Seroprevalence of *Toxoplasma gondii* infection in dogs at the Chattogram Metropolitan Area, Chattogram

Shaheda Banu

Roll No. 0118/05

Registration No. 519

Session: 2018 – 2019 (January – June)

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Department of Medicine and Surgery

Faculty of Veterinary Medicine

Chattogram Veterinary and Animal Sciences University

Chattogram – 4225, Bangladesh

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examiners will be sent for examination

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(Prof. Dr. Md. Rayhan Faruque) **(Prof. Dr. Himel Barua)**

Supervisor **Co-Supervisor**

(Prof. Dr. Md. Yousuf Elahi Chowdhury)

Chairman of the Examination Committee

Department of Medicine and Surgery
Faculty of Veterinary Medicine

Chattogram Veterinary and Animal Sciences University
Chattogram – 4225, Bangladesh

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Authorization

The work presented in this thesis is entirely my own and I hereby declare that I am the sole author of the thesis entitled 'Seroprevalence of *Toxoplasma gondii* infection in dogs at the Chattogram Metropolitan Area, Chattogram'. I also declare that it has not been previously submitted to any university for the award of a degree.

I, the undersigned, and author of this work, declare that the electronic copy of this thesis provided to the CVASU Library, is an accurate copy of the print thesis submitted, within the limits of the technology available.

Shaheda Banu

DEDICATED TO MY RESPECTED

AND BELOVED PARENTS AND

TEACHERS

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The Author

Table of Contents

List of Tables

List of Figures

List of abbreviations and symbols

CF	Complement Fixation
CI	Confidence Interval
CMA	Chattogram Metropolitan Area
DT	Dye Test
ELISA	Enzyme Linked Immunosorbent Assay
IFAT	Indirect Fluorescent Antibody Test
IHA	Indirect Haemagglutination
LAT	Latex Agglutination Test
MAT	Microscopic agglutination test
OR	Odd Ratio
PAS	Periodic Acid-Schiff
PRTC	Poultry Research and Training Centre
PVM	Parasitophorous Vacuole Membrane
RPM	Rotation Per Minute
SAQTVH	Sahedul Alam Quadery Teaching Veterinary Hospital
UV	Ultra Violet

Abstract

Infection by the protozoan parasite, *Toxoplasma gondii* is wide rife in humans and animals throughout the globe. Transmission takes place principally by intake of raw or undercooked meat that contains parasite cysts or by intake of oocysts excreted in cat faeces, which might contaminate water and meat. Dogs play a crucial role in human infection attributable to their intimate relationship with humans. This study was designed to see the prevalence of infection in dogs at the Chattogram Metropolitan Area (CMA) wherever no such work has been conducted previously. For this study, sixty serum samples were collected from dogs referred to Sahedul Alam Quadery Teaching Veterinary Hospital (SAQTVH) of Chattogram Veterinary and Animal Sciences University (CVASU). The samples were then tested by indirect enzyme-linked immunosorbent assay (ELISA). Epidemiological data were obtained from the pet owners employing a questionnaire. Overall seroprevalence in dogs was found 25% (95% CI: 14.7–37.8) (15/60). Logistic regression and chi square tests were used for evaluating of risk factors. The prevalence is considerably higher in dog with presence of cats (50%) within the house than absence of cats (20%) p = 0.046. The prevalence of infection was considerably high in female (OR=4.34,95% CI, 1.03,18.36; p=0.046) than male. However, no statistically significant association was found with dogs' age, breed, access to outside, deworming, cooked meat, hunting practices and purpose. Overall, the results showed a comparatively high seroprevalence of *T. gondii* infection in dogs at Chattogram metropolitan area and proved association of *T. gondii* prevalence rates with the dogs' sex, presence or absence of cats. The high detection of antibodies of *T. gondii* parasite confirms the dogs in the CMA, which is a potential hazard not just for dogs, however conjointly for public health. Considering disease importance, the current study indicates that *Toxoplasma gondii* is widespread in dogs in CMA which may have important implication for public health.

Keywords: Seroprevalence, *Toxoplasma gondii,* dogs, ELISA

Chapter I: Introduction

Toxoplasma gondii is a parasitic disease widely prevalent in all warm-blooded animals including humans (Dubey *et al.,* 2009). It is zoonotically important because it causes congenital defects or abortion and fatal disease in humans. It also causes different respiratory, alimentary, neurological and muscular problem in conjunction with viral infection and stress (Li, B. *et al.,* 2012). The possible way to infection in human and other animals by consuming tissue cysts from undercooked meat or frozen food or by drinking contaminated water with oocysts shed in cat faeces.

Dogs play a crucial role in the mechanical transmission of *T. gondii* gametocyte to human by swallowing cat's dejection (Frenkel *et al*., 2003). The amount of parasite contamination in the environment may reveal by seroprevalence of infection in dogs (Alvarado *et al*., 2014). Therefore, dogs are considered as sentinel animals for *T. gondii* infection because of their close contact with humans. Generalized infection could occur in dogs beneath one year and is characterised by expulsion, fever, diarrhea, inflammation, icterus, and dyspnoea (Dubey *et al*., 2009). In some cases, clinical infection in dogs may be misdiagnosed as distemper infection because it can infect and cause sickness in immunological disorder patients (Hosseininejad *et al*., 2011).

Dogs are considered as intimate and devoted friends of human. Some reports revealed that the oocysts eaten via contaminated food or water can pass from digestive tract through faeces in dog (Lindsay *et al*., 1997). The presence of dog in house or housing space contemplate as a risk issue for *T. gondii* infection (Sroka *et al.*, 2010). However, it causes visual losses, deaths and high morbidity in fetuses (Frenkal *et al*., 1995) and immunocompromised patients (Passos *et al*., 2000).

