates (Goodwin *et al*., 2008; Dubey, 2010; Fritz *et al*., 2012).

3.7. Questionnaire survey

Structured questionnaire was administered for each farm owners regarding herd size, age, sex (female/male), management system (intensive/extensive), feed type (with/without animal product), presence of domestic cats (yes/no), way of life of domestic cat (totally outdoor/mixed), presence of rodents on the farm (yes/no), access of pigs to dead animals (yes/no), water source (pipe/mixed) and history reproductive failure (abortion, still birth, neonatal mortality, mummification) were recorded during sample collection to assess potential risk factors for *T. gondii* infection in pigs. Furthermore two people were interviewed from each 13 farms to assess farm attendant or owners knowledge about toxoplasmosis as a zoonosis (Annex 4).

3.8. Data analysis

Data generated from questionnaire survey and laboratory investigations 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 seropositive samples divided by the total number of samples tested. To identify predictors of seropositivity, first the association of the potential risk factors (origin, age, sex, management system, presence of domestic cat, presence of dog, way of life of domestic cats, pig feed type, access to dead animal and water source) were analyzed by univariable logistic regression. Then all non- collinear variables with P-value ≤ 0.25 in univariable logistic regression analysis were included in the final multivariable logistic regression model to construct the likely model (P < 0 .05). The model was reduced by backwards elimination of non-significant variables (P > 0.05) based on likelihood ratio test to define the model that would best fit the data. In line with this, except management system, type of pig feed, presence of dog and age, all variables were dropped due to collinearity. Variables with small and uncomparable frequencies in the contingency table were not considered.The agreement between microscopic cyst detection and MAT used for the diagnosis of *T. gondii* infection in mice was done using kappa test and interpreted according to the recommendations of Dohoo *et al.* (2003) who states Kappa values as: <0.2: slight agreement, 02–0.4: fair agreement, 0.4–0.6: moderate agreement, 0.6–0.8: substantial agreement and >0.8: almost perfect agreement. The 95% confidence interval and a significance level of $\alpha = 0.05$ were used.

4. RESULTS

4.1. Characteristics of farm attendant and knowledge about health risks of cats

Among 26 farm attendants interviewed during the study 14 (53.8%) have completed university education whereas only 2 (7.7%) were illiterate. Sixteen (61.5%) and 18 (69.2%) of study participants do not have the knowledge about the role of cats in transmitting zoonotic diseases to humans and animal respectively (Table 2).

Table 2. Educational status and knowledge about health risks of cats among farm attendants (n=26).

Variable	Category	Frequency (%)
Level of education	Illiterate	2 (7.7)
	Primary School	4 (15.4)
	Secondary school	6 (23.1)
	University	14 (53.8)
Knowledge of health risk of cats to humans	Yes	10 (38.5)
	No	16 (61.5)
Knowledge of health risk cats to animals	Yes	8 (30.8)
	No	18 (69.2)

4.2. Farm characteristics

Out of 13 pig farms considered for this study, 12 (92.3%), 10(76.9) and 9(69.2%) of the farms reported the presence of neonatal mortality, weak birth and history of abortion among herds in their farms respectively. Nine farms (69.2%) do not practice rodent control in their farms (Table 3).

Table 3. Farm characteristics and reproductive problems in pig farms in study area (in percentage) n=13

Variable	Frequency (%)	
	Yes	**No**
History of abortion (s) in herd	9 (69.2)	4 (30.8)
Presence of stillbirth among herd	8 (61.5)	5 (38.5)
Neonatal mortality among herd	12 (92.3)	1 (7.7)
Presence fetal mummification	9 (69.2)	4 (30.8)
Presence of weak births	10 (76.9)	3 (23.1)
Contamination of stored animal feed by cat feces	12 (92.3)	1 (7.7)
Presence of feral cats	13 (100)	0 (0)
Presence separate cat house	0 (0)	13 (100)
Pig mortality in farms	10 (76.9)	3 (23.1)
Presence of rodent control	4 (30.8)	9(69.2)

4.3. Overall seroprevalence

An overall seroprevalence of *T. gondii* in the study area was 32.7% (182/557) (95% CI: 0.29-0.37). Among 402 pigs considered for sero-epidemiological study of risk factor assessment seroprevalence of 32.1% (129/402) (95% CI: 0.28-0.37) was found. Highest animal level sero-prevalence was observed in Addis Ababa Kolfe Keraniho Sub-city (43/75, 57.3%) followed by Addis Ababa Akaki Kaliti Sub-city (11/24, 45.8%), Ambo (19/57, 33.3%) and Bishoftu (56/246, 22.8%). This result is significantly different between study areas ($P<0.001$) (Table 4).

4.4. Risk factors

4.4.1. Univariable logistic regression analysis

The variable "presence of animal feed contamination with cat feces" was not considered in logistic regression because of less frequency. History of abortion, presence of still birth, neonatal mortality, presence of fetal mummification, presence weak births and pig mortality were also excluded from analysis because of lack of information on individual animals. (Table 3).The potential risk factors for seropositivity of pigs according to farm characteristics and management systems with the corresponding univariable analysis are shown in Table 4.

Pigs reared on farms that had access to dog (OR=2.56, 95% CI: 1.61, 4.08) had significantly higher seroprevalence than pigs reared in farms where dogs were not present. In this study no significant difference ($P > 0.05$) was observed in the seroprevalence of *T. gondii* infection between male (34.0%, 55/162) and female (30.8%, 74/240) pigs as well as between extensive and intensive management systems. Pig which drank water from mixed sources (pipe, river and pond) had a higher risk to become infected with *T. gondii* than those pigs which drank pipe water (OR = 1.16, 95% CI: 0.67, 2.0). The presence of rodents on farms did not significantly influence seropositivity of animals ($P>0.05$) (Table 4).