The prevalence of this parasite includes a wide variation reckoning on animals, geographic factors, climate, social and cultural habits (Garcia *et al*., 2006). The distribution of *T. gondii* in dogs is worldwide, with prevalence ranging from 20 to 91% in different countries (Ali *et al*., 2003; Azevedo *et al*., 2005; Dubey *et al*., 2007; Silva *et al*., 2002). Clinical infection in dogs is commonly related to immunological

disorder induced by distemper infection (Hosseininejad *et al*., 2011). Clinical manifestations of infection are considerably totally different in every infected dog having neuro-muscular, digestive, respiratory or skin disorders. However clinical symptoms in the mainly seen in respiratory system that is because of reactivation of concealed infection (Hoffmann *et al*., 2012). In one experiment speculated oocysts in dogs' dejection were determined 2 days after ingestion of *T. gondii* gametocyte and is also believed that infected dogs play role in transmission of parasite to their house owners with licking (Schares *et al*., 2005). One of the vital routes of *Toxoplasma* infection in dogs is ingestion raw animal meat that will contain tissue cyst (Alvarado-Esquivel *et al.*, 2014).

The best way of disease confirmation tests like indirect hemagglutination assay (IHA), enzyme-linked immunosorbent assay (ELISA) and indirect fluorescence antibody test IFAT (Carlier *et al*., 1980; Watson *et al*., 1982). However, ELISA shows a reliable sensitivity and specificity in identification of *Toxoplasma* infection (Hosseininejad *et al.*, 2011).

Therefore, the study was designed with following objectives:

- To determine seroprevalence of toxoplasmosis in dogs at the Chattogram Metropolitan area
- To find out risk factors related to toxoplasmosis in dogs

Chapter-II: Review of Literature

2.1. History

Toxoplasma gondii was initially discovered by Nicolle and Manceaux (1908) in tissues of a gnawing animal like rodents, the gundi (*Ctenodactylus gundi*), that was being employed for *leishmania* infection analysis within the laboratory of Charles Nicolle at the biologist Institute in Tunis. The name *Toxoplasma gondii* was coined by Nicolle and Manceaux (1908) supported the crescent form of the tachyzoites (In Greek: *toxo* = arc or bow, *plasma* = type or life). At similar time, Splendore (1908) working in Sao Paulo, Brazil, discovered an identical parasite in rabbits mistakenly distinctive as *Leishmania*, however he didn't name it (Dubey, 2008). The medical importance of *T. gondii* remained unknown till 1939 and it was known in tissues of a congenitally infected child presenting with the classic triad of symptoms, specifically hydrocephaly, retinochoroiditis and intracranial calcification (Dubey, 2008; Innes, 2010). The veterinary importance of *T. gondii* became known when it was found to cause abortion storms in sheep in 1957 (Dubey, 2008).

2.2. Biology of *Toxoplasma gondii*

Toxoplasma belongs to the phylum *Apicomplexa*, that includes intracellular parasites which have a commonly polarized mobileular shape and a flowery cytoskeletal and organellar association at their apical part (Dubey *et al*., 1998). Several different protozoan parasites of medical and veterinary or economic importance exist among the phylum *Apicomplexa*, with variable degrees of biological similarity to *T. gondii.* Within the taxonomy of *Apicomplexa*, *Toxoplasma gondii* is categoryified into class Coccidia, order Eimeriida, and family Sarcocystidae. Different members of this phylum embody human pathogens (*Plasmodium*: the explanation for protozoal infection, Cryptosporidium: animal parasite and an expedient microorganism of humans, *Babesia, Cyclospora, Isospora*), and animal pathogens (*Eimeria*: the tributary agents of chicken infestation, *Theileria:* tick-borne parasites of bovine, *Neospora*: major cause for 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*

2.2.1. Tachyzoites

The tachyzoite is usually crescent shape and is just about a pair of 2 μm x 6 μm, the scale of a red blood corpuscle. The anterior part of the tachyzoite is pointed and therefore the posterior part is spherical (Hill *et al*., 2005). It has a subpellicular microtubules, a polar ring, a conoid, rhoptries, micronemes, mitochondria, endoplasmatic reticulum, cyst, ribosomes, rough surface endoplasmatic reticulum, micropores and a centrally set nucleus (Figure 1). The conoid, the rhoptries and therefore the micronemes area unit characterized a structure of this parasitic type. The pellicle consists of 3 membranes. The inner membrane is discontinuous in 3 areas, at the polar ring (anterior), at the micropore (lateral) and therefore the posterior end. The polar ring is associate in osmiophilic thickening of the inner membrane at the anterior part of the tachyzoite. The round shape is found at the polar point. It's a cylindrical cone that consists of six to eight fibrillary components organized sort of a compressed spring. This structure is perhaps related to the penetration of the tachyzoite through the membrane of the host cell. Terminating among the round shape area unit the rhoptries. These area unit four to 10 secretory organ like structures with associate in anterior slim neck and posterior-sac-like part of reaching as so much because the nucleus. Every compartment has its' own complement of proteins whose operate is in keeping with the temporal arrangement of them unharness: microrems release their contents early throughout the attachment-invasion method and so the rhoptries area unit discharged as invasion income. Finally, the dense granules discharge their

contents throughout and when the formation of the PV, modifying the PV surroundings for animate thing survival and replication of the parasite and this happens once invasion is basically complete. Once the parasite has connected to the host cell, their contents area unit discharged through the round shape. The micronemes area unit tube like structures at the anterior end of the organism. They're sometimes fewer than a hundred placed at the conoidal endpoint of the parasite and also are concerned in invasion of the host cell. The micropores area unit sites specialised for the uptake of nutrients through endocytosis. After access into the host cell, the parasite is surrounded with the aid of using a parasitophorous vacuole membrane (PVM) (Black and Boothroyd, 2000, Saleh, 2006). The PVM provides a secure surroundings for the tachyzoites to multiply because it is resistant against acidification and lysosomal fusion. Generation time of tachyzoites is 6 to 8 h (in vitro) and therefore the parasites exit the cell, sometimes when 64 to 128 parasites have accumulated per cell (Radke and White, 1998).

This stage features a high rate of multiplication associate needs an animate thing environment to survive and multiply. Tachyzoites enter the cells by direct penetration or by body process and multiply by endodyogeny among the host cell. The tachyzoites area unit associated to the acute part of infection, throughout that they invade host cells. when invasion, the tachyzoite still divide till the host cell is full of parasites and type rosettes. Cell protoplasm becomes full of parasites resulting in cell disruption, upon that the discharged tachyzoites invade contiguous cells by active invasion of the host semipermeable membrane or by body process (Hill *et al.*, 2005; Sousa, 2009).