The data obtained from the questionnaires survey showed that all pigs in the study area had access to feral cats. Moreover, separate cat housing system was not practiced for domestic cats. Domestic as well as feral cats get free access to pig farms. These variables were not subjected to statistical analysis since they are common for all farms (Table 3).

Table 4. Results of univariable analysis of potential risk factors associated with *Toxoplasma gondii* seropositivity

Variable category	Number tested	Test Pos. (%)	χ^2	*P* value	Univariable OR (95% CI)	*P* value
Origin			33.9	<0.001		
Bishoftu	246	56 (22.8)			1.0	
Ambo	57	19 (33.3)			1.7 (0.91-3.17)	0.098
Akaki Kaliti	24	11 (45.8)			2.87 (1.22-6.76)	0.016
Kolfe Keraniho	75	43 (57.3)			4.56 (2.64-7.87)	<0.001
Sex			0.43	0.511		
Female	240	74 (30.8)			1.0	
Male	162	55 (34.0)			1.15 (0.75-1.76)	0.511
Age			2.27	0.039		
<12 months	170	45 (26.5)			1.0	
≥12 months	232	84 (36.2)			1.58 (1.02-2.43)	0.039
Management			2.18	0.140		
Intensive	334	102 (30.5			1.0	
Extensive	68	27 (39.7)			1.5 (0.87-2.57)	0.142
Presence of cat			0.03	0.864		
No	61	19 (31.2)			1.0	
Yes	341	110 (32.3)			1.05 (0.58-1.89)	0.864
Presence of dog			16.2	<0.001		
No	157	32 (20.4)			1.0	
Yes	245	97 (39.6)			2.56 (1.61-4.08)	<0.001
Way of life of domestic cat			0.26	0.607		
Totally outdoor	138	42 (30.4)			1.0	
Mixed*	264	87 (33)			1.12 (0.72-1.75)	0.607
Pig feed type			26.96	<0.001		
Without animal product	327	86 (26.3)			1.0	
With animal product	75	43 (57.3)			3.77 (0.24-6.33)	<0.001
Access to dead animals			10.22	0.001		
No	230	59 (25.7)			1.0	
Yes	172	70 (40.7)			1.99 (1.30-3.04)	0.001
Water source			0.28	0.599		
Pipe	333	105 (31.5)			1.0	
Mixed**	69	24 (34.8)			1.16 (0.67-2.0)	0.599
Rodent presence			1.35	0.245		
No	12	2 (16.7)			1.0	
Yes	390	127 (32.6)			2.41 (0.52-1.18)	0.260

*= indoor and outdoor, **= pipe, river and pond

4.4.2. Multivariable logistic regression analysis

For multivariate logistic regression non-collinear variables with univariate *P*-value <0.25 were considered. Selection of one of the collinear variable was done on the basis of biological ground to reason out scientifically the hypothesized risk factors in a better way. Sex, presence of domestic cats, way of life of domestic cats, source of water and presence of rodent were variables excluded from final model due to univariable *P*- value > 0.25. Whereas origin and presence of pig access to dead animal were removed due to collinearity. Finally, management system, age category, type of pig feed and presence of dogs were entered into multivariable logistic regression model and the results are depicted in Table 5.

In multivariate logistic regression analysis management system and type of feed were the only risk factors significantly associated ($P < 0.05$), with pig seroprevalence (Table 5). The risk of acquiring toxoplasmosis for pigs raised on extensive farms was 1.91 times higher than pigs raised on intensive farms. Type of feed for pigs was also a risk factor associated with pig toxoplasmosis that pigs feeding on additional animal byproduct (slaughterhouse leftover, dairy product (whey)) as feed supplement had higher chance of acquiring *T. gondii* infection than pigs that were not supplemented with such animal product.

Table 5. Results from multivariable logistic regression analysis of predictors of *T. gondii* infection in pig of study area

Variable category	**Adjusted OR(95% CI)**	***P*-value**
Management	1.91(1.01-3.63)	0.047
Type of pig feed	3.84(2.01-7.36)	<0.001
Presence of dog	1.48(0.84-2.61)	0.172
Age	1.12(0.68-1.84)	0.665

4.5. Bioassay in mice

4.5.1. Toxoplasma gondii isolation

Viable *T. gondii* was isolated from 24 of 50 (48%) seropositive hearts bioassayed. Three MAT positive mice gave cyst negative result. Brain tissue cysts were detected from two MAT negative mice. Viable *T. gondii* tissue cysts were also isolated from two groups of seronegative pooled tissue samples (P1 (three pigs) and P2 (four pigs). During follow-up period 28 mice were dead within 1-4 days post inoculation. One isolate from Bishoftu area killed four mice between 28 and 30 days. Another isolate from Addis Ababa killed one mouse on the 6th day post inoculation. Most of the survived mice showed clinical signs such as tachypnea (12 mice), inappetance (18 mice), starry hair and dullness (75 mice) but become normal until at the end of examination. A sample from Adama area (pig number AA130) showed neurological sign (circling) in one mouse (Table 6).

Table 6. Summary of isolation of *T. gondii* from selected seropositive pigs in study area

Origin	Animal Id	Reciprocal MAT titer of seropositive pig	Bioassay in mice			
			Seropos mice/mice exam.	Cyst pos mice/mice exam.	Mice dead/mice inoculated	Days of mice death PI (no. mice)
Adama area	AA112	≥1620	0/5	0/5	0/5	Survived
	AA113	≥1620	0/4	0/4	1/5	2(1)
	AA117	18000	2/2	2/2	3/5	2(1), 3(2)
	AA118	≤6000	0/3	0/3	2/5	2(2)
	AA119	≤6000	1/3	0/3	2/5	4(2)
	AA130	≤6000	1/4	1/4	1/5	2(1)
	AA236	≥1620	0/5	0/5	0/5	Survived
	AA249	≥1620	0/4	0/4	1/5	2(1)
	AA261	≥1620	2/5	0/5	0/5	Survived
	AA263	≥1620	1/5	2/5	0/5	Survived
	AA268	180	0/5	0/5	0/5	Survived
Bishoftu	AA42	18000	1/3	1/3	2/5	2(1)
	AA146	≥1620	0/5	0/5	0/5	Survived
	AA256	≤60	0/5	0/5	0/5	Survived
	AA270	≤60	1/5	1/5	0/5	Survived
	ALP2	≥1620	1/1	1/1	4/5	28(3),29(1)
Addis Ababa	AA45	≤60	0/2	0/2	3/5	2(3)
	AA66	≤6000	5/5	5/5	0/5	Survived
	AA67	≥1620	3/5	3/5	0/5	Survived
	AA68	≤6000	2/4	1/4	1/5	3(1)
	AA69	≤6000	0/4	0/4	1/5	4(1)
	AA70	≥1620	1/5	1/5	0/5	Survived
	AA71	≤6000	2/5	2/5	0/5	Survived
	AA72	162000	5/5	5/5	0/5	Survived
	AA77	≥1620	0/4	0/4	1/5	6(1)
	AA78	≤6000	0/5	0/5	0/5	Survived

Table 6. Summary of isolation of *T. gondii* from selected seropositive pigs in study area (*continued*)

	AA83	162000	0/5	0/5	0/5	Survived
	AA100	≥1620	0/4	0/4	1/5	2(1)
	AA101	≥1620	1/4	1/4	1/5	2(1)
	AA104	≥1620	0/5	2/5	0/5	Survived
	AA105	≥1620	0/5	0/5	0/5	Survived
	AA155	≤6000	1/5	1/5	0/5	Survived
	AA156	≥1620	1/5	1/5	0/5	Survived
	AA158	≥1620	1/5	1/5	0/5	Survived
	AA160	≥1620	0/3	0/3	2/5	1(2)
	AA169	≥1620	0/4	0/4	1/5	1(1)
	AA180	≥1620	0/5	0/5	0/5	Survived
	AA219	≤6000	0/5	1/5	0/5	Survived
	AA220	≥1620	1/5	1/5	0/5	Survived
	AA222	≥1620	2/5	2/5	0/5	Survived
	AA225	≥1620	1/5	1/5	0/5	Survived
	AA226	≥1620	1/5	1/5	0/5	Survived
	AA249	≥1620	0/4	0/4	1/5	3(1)
	AA250	≥1620	0/5	0/5	0/5	Survived
	AA252	540	0/5	0/5	0/5	Survived
	AA254	540	0/5	0/5	0/5	Survived
	AA255	540	0/5	0/5	0/5	Survived
	AA274	≥1620	0/5	0/5	0/5	Survived
	AA275	≤60	3/5	3/5	0/5	Survived
	AA279	≤6000	1/3	1/3	2/5	3(2)
Others	P1*	-	1/1	1/1	4/5	2(2), 3(2)
	P2*	-	2/5	2/5	0/5	Survived

*= pooled samples (P1 from Addis Ababa (sample number AA154, AA161 & AA162) and P2 from Addis Ababa and Adama area (sample number AA266, AA269, AA276 & AA277)

4.5.2. Predictors of brain cyst count

The isolation of viable *T. gondii* parasites generally showed a significant increment with anti-*T. gondii* antibody end titer of the pig. *Toxoplasma gondii* was isolated from 34.4% of seropositive pigs with end titer (≥6000) as compared to 13.6% isolation rate from the hearts of pigs with antibody end titers of ≤1620. Mice with mean weight difference of ≥7 g had a significantly lower *T. gondii* isolation rate (11.46%) as compared to mice with mean weight difference of < 7 g (25.8%) (Table 7).

Table 7. Results of the rate of mice brain cyst positivity in relation to potential predictors of cyst positivity using Chi-square test

Variable category	No of mice	No of cyst positive mice (%)	χ^2	*P*-value
Study area			1.0933	0.579
Adama area	42	6 (14.3)		
Bishoftu	18	3 (16.7)		
Addis Ababa	156	33 (21.2)		
Sex			0.9307	0.335
Female	107	18 (16.8)		
Male	109	24 (22.0)		
Age			0.847	0.357
<12months	64	10 (15.6)		
≥12months	152	32 (21.1)		
Mice serostatus			156.02	<0.001
Negative	176	6 (3.4)		
Positive	40	36 (90.0)		
MATRETP			12.18	<0.001
≤1620	155	21 (13.6)		
>6000	61	21 (34.43)		
Mice Wt diff.			7.04	<0.008
≥7.1(mean)	96	11 (11.46)		
<7.1g	120	31 (25.8)		

MATRETP= modified agglutination test reciprocal end titer of pig, Mice wt diff = difference in body wt in gm between final and initial wt., diff.=difference, wt.=weight

The mean summary of cyst count in the study area was depicted in Table 9. The overall mean cyst count recorded in this study area was (157.2 ± 188.96) in the range of 29-686 cysts per brain of mice.