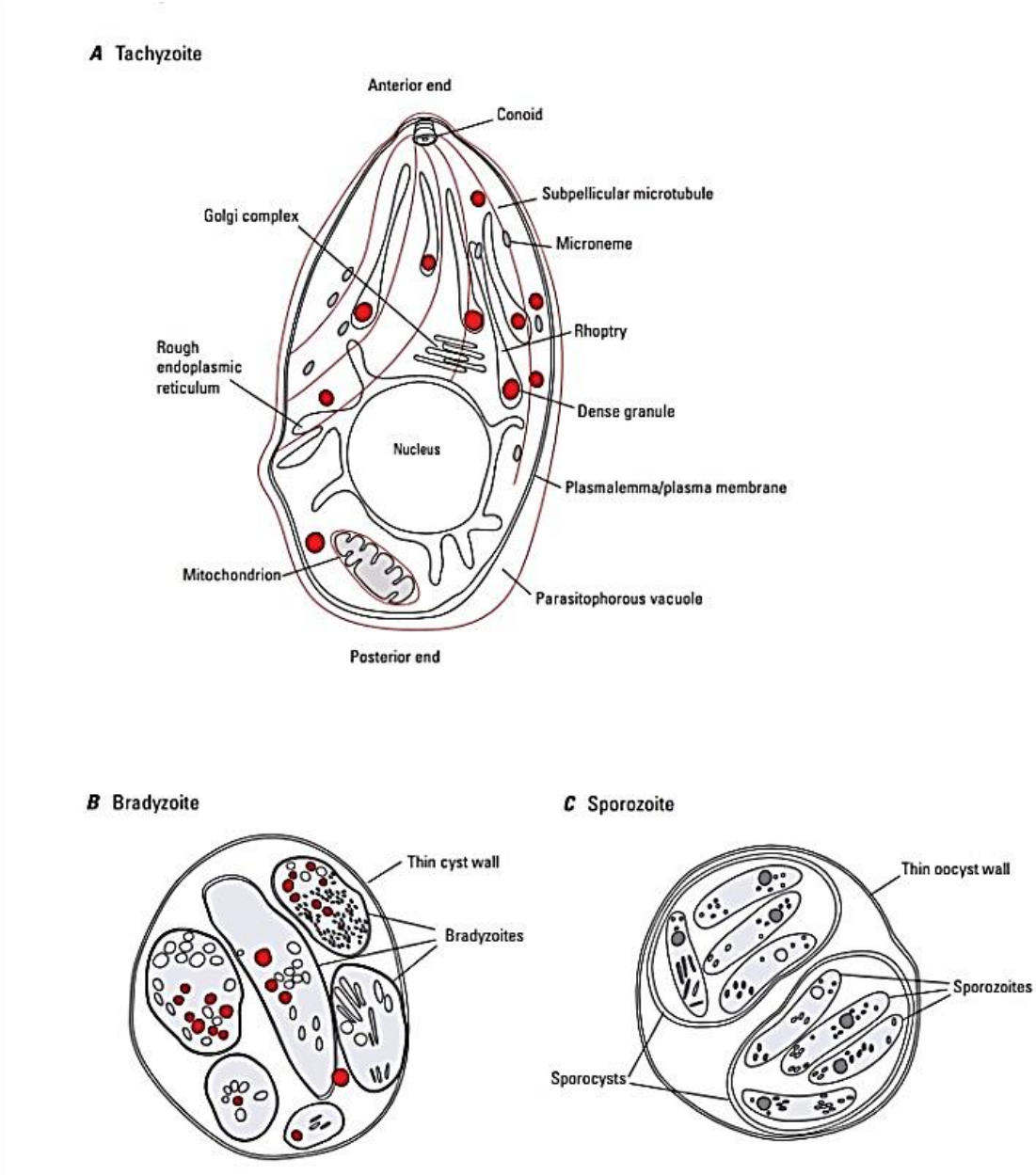


Figure 1**.** Infectious stages of *T. gondii*: *A*, tachyzoite, *B*, bradyzoite, and *C*, sporozoite. (Ajioka *et al.*,2001)

2.2.2. Bradyzoites and tissue cyst

Bradyzoites result from the conversion of tachyzoites into a slow-dividing stage and form tissue cysts (Figure 2). These cysts unit heaps of or less ellipsoid of revolution in brain cells or elongated in muscular cells. They vary in size from 10 μm for the younger cysts, containing exclusively two bradyzoites, to up 10 μm to 100 μm for the older ones, containing voluminous or thousands of densely packed bradyzoites (Robert-Gangneux and Dardé, 2012). Although tissue cysts may develop in visceral organs,

including lungs, liver and kidneys, they seem to be a heap of prevailing inside the neural and muscular tissues including the brain, eyes, skeletal and cardiac muscles. Intact tissue cysts possibly do not cause any injury and may persist for the period of time of the host whereas not inflicting a bunch inflammatory response (Dubey *et al*., 1998). Bradyzoites appear structurally nearly like tachyzoites by light-weight analysis, but ultra-structurally bradyzoites have a heaps of posterior nucleus, heaps of cellular rhoptries than tachyzoites, and contain amylopectin granules (Weiss and Kim, 2000) (Figure 1). A selected morphology of bradyzoites is that their parasitophorous cavity becomes thickened forming the tissue cyst wall. The cyst wall is rich in sugar and stains with varied lectins (Zhang *et al*., 2001). They are slender and fewer prone to destruction by chemical process than tachyzoites. Bradyzoites have a latent metabolism, well-tailored to long survival. The death of the host cell may trigger the disruption of the cyst wall and so the sequent liberation of bradyzoites. The resistance of bradyzoites to the acid accelerator (1- 2 hrs survivals into pepsin-HCl) permits their transmission through consumption. Intact tissue cysts possibly do not cause any injury and cysts remain intracellular throughout their life in the host (Dubey *et al*., 1998; Hill *et al*., 2005; Robert-Gangneux and Dardé, 2012). Bradyzoites is discharged from tissue cysts to form tachyzoites again, inflicting a reactivated infection in upset hosts (Montoya and Liesenfeld, 2004). Modifications of the environmental hydrogen ion concentration, shifting the temperature, IFN-γ treatment, or the inhibition of the mitochondrial metastasis chain induces transition from the tachyzoite to the bradyzoite stage (Tomavo, 2001). Oocyst consumption by host will even result in bradyzoites formation and to alittle extent with tachyzoites or contaminated meat with tissue cysts. It's 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).

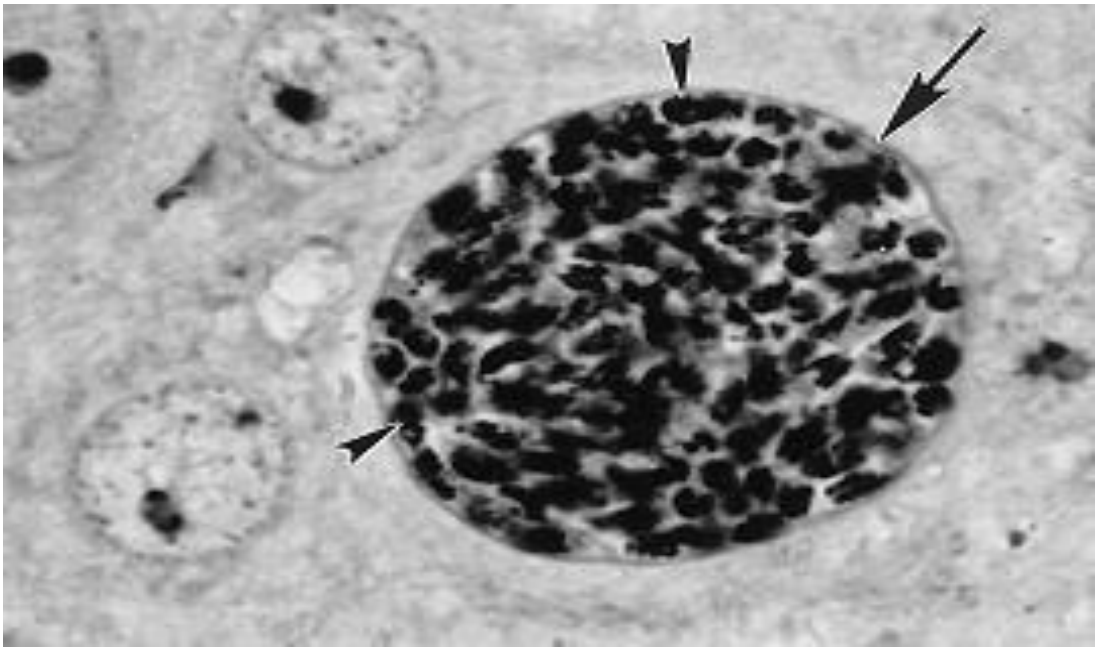


Figure 2. Tissue cyst with numerous periodic Acid-Schiff (PAS) positive bradyzoites (arrowheads) enclosed in a (PAS) negative cyst wall (arrow). Source: (Dubey *et al.,*1998).

2.2.3. Oocyst

Unsporulated Oocysts are subspherical to spherical with 10 μm x12 μm in diameter (Figure 3). Sporulated oocysts are subspherical to ellipsoid with 11 μm x13 μm in diameter. Each sporulated oocyst contains two sporocysts measuring 6 μm x8 μm. Each sporocyst divides into four sporozoites, live 2 μm x 6 μm to 8 μm. Thus, the sporulated oocysts contain eight sporozoites. Oocyst wall of sporulated oocysts embody three layers. One electron-dense outer layer, associate degree electron-lucent middle layer and a moderately electron-dense inner layer (Dubey *et al*., 1998). Oocysts sporulation depends on the temperature and oxygen and will take 1 to 21 days. Sporulation takes place in 2 to 3 days at 24ºC, 5 to eight days at 15ºC and 14 to 21 days at 11ºC. Sporulated oocysts of *T. gondii* are resistant to environmental conditions. They survive short periods of cold and dehydration and keep infectious in wet soil or sand for up to eighteen months. They are extraordinarily impermeable and, therefore, are also very resistant to disinfectants (Petersen and Eaton, 2000). Ultraviolet (UV) treatment is effective 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)

2.3. Life cycle of *Toxoplasma gondii*

Toxoplasma gondii is transmitted in numerous ways and its life cycle is commonly delineated as ‘complex’. However, finishing the life cycle isn't necessary for its existence. *T. gondii* reproduces each asexually and sexually, lies dormant within the hosts beneath the management of the hosts’ immune responses, and survives within the surroundings (Jokelainen, 2013). Life cycle of *T. gondii* includes definitive and intermediate hosts. The sexual and vegetative cycle of the parasite will ensue in enteral vegetative cell of the cat (definitive host), however within the host solely vegetative cycle takes place (Dubey, 2008).