Table 8. Summary of cyst count in mice experimentally infected with extracts from hearts of seropositive pigs

Origin of pigs	**No. seropos. pigs used for bioassay**	**No. cyst pos.pigs**	**No. of cyst positive mice / Tot. mice exam.**	**Cyst count/brain**	**Range**
				Mean ±SD	
Adama area	10	3	6/11	209.3 ± 266.52	29-552
Bishoftu	5	3	3/9	128.3 ± 171.5	29-326
Addis Ababa area	35	18	33/86	150.4 ± 179.4	30-686
Overall	50	24	42/106	157.2 ± 188.96	29-686

SD= Standard Deviation; No. = Number; pos.= positive; seropos.= seropositive; Tot.=total; exam.=examined

The agreement between modified agglutination tests versus microscopic cyst detection in mice was calculated by using kappa value as shown in Table 9.

Table 9. Qualitative comparison of results of MAT and microscopic cyst detection in mice

		Cyst		
		Negative	**Positive**	**Total**
MAT	Negative	170	6	176
	Positive	4	36	40
	Total	174	42	216

Kappa = 0.85 ($P < 0.0001$)

5. DISCUSSION

5.1. Knowledge about health risks of cats

The survey results indicated that most of the respondents had no knowledge and awareness with regards to the role of cats in the transmission of diseases either to humans (61.5%) or animals (69.2%) respectively**.** Therefore, lack of knowledge regarding the common zoonotic diseases from cats in this study might contribute towards the transmission of various zoonotic diseases including toxoplasmosis. Several studies have indicated that contact with cats' faeces to be the major risk factor for pregnant women in acquiring *T. gondii* infection (Ayi *et al.,* 2009; Babaie *et al.,* 2013). Other studies have also indicated that a variety of non parasitic zoonotic diseases may be associated with pet animals, including cat scratch disease (bartonellosis), cat bite abscesses, rabies, leptospirosis, *Clostridium difficile*-associated diarrhea, salmonellosis, avian chlamydiosis, campylobacteriosis, dermatophytosis, and blastomycosis (Weese *et al.,* 2002). Furthermore, animals and humans can be infected by ingesting soil, water, or plant materials contaminated with *T. gondii* oocysts shed by infected cats *(*Pereira *et al.,* 2010). In order to understand the transmission dynamics of zoonotic parasitic infections to humans, it is essential to have knowledge on the life cycle and prevalence of infection in both domestic and wild animals (Lavikainen, 2010). It is believed that educated people are much more dedicated to their health than non- educated people and in most of the cases; they can have a tendency to gather information about factor which are risky for their health and also its prevention (Babaie *et al.,* 2013).

5.2. Farm characteristics

The results of the questionnaire revealed widespread presence of neonatal mortality, history of abortion and presence of weak birth among pig herds of the study area. Several previous studies showed that *T. gondii* infections result in tremendous problems for livestock husbandry and cause huge economic losses due to reproductive wastage (Boughattas *et al.,* 2011; Yang *et al.,* 2012). Among domestic animals, abortion due to *T. gondii* in goats and sheep and high mortality rates in swine populations are widely noted (Dubey and Lindsay, 1996; Dubey, 2009). Mortality due to *T. gondii* infection is more common in young pigs than in adult pigs (Hill *et al.,* 2005). Pigs infected transplacentally

with *T. gondii* may be born premature, dead, or weak, or may die soon after birth (Lindsay *et al.,* 1999). Recent report from Korea has also demonstrated higher abortion rates, (up to 44%) and unusually high sow mortality rates, (up to 19%), that were primarily associated with toxoplasmosis (Kim *et al.,* 2009). Even though, the role rodent as a reservoir for *T. gondii* has not been yet studied in domestic animals including pigs in Ethiopia, the absence of rodent control in pig farms in this study might be an important factor for the transmission of pig toxoplasmosis. Kijlstra *et al. (*2008) suggested that rodents can act as a reservoir for transmission of *T. gondii* and inadequate rodent control is considered to play a key role in *T. gondii* infection of pigs.

5.3. Seroprevalence

In the present study, the overall prevalence of *T. gondii* infection was found to be 32.7% using MAT. Even though, no reports of toxoplasmosis from pig have been made in Ethiopia, the results of the present study was much higher than reports from Brazil (12.5%) using the indirect fluorescent antibody test (IFAT) (Samico Fernandes *et al.,* 2012), Central Italy (16.14%) with IFAT (Veronesi *et al.,* 2011), Serbia (9.2%) with MAT (Klun *et al.,* 2011), Southern Italy (16.3%) (Villari *et al.*, 2009) using Enzyme-Linked Immunosorbent Assay (ELISA) and Portugal (15.6%) with MAT (Sousa *et al.,* 2006). In contrast, the present result was lower than the report from Egypt (56.6%) using MAT (El Moghazy *et al.,* 2011). However, this result was comparable with the reports from Northwestern Taiwan (28.8%) (Fan *et al.,* 2004), South-west China (30.6%) (Wu *et al.,* 2012), Peru (32.3%) (Suar´ez-Aranda *et al.,* (2000), Netherlands (31%) (Van Knapen *et al.,* 1995) and in Switzerland (32%) (Wyss *et al.,* 2000).

The difference in seroprevalence between the present study and the aforementioned reports might be associated with the type of serological technique employed (Huang *et al.,* 2010), cut-off values used, sample sizes and sampling techniques (Halova *et al.,* 2012), climatic variations, density of felines and rodents control in the farms (Heidari *et al.,* 2013; Gharekhani, 2013). The high seroprevalence of *T. gondii* in the present study might be associated with farm management system, and access of free roaming cats to pig farms. According to Arko-Mensah *et al.* (2000) the main possible risk factors influencing the prevalence of antibodies against *T. gondii* in Ghana was the age of the animals, breed, environmental conditions, and management practices.