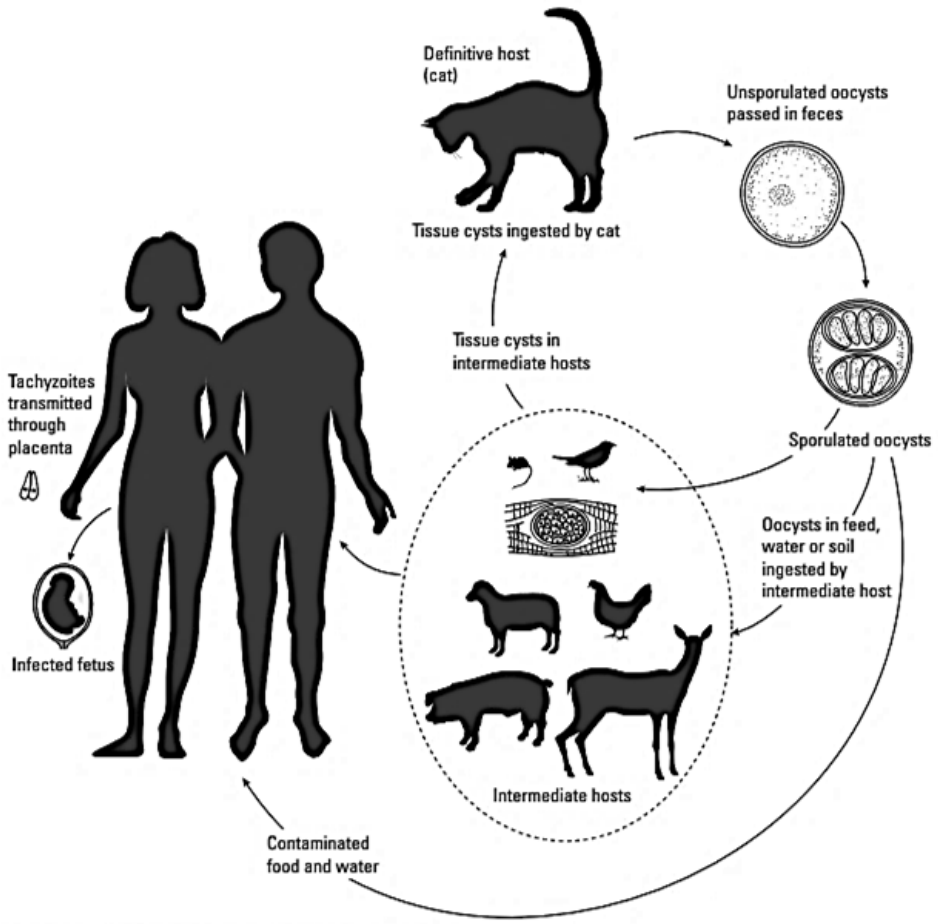


Figure 4. Life cycle of *T. gondii.* (From Dubey and Beattie, 1988)

2.3.1. Life cycle in the intermediate hosts

Toxoplasma gondii contains a heteroxenous biological life cycle and may nearly infect all species of warm-blooded animals (mammals and birds), beside humans as intermediate hosts and felines as final hosts (Dubey *et al*., 2004; Dubey *et al*., 2007). Once host ingests oocysts, sporozoites unit free into the gut lumen and join up with the gut tissue to enter cells at intervals the plate propria. Simply just in case academic degree host ingests tissue cysts, the free bradyzoites behave equally to sporozoites at intervals that upon activity every sporozoites and bradyzoites invade the internal organ tissue, differentiate into the rapidly growing tachyzoite and disperse throughout the body. Every sporozoites and bradyzoites transform into tachyzoites that enter variety cell where they divide rapidly until the cell bursts (Weiss and Kim, 2000; Opsteegh, 2011). Tachyzoites can infect nearly any cell organ cell type, although a response

positive as shooting cell types (for instance retinal tube animal tissue cells) has been reported (Smith *et al*., 2004). Once offensive a cell, the tachyzoites can divide and once death of the host cell they go to invade adjacent cells or once traveling through the blood stream attach to cells elsewhere at intervals the body. The tachyzoite stage can rework into a slowly dividing bradyzoite (Klaren and Kijlstra, 2002) as a results of as immunity develops, replication of tachyzoites decreases and tissue cysts develop that do not commonly have host reaction (Saavedra, 2003).

2.3.2. Life cycle of *Toxoplasma gondii* in definitive hosts

Cats and wild felids are the only definitive hosts which can pass oocysts with their faeces and play a vital role in epidemiology of infection (Bayarri *et al*., 2012). Although, domestic cats play the foremost important role at intervals the dissemination of *T. gondii*. Cats acquire the infection in 1 or 2 main ways: via consumption of cysts contaminated organs or tissues of a chronically infected host prey or via activity of oocysts within their food or water. Once activity of tissue cysts, the cyst wall is eatable by stomachic acid, juice and lytic enzymes of the upper gastrointestinal tract, that ends in bradyzoites and additionally the free bradyzoites, invade the internal organ tissue. Besides general dissemination once conversion to the invasive tachyzoite stage, some organisms at the tissue of host endure five fully completely different organic process stages that reproduce asexually by endodyogeny, where two daughter cell created at intervals one and by schizogeny to differentiate into little and macro gametocytes within a pair of days of infection and involving the formation of multiple protozoan cells around a previously divided nucleus. The gametes fuse to make a cell, that later secretes a cyst wall to rework oocysts. Oocysts rupture the internal organ tissue cells to disperse into the lumen and plenty of millions unit excreted for days or weeks. Oocysts endure reproduction outside of the body to become infective to completely different hosts (Dubey *et al*., 1998; Dabritz and author, 2010). The persistence of oocysts at intervals the setting can increase the probability of transmission to humans or animals. Cats with infection sometimes show no signs of the ill health (Elmore *et al*., 2010). The shortest pre patent quantity, i.e. the time from infection until the shedding of oocysts is 3 to 10 days once activity of tissue cysts. The pre patent quantity is 13 days or extra once overwhelming tachyzoites and eighteen days or extra once oocysts activity (Dubey, 2008). The cats shed oocysts once tissue cysts activity whereas however 30-

50% of cats shed oocysts once activity of tachyzoites or oocysts (Dubey *et al*., 2009). The prevalence of *T. gondii* infection in cat populations not entirely rely on the provision and create contact with infected prey species, where infection levels rely on access to and activity of oocysts and infected tissue but jointly transmission of the infection via a congenital route. Once a primary infection cats become immune, and do not generally discharge oocysts over again if re-infected. This immunity can keep for up to 6 years and some cats can shed oocysts over again if re-infected. However, entirely in smaller amounts than once the primary infection (Dubey, 1995).

2.4. Transmission

It was not until the invention of the Sabin-Feldman dye test that the life cycle and transmission routes of *T. gondii* were able to be explored (Weiss and Dubey, 2009). The invention of this sensitive and specific test allowed researchers to research the characteristics of the parasite in humans and variety of animals. This prompted a lot of investigations into the potential transmission routes of *T. gondii*. It's presently known that the wide unfold distribution of *T. gondii* are going to be attributed to the various mechanisms of transmission that *T. gondii* are going to be transmitted horizontally and vertically. Horizontal transmission can arise from tissue cysts of intermediate hosts, the oocysts contaminated water, soil and organ transplants and blood transfusions (Martin, 2001; Montoya and Liesenfeld, 2004). Vertical transmission happens once tachyzoites unit transmitted across the placenta during gestation period of affeceted female. Possibility of horizontal and vertical transmission routes reported in different species including dogs, cats, grey kangaroos and humans (Al-Qassab *et al*., 2009; Parameswaran *et al*., 2009; Powell *et al*., 2001; Wilson *et al*., 1980).

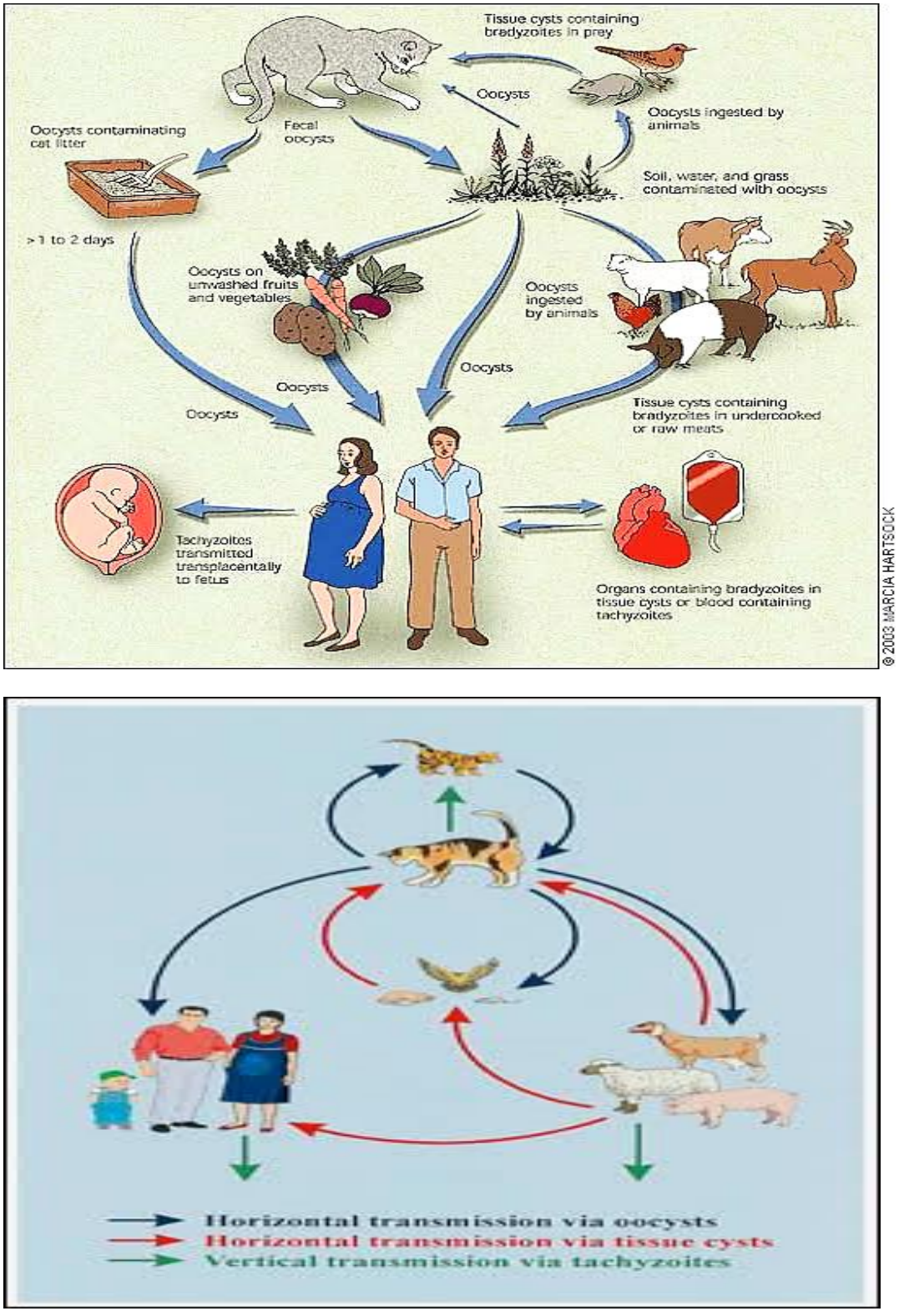


Figure 5. Pathways for *Toxoplasma gondii* infection [Adapted from: Jones *et al.*, 2003 and Tenter *et al.*, 2001]

2.5. Risk factors for toxoplasmosis

Seroprevalence of *T. gondii* is extremely variable among different geographic regions. Even for identical continent, deep variations area unit typically found for the assorted countries. Several reasons may explain this reality such as: diet, preparation of food, hygiene, environmental conditions, host population (wild and domestic Felidae) and completely different laboratory techniques used for sero-diagnosis (Sousa, 2009). The variation in climate contains a marked influence on the environment of *T. gondii* as an example, an elevation in shut temperature and precipitation can modify the soil humidity, so as that the sporulated oocysts persist for an extended time viable inside the wet setting (Meerburg and Kijlstra, 2009). Prevalence in very little ruminants is usually really high because of the continual contamination of pastures (Cenci - Goga *et al*., 2013). This might reflect epidemiologic factors like different types of confinement, hygiene of stables and different types of feed. Against this, seropositivity is usually high in dogs, indicating their continuous exposure to a natural setting and additionally the additive impact age. All of these animals may harbor a considerable number of tissue cysts in their organs, alongside skeletal muscles, and so have importance in food-borne transmission to humans via consumption of meat (Tenter *et al*., 2001).

2.5.1. Age

It has been reported that age of the animals was positively associated with the seroprevalence of toxoplasmosis. Adult animals had comparatively higher prevalence of toxoplasmosis infection compared to young animals (Ramzan *et al*., 2009; Rossi *et al*., 2011; Ahmad *et al.*, 2015). It is due to exposure of adult animals to the risk factors for longer period of time than the younger ones (Van der Puije *et al*., 2000).