Among study areas pigs from Addis Ababa Kolfe Keraniho sub-city origin were found to be with the highest seroprevalence of *T. gondii* infection. The likely reasons for differences in seroprevalence among study areas might be associated with the environmental temperature. Kolfe Keraniho sub city of Addis Ababa is characterized by warm and moist agro climate compared to the midhighlands of Ambo and the hot and arid climate in the lowland of Adama and Bishoftu. The influence of the environment on the epidemiology of toxoplasmosis has been well documented (Tenter *et al.,* 2000; Dubey, 2004). It has been suggested that a warm and moist climate is associated with a high prevalence of *T.gondii* infection than hot dry ones (Dubey, 2010). This is due to the fact that the oocysts of *T. gondii* survive and sporulate better under moist and humid environment (Villari *et al.,* 2009) where as dry climate has an deleterious effect on the persistence and dissemination of *T. gondii* oocysts which inturn likely to decrease the chance of oocyst survival, generally resulting in a low prevalence of toxoplasmosis (Dubey, 2010).

5.4. Risk factors analysis

Even though, in our study, the seroprevalence of toxoplasmosis was not significantly associated with presence of cats in the pig units/ farms, all pig farms in the study area are accessed either by domestic or feral cats. Domestic cats don't have separate housing system and have frequent outdoor access. Feed storage areas are easily accessed by feral and domestic cats leading to the possible contamination of pig feed and environment with oocysts shed in their faeces. This finding was corroborate with the report of Fajardo *et al.* (2013) who suggested that in most cases since cats spending time looking for rats in the feed storage locations, infected cats with *T. gondii* can defecate on the feed and contaminating it with oocysts. Presence of cats is considered as one of the main risk factors for seropositivity in pigs, especially in animals kept in outdoor facilities. This is due to the fact that only few oocysts (one oocyst) are sufficient to produce infection in pigs (Dubey, 2006; Dubey, 2009; Veronesi *et al.,* 2011). Furthermore, most farm workers don't use boots and coveralls or footbaths before entering the stables, thus contributing to the introduction of the oocysts collected from the environment inside the finisher units. This could mask the influence of exposure to cats as risk factor (Veronesi *et al.,* 2011). Therefore, the transmission of toxoplasmosis to pigs might rely on the presence of cats, either feral or domestic, in the pig farm. In America, the presence of cats on the farm was

confirmed to be an important risk factor for seropositivity of *T. gondii* infection in sows (Assadi-Rad *et al.,* 1995; Dubey *et al.,* 1997a; Lehmann *et al.,* 2003).

There is a meager data regarding the epidemiology of *T. gondii* infection in cats of the study area. A recent report showed a very high *T. gondii* seroprevalence (85.4%) and oocyst shedding (22.2%) in feral cats of Addis Ababa, Ethiopia suggesting a high environmental contamination with oocysts of cats which might also serve as source of infection for other animals (Dubey *et al.,* 2013, Tiao *et al.,* 2013). Several studies showed that a high seroprevalence of *T. gondii* infection in pigs in farms with high soil contamination by *T. gondii* oocysts and presence of a high number of cats (Meerburg *et al.,* 2006; Du *et al.,* 2012). Therefore the possible risk factors for pig infection with *T. gondii* in the present study area might be associated with exposure of pig farms to cats.

The final multivariable logistic model showed that the types of pig feed containing animal byproducts and extensive management system were independent predictors of toxoplasmosis in pigs. Studies have indicated that the prevalence of *T. gondii* infection in pigs is influenced by management systems and was as high as 68% in poorly managed non-confinement system and also the prevalence may vary strongly from one locality to other due to difference in certain ecological factors and breeding systems (Gamble *et al.,* 1999). Similar results were observed by Dubey, (2009) who stated that raising pigs indoors in confinement has greatly reduced *T. gondii* infection than that of outdoors and organic farmed pigs. Earlier research done in the Netherlands reported that a significantly higher risk of seropositivity for *Toxoplasma* antibodies in free range pigs than for those on an intensive pig unit (Kijlstra *et al.,* 2004b; van der Giessen *et al.,* 2007). In contrast, study in Switzerland showed that conventional fattening pigs under confined condition and free-range pigs surprisingly exhibited the same prevalence (2.0%; 95% CI: 0.2–7.0%) (Berger-Schoch *et al.,* 2011b). A possible explanation for the high seropositivity of extensively managed pigs in the present study might be related to scavenging in areas contaminated with either cat faecal material containing oocysts, or carcasses containing infective bradyzoites. Moreover, free-roaming behavior in extensively managed pigs favor contact with rodents increasing the probability of ingestion of tissue cysts (Venturini *et al.,* 2004; Thomas *et al.,* 2013).

In this study the type of feed consumed by pig was more likely to be a notable source of *T. gondii* infection as it frequently includes slaughter byproduct which might contain tissue cysts. In addition, feed supplements such as concentrates, forage, household leftover, raw vegetables, and fruits might be contaminated with cat feces containing oocysts.

Although, presence of dogs in pig farms had no significant association with toxoplasmosis in the final model, it was significantly associated with *T. gondii* infection during univariable analysis. Many dogs were seen roaming around pig feed (household leftover, animal byproducts like cow whey, slaughterhouse leftover). These dogs might contaminate pig feed with oocysts of *T. gondii* from cat feces. Although dogs do not produce *T. gondii* oocysts like cats, it has been suggested that outdoor dogs might contribute to *Toxoplasma* transmission in two ways: fur contamination and oocyst reshedding near houses following ingestion of infected cat feaces (Frenkel and Parker, 1996; Lee *et al*., 2008). Other studies also revealed that under the laboratory conditions, dogs have been shown to eliminate viable oocysts after ingesting sporulated oocysts in cat feaces and the fur of dogs that have come in contact with cat faeces may be a vector for transmission of oocysts to humans (Lindsay *et al.,* 1997). Data in US swine operation indicated that it is difficult to eliminate *T. gondii* infection from swine herds which allow cat or dog access to swine facilities (Hu *et al.,* 1997).

Univariable logistic regression analysis of the present study revealed significantly higher seroprevalence (36.2%), (OR= 1.58; 95% CI: 1.02-2.43) in pigs of ≥ 12 months of age compared to those pigs <12 months. Similar finding was reported from elsewhere (Dubey *et al.,* 2002a; Damriyasa *et al.,* 2004; Villari *et al*., 2008; Halova *et al*., 2012). Recent study from Slovakia also identified that the seroprevalence of toxoplasmosis in sows (4.26%) was two times higher than that in slaughter pigs (2.06%) (Turčeková *et al.,* 2013). The relatively higher seroprevalence in pigs of ≥ 12 months of age as compared to pigs of < 12 months of age might be due to the longer contact time of older animals with potentially infected environment containing *T. gondii* oocysts and / or tissue cysts (Villari *et al.,* 2009).

5.5. Bioassay in mice

In the present study, *T. gondii* tissue cysts were isolated from 48% of seropositive pigs in mice which is higher than 31.7% of isolation rate from Iowa, USA (Dubey *et al.,* 1995b) and 25% isolation rate from Brazil (Dos Santos *et al.,* 2005). The present isolation rate is comparable with the findings of Sousa *et al.* (2006) from Portugal (47.5%). The variation in rate of isolation might be due to the difference in the type of bioassay used (cats versus mice) and the number of mice used for bioassay (10 or 2 versus 5 mice), the amount of tissue sampled (50 g versus 100 g). The MAT has been found to be the most specific and sensitive test to detect *T. gondii* infection in pigs (Dubey *et al.,* 1995b; Gamble *et al.,* 2005, Sousa *et al.,* 2006). Further, the variation in the isolation rate of *T. gondii* might also be due to the type of mice used and the strains of *T. gondii* as some strain do not readily produce tissue cyst (Type I) (Sibley and Boothroyd, 1992) or give rise only to small number of tissue cyst burden in mice (Type III) (Gatkowska *et al.,* 2012) which could easily be missed during microscopic examination.

Increased isolation rate of viable *T. gondii* with an increase in anti-*T.gondii* antibody end titer of the pig of this study are consistent with studies conducted in USA which stated that the rate of isolation increase with antibody titre in the pig (Dubey *et al.,* 1995b, 2002a; Dubey and Jones, 2008). However, in our study *T. gondii* was also isolated from two different pooled samples of seronegative pigs (P1 and P2). Other studies also revealed that antibody titre that should be considered indicative of latent infection in pigs is not always certain because viable *T. gondii* has been isolated from seronegative pigs (Dubey *et al.,* 2002a, 2008; Sousa *et al.,* 2006). On the other hand, study done in Canadian market-aged pigs was unable to successfully isolate viable parasite from pigs with low levels of antibody (Gajadhar *et al.,* 1998). These results indicate that either these pigs were recently infected and had not yet developed *T. gondii* antibodies, or that the antibody titres had declined to undetectable levels (Dubey *et al.,* 1995b). On other hand, isolation of *T. gondii* from IgG negative pigs is possible because seroconversion during *T. gondii* infection requires 2 -3 weeks (Denkers, 2003), but tissue cysts containing bradyzoites can be detected within six to seven days following infection with oocysts or tissues cysts (Dubey *et al.,* 1998). Therefore, if pigs were sampled between infection and seroconversion, there might be a chance of getting cyst positive results in mice. High cyst count was observed in mice as the mean weight difference of mice decreases. Previous studies have also indicated

that infection with *T. gondii* regardless of the type was shown always to result in a loss of weight in mice. However, the onset of the response was different depending on the dose and the genotype of *T. gondii*. Infection by clonal type I resulted in an earlier and more pronounced weight loss than observed in mice infected with types II or III. Also, mice infected with types II or III always showed a period of weight gain after initial weight loss, but never reached their pre-infection weight (Herrmann, 2012).

The majority of the isolates recovered from pigs in this study were predominantly avirulent to Swiss Albino mice except one one sample obtained from Bishoftu (pig sample number ALP2) which killed four mice on 28th and 29th day p.i probably associated with intermediate virulent *T. gondii* strains and ingestion of such strains by humans with raw or undercooked pork might lead to more serious toxoplasmsosis (Cook *et al.,* 2000). According to Dubey *et al. (*2002b; 2007b) *T. gondii* isolates differ markedly in their virulence to out bred mice. Further, the avirulent strains were defined as no mortality at any dose, whereas a "low-dose survivability" phenotype was defined by survival time after injection of 100 parasites (Dardé, 2008). Even though, it has been suggested *T. gondii* virulence in mice depends on several factors, including the stage of the parasite, route, dose, types of mice used, host and the strain of the parasite (Dubey *et al.,* 2004a; 2007b).

The observed clinical signs in mice in this study were supported by Kyan *et al.* (2012) who observed that clinical conditions varied across the seropositive mice and include cowlick, depression and forced breathing and most of these symptomatic mice recovered to a normal condition. Study of toxoplasmosis in experimentally infected mice indicated that mice with acute toxoplasmosis were less active, had erectile body hair and seldom took food or water resulting in weight loss and all mice died within 7 days post-inoculation (Waree *et al.*, 2007).