2.5.2. Gender

It has been reported that female is more susceptible than males to toxoplasma infections (Alexander and Stimson, 1988; Ahmad *et al*., 2015). It may be explained by the fact that immunity in females is reduced by various factors such as pregnancy, nutrition and lactation (Messingham *et al*., 2001). Although there are so many published reports they reported that there was no significant correlation between toxoplasma infection and the gender of the animals (Cavalcante *et al*., 2008; Gebremedhin and Gizaw, 2014).

2.5.3. Cat presence

Cats are the definitive hosts of the parasite and play a vital role in infecting other animals by shedding oocysts in the environment (Lopes *et al*., 2010). Presence of cat specially the free roaming cats increase the risk of transmission of the infection in animals. Cats increase the oocysts load on nearby pastures resulting in contamination of environment. These oocysts when ingested along with food and water result in postnatal infection. There were so many reports that presence of cat play vital role in transmission of toxoplasmosis in animal (Lopes *et al*., 2010; Ahmad *et al*., 2015).

2.5.4. Raw or undercooked meat consumption

An association between raw meat consumption and *T. gondii* seropositivity (Mengesha *et al*., 1984; Masresha, 2012; Walle *et al*., 2013) was reported, but species of the animal used as source of raw meat was not mentioned (Negash *et al*., 2008) and Yibeltal (2008) reported consumption of raw or undercooked mutton as risk factors. Raw or undercooked beef consumption was also associated with a high IgG seroprevalence (96.77%) of Toxoplasma infection in a study on 279 abattoir personnel in Addis Ababa (Yimer *et al*., 2005).

2.5.5. Climate

A significantly higher seroprevalence of toxoplasmosis was reported in warm humid climatic zones as compared to drier areas (Guebre-Xabier *et al*., 1993).

2.6. *Toxoplasma gondii* detection methods

Clinical symptoms of infection are non-specific and are not sufficiently characteristic for a definite diagnosis of *Toxoplasma*. The direct demonstration of *Toxoplasma* tachyzoites in cerebral tissues is the technique for definitive detection of cerebral infection (Pereira -Chioccola *et al*., 2009). Serodiagnosis can be a useful and adequate tool to diagnose *Toxoplama* infection in every man and animals (Hashemi-Fesharki, 1996). Serodiagnosis is foremost commonly used technique for clinical designation of infection. Serologic tests such as Sabin--Feldman dye test (DT), complement fixation (CF), indirect fluorescent antibody test (IFAT), indirect hemagglutination (IHA), Enzyme linked immunosorbent assay (ELISA) and agglutination (AG) tests used for the detection of Toxoplasmosis.

Bioassay involves the inoculation of animals or cell cultures with suspected *T. gondii* infected tissue (s) or suspected *T. gondii* oocysts (Dubey and Lappin, 1998). Both mice and cats are natural hosts of *T. gondii* and they can serve as a biological incubator to produce a large number of parasites to ease the identification of infection. Molecular methods rely on PCR for the specific detection or analysis of *T. gondii* DNA (Su *et al*., 2010). Molecular methods have become indispensable and reliable tools (Williams and O'Donovan, 2009; Su and Dubey, 2010), not only in the diagnosis but also in the understanding of the epidemiology of *T. gondii* (Su and Dubey, 2010). Restriction Fragment Length Polymorphism (RFLP) analysis of specific genetic loci has been widely used for *T. gondii* genotyping. RFLP markers are amenable to high throughput analysis using PCR amplification, followed by restriction digestion and gel electrophoresis (Sibley *et al.,* 2009), which is extremely valuable for performing population surveys. All of the above methods sequence--based methods provide the best approach for detecting polymorphisms in new isolates or from previously unsampled populations (Sibley *et al.,* 2009). The obvious disadvantage of sequence based typing is its high cost and the need for access to sophisticated technology (Sibley *et al.,* 2009).

2.7. Control and prevention methods

Control of *T. gondii* infection in animals depends on management, legislation, hygiene and vaccination. All feed and water need to be free from soiling as far as possible. Completely different measures to cut back environmental contamination by oocysts need to be aimed toward reducing the amount of cats capable of shedding oocysts. These include indoor keeping of cats, prevent hunting, feeding only canned or dried food or well-cooked meats (Frenkel, 1974).

2.8. Prevalence in Dogs

Toxoplasma gondii prevalence in dogs has been recorded in world wide. In Pakistan seroprevalence was 78.5% in stray dogs and 34.6% in owned dogs (Ahmad *et al*., 2001). In Iran, a seroprevalence of 31.2% in stray dogs and 9.03% in owned dogs was reported by (Hosseinininejad *et al*., 2011). In Korea, the prevalence of antibodies in stray dogs with owned dogs, found ensuing proportion in stray dogs 18.5% than in owned dogs 5.1% (Nguyen *et al*., 2012). Different researchers, although not scrutiny two population of dogs (stray vs. owned) reported high positivity in stray dogs 51.3%

in Turkey (Hosseinininejad *et al*., 2011) and 40.3% in an urban rural gradient in China; 67.4% in Sri Lanka (Dubey *et al*., 2007); 38.0% in Portugal (Lopes *et al*., 2011). 50.5% in Brazil (Dubey, 2008) and 16.8% in Republic of Colombia (Hosseinininejad *et al*., 2011). An occasional seropositivity 10.81% has been found in own dogs in Northwest China (Wu, *et al*., 2011); 3.50% in Southwestern China (Liu, Q. *et al*., 2012) and 25.9% in Czech Republic (Oncel *et al*., 2007). High prevalence (25%) of *T. gondii* infection in dogs was found in Nigeria (Kamani *et al*., 2010). Seroprevalence in Greneda, West indies was 25% in dogs (Sharma *et al*., 2014).

Chapter III: Materials and Methods

3.1. The study area

Chattogram is one of the largest cities of Bangladesh located 22º22'0"N and 91º48'0"E. The tropical monsoon climatic condition characterizes by annual average temperature of 13ºC to 32ºC, humidity of 70-85% and rainfall of 5.6 mm to 727.0 mm (Source: Bangladesh Meteorological Department, www.bmd.gov.bd).

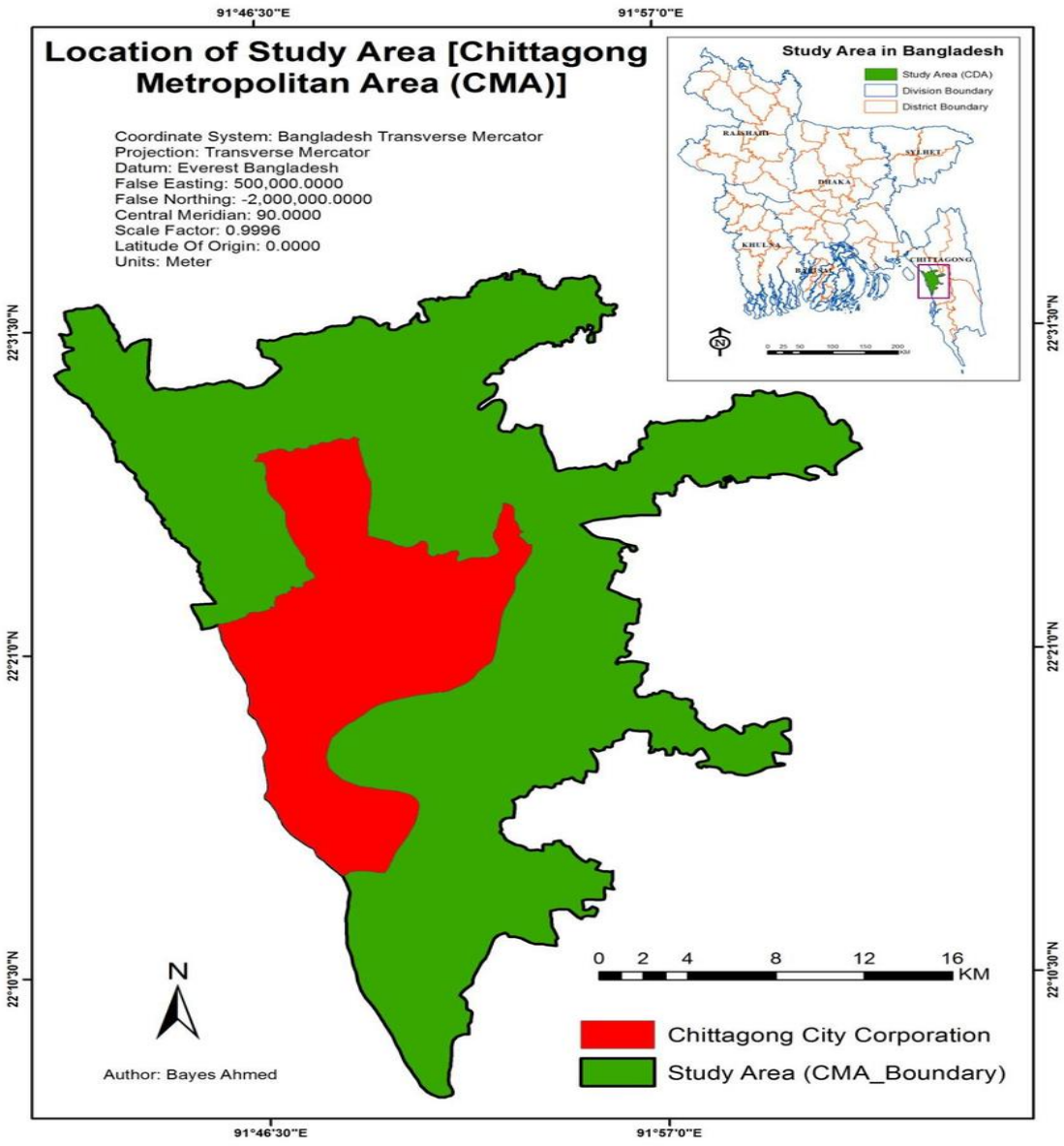


Figure 6. Chattogram metropolitan area [www.researchgate.net/profile/figure/fig4]

3.2. Study Animals

In this study, a total of 60 dogs (healthy=20 and sick=40) from completely different places of Chattogram metropolitan area, visited to SAQTVH (Sahedul Alam Quadery Teaching Veterinary Hospital), CVASU were tested for the presence of *T. gondii* infection. The study was conducted throughout the month of February to April 2019.

3.3. Questionnaire survey

A form for owners of dogs was designed to gather information for risk factors analyses. Data enclosed signalment (age, sex, breed, deworming), surroundings (outdoor access, presence of cat) and diet (raw meat, cooked meat). (Appendix- I).

3.4. Blood collection and sera separation

Approximately 3 ml of blood was collected from the cephalic vein by disposable vacutainer tubes and needles (BD Vacutainer Systems, Plymouth, UK). Samples were tagged and left for a number of hours to clot. The samples were then centrifuged at 4000 RPM for 5 minutes. The sera were collected in 1.5 ml Eppendorf tubes (Eppendorf-AG, Hamburg, Germany) and transported to the PRTC (Poultry Research and Training Centre), CVASU with the ice box and keep at -20°C till tested.

3.5. Serological examination (ELISA)

The enzyme-linked immunosorbent assay (ELISA) was done using ID screen Toxoplasmosis Indirect multi-species ELISA kit in keeping with the producer's directions (ID Vet, Grabels, France). The protocol is following:

1. Allowing all reagents to come room temperature ((21^0 C ± 5^0 C) and homogenize by vortex or inversion
2. Adding:
    - 90 μl of dilution buffer 2 in every small well
    - 10 μl of the negative control to wells A1 and B1
    - 10 μl of the positive control to wells C1 and D1
    - 10 μl of every sample to be tested to remaining wells
3. Incubation 45 min ± 4 min at 21^0 C (± 5^0C)
4. Emptying the wells and wash 3 times with 300 μl of wash solution (1X)

5. Preparation of conjugate 1X by diluting the concentrated conjugate at 10X to 1/10 in dilution buffer 3
6. Adding 100 μl of the conjugate 1X to every well
7. Emptying the wells and wash 3 times with 300 μl of wash solution