The mean cyst count per brain of mice in the present study (157 ± 188.96) was higher than the previous report (25 mean cyst count per brain) reported by Yunus (2013) from camel at 1/4000 screening dilution of MAT. Similarly this result was higher than (57.4 mean cyst count per brain) and the overall range of mean cyst count per brain (0-352) reported by Tesfamaryam (2013) in free range chicken from Ada'a Liben, Central Ethiopia. This might suggest more isolation rate of *T. gondii* from pigs than chicken and camel.

In the present study the agreement observed between MAT and microscopic cyst detection was almost perfect (Kappa =0.85). Previous studies have indicated that in most animal species, including pigs and wild boars, infection with *T. gondii* is persistent and an antibody response remains detectable for prolonged periods, probably for the lifetime of the animals (Deksne and Kirjušina, 2013). On the other hand, the MAT is safe and does not require species specific conjugate and can be used on any species (Mainar-Jaime and Barberán, 2007). Furthermore, good correlation between the serological detection of antibodies and the isolation of *T. gondii* has been reported previously (Dubey *et al.,* 1996; Dubey *et al.*, 1997b).

In general the widely distributed *T. gondii* infection and isolation rate demonstrated in this study coupled with currently increasing trends of pork consumption in Ethiopia might brings serious consequences of toxoplasmosis particularly in vulnerable groups like pregnant women, very young children and immunodeficient patients such as patients with HIV. Butchers and slaughterhouse workers that handle carcass and organs infected with *T. gondii* are also at risk of getting infection with toxoplasmosis.On the other hand pig carcasses fed either to domestic or feral cats, may also contribute to the disease transmission between animal species and thus indirectly to the humans. Furthermore, though the role of toxoplasmosis with pig abortion is not known in Ethiopia, the occurrence of high prevalence of pig toxoplasmosis in the present study could cause a decrease in reproductive performance of sows which can greatly affect pig production and this warrants further investigations in the pig farms of the country. In this study, there were certain limitations. Although some farms of the study area allow us to take blood samples from live animals at farm level, there were some restrictions in the farms to include animals of all age group and to the abattoir for tissue sample to isolate of the parasite from all study farms. Other limitation was that seroprevalence of pigs under extensive management system might be under estimated due to sampling of few pigs compared to the intensive management system.

6. CONCLUSION AND RECOMMENDATIONS

The study has shown that *T. gondii* infections in pigs are widely prevalent in Central Ethiopia. The potential risk factors identified for *T. gondii* infection include extensive management system and pig feed type containing animal byproducts. The high sero-prevalence rate of toxoplasmosis in pigs is suggestive evidence for the high level of environmental contamination with *T. gondii* oocysts due to free access of cats to premises of pigs. The high seroprevalence and the high isolation rate of tissue cysts from edible organs are suggest that pigs might be a potential source *T. gondii* infection for humans.

Based on this conclusion the following recommendations are forwarded.

- ✓ Strategies to prevent exposure of pigs to *T. gondii* should focus on improvement of management of pigs, proper farm sanitation, feed hygiene, rodent control and prevention of access of cats to pig farms.
- ✓ Feeding pigs with cooked animal byproducts should be practiced.
- ✓ Creating public awareness about toxoplasmosis should be promoted
- ✓ Studies on the role of toxoplasmosis in reproductive performance of sows should be conducted.
- ✓ Further large scale studies should be conducted in order to identify the genotype and population structure of *T. go*ndii strains.

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8. ANNEXES

Annex 1. MAT Procedure

1. Serum samples and control sera (R4&R5) will be diluted in test tubes
    - 1/20: 100ml of serum + 1.9ml of PBS (reconstituted R6)
    - 1/2000:25ml of the 1/20 + 2.5ml of PBS (Reconstituted R6)

2. Pig sera will be located on the report sheet and 25ml of each dilution dispensed into 2 wells provided

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

4. 50ml of R1 antigen suspension diluted to each well

Allow 1 well for the antigen control:

- 2-mercaptoehtanol (0.2mol/l) 25ml
- PBS 25ml
- Antigen (R1) diluted 1/5 50ml

5. Homogenize using the vibrator or kline rotator

6. Cover with a self-adhesive sheet. Leave for 5-18 hours at room temperature away from vibration and drying

7. Perform reading

Readings

Antigen control: sedimentation of the Toxoplasma in button or ring

Positive reaction: (e,g. positive control serum R4 at 1/40) Agglutination of the Toxoplasma in a mat covering about half of the wells base. The mat show slight shrinking around the edges (irregular shape).

Negative reaction: (e.g. Negative control serum R5) sedimentation of the Toxoplasma in buttom or ring

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

Annex 2. Bioassay of pig tissues for *T. gondii* in mice (Dubey, 2010).

First connective tissue and fat from heart tissues was trimmed using nonporous, hard plastic cutting boards and put in plastic bags. Fifty gram (50g) of the myocardium of each pig was chopped on nonporous, hard plastic cutting boards and gently ground in a blender without any fluid after removing connective tissue, fat, auricles and blood. Aqueous saline solution (125 ml, 0.85% NaCl) was then poured into the blender and homogenized for 30 seconds at top speed. To the prewarmed (37 ^{o}C) homogenate 250 ml of freshly prepared, prewarmed (37^{o}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 was added and incubated at 37 ^{o}C in a shaking water bath for 60 min. Then the homogenate was filtered through two layers of gauze and 250 ml of filtered homogenate centrifuged in falcon tube 1,200 X g (3200 RPM) for 10 min. After pouring off the supernatant the sediment was neutralized with 20 ml freshly prepared 1.2% sodium bicarbonate (pH 8.3) and centrifuge at 1200 X g (3200 RPM) for 10 min after mixing with gentle shaking. Following this the supernatant was pouring off and 5-10 ml of saline was added with 7.5ml antibiotic solutions that contain 1,000 units penicillin and 100µg of streptomycin per ml (wait for 30 minutes for antibiotics to act). Finally, the homogenate was inoculated (1ml/mouse) intrapeitonealy (i.p.) into female Swiss albino mice weighing 20-25 g using a 4-cm long 21-23-gauge needle (five mice for each study animal tissues). *T. gondii* stages (tachyzoites, bradizotes) in tissues are killed by water and by heating to 60 ^{o} C , and so blenders , cutting boards and other materials was always cleaned with soap and hot water, then rinsed with cold water and finally with saline before using for the next specimen. The inoculated mice will be 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) will be recorded. The mice will be fed with pelleted feed and municipal chlorinated water ad libitum. Surviving mice will be bled on day 60 after i.p inoculation. Serum samples will be tested for antibodies of *T. gondii* using MAT and brains of all mice will be examined for tissue cysts. The inoculated mice will be considered infected with *T. gondii* when tissue cysts were found in the brains of mice or mice sera reacted positively for MAT (Annex 1). Non-infected mice were kept separately as negative control.

Annex 3. Data collection sheet for monitoring mice infected with heart tissue homogenate of seropositive pigs

SN	Date	Mice Wt (g)	Init Wt (g)	General signs							Neurological signs				Other signs	Remark
				Rough coat	Inappetance	Dullness	Wt loss	Ascites	Arched back	Tachypnea	Circling	Paralysis	Seizure	Lethargy		
1																
2																
3																
4																
5																
6																
7																
8																
9																
10																
11																
12																
13																
14																

Annex 4. Questionnaire survey to investigate potential Risk Factors for *Toxoplasma gondii* infection in pigs

1. General Information:

a. District _____ Kebele_____Name of the farm_____

Owner s name_____code_____

C. Herd Flock: _____No. males_____Females_____

D. Residential place: a. urban ☐ b. semi-urban ☐ c. Rural ☐

H. Do you have cat (s)? Yes ☐ No ☐

I. No. of cats, if present _____

H. How many of the cats are kitten (young) _____

I. Are there feral cats in the area? Yes ☐ No ☐

J. Do you have dogs: Yes ☐ No ☐ Number of dogs _____

2. Level of education:-

	Attendant	Owner
Illiterate	☐	☐
Primary school	☐	☐
Secondary school	☐	☐
Tertiary /university	☐	☐

3. Way of life of cats: a. Totally indoor ☐ b. Totally Outdoor ☐ c. Mixed ☐

4. Cat housing: Do you have separate house (kennel) for your Cat? Yes ☐ No ☐

5. Cat feeding practices: Indicate the type(s) of feed for your cat

a. Raw animal products ☐ b. Cooked animal products ☐

c. Household left over ☐ l. Others, please specify_____

6. Do you know the health risks cats have to owners and community? Yes ☐ No ☐

7. If your answer to question No. 6 is yes, what type of risk?_____

_____

8. Do you know that cats have health risks to other animals? Yes ☐ No ☐

9. If your answer to question No.8 is yes, what type of risk? _____

10. Has there been history of abortion(s) in your herd? Yes ☐ No ☐

11. If yes (question 11) how many of pigs aborted over the last one year? _____

12. What is the approximate age of fetus at the time of abortion?

a. Below 60 days (early gestation) ☐ c. 120 days (late gestation) ☐

b.60 – 120 days (mid gestation) ☐

13. What was the average flock size i.e., females above puberty for pigs_____

14. Has there been stillbirth among your herd? Yes ☐ No ☐

15. Has there been neonatal mortality among your herd? Yes ☐ No ☐

16. Have there been cases of mummification of fetus among your flock? Yes ☐ No ☐

17. Has there been weak births over the previous one year? Yes ☐ No ☐

18. Do you think that there is possibility of contamination of stored animal feed by Cat feces? Yes ☐ No ☐

19. Sanitary system of the farm: Poor ☐ Fair ☐ Good ☐

20. What do you feed your pigs?

a. Forage alone ☐ b. Forage +mineral (salt) + feces of poultry ☐

c. Forage +salt +"atella"+ feces of poultry ☐ d. Forage +concentrate+ feces of poultry ☐

e. Over left food from hotels and others ☐

Other, specify_____

21. Do you think that your pigs have access to dead animal's carcass (chicken, sheep, goats, cattle, horse, mule, donkeys, etc)? Yes ☐ No ☐

22. What is the management system of the flocks?

a. Extensive ☐ b. Semi-intensive ☐ c. Intensive ☐

23. Indicate the water sources for your pigs.

a. Pipe water ☐ b. Wells ☐ . Pond ☐ d. River ☐

24**.** Grazing land:

a. Mountainous ☐ b. Sloppy ☐ c. Plain ☐ Water logged ☐

25. Supplement feeding troughs:

a. No supplement (trough) ☐ b. Concrete feeding trough ☐ .Wooden boxes ☐

d. Plastic trough ☐ e. Other, specify_____

26. Have you encountered pig mortality in your farms? Yes No

27. What do you do with dead pigs? a. Buried ☐ b.Feeding for cats and dogs ☐

c. Home consumption ☐ d. Throw way in the field ☐ e. Feed to pigs ☐

28. Is there any rodent (rat, mice) in or around your farm? Yes ☐ No ☐

29. What do you use to control rodents? a. keeping cat ☐ b. chemicals ☐ c. other__

30. If you use chemicals, mention the type of rodenticide _____

31. Is there any wild feline coming around the farm? Yes ☐ No ☐

32. Mention the type of wild feline? _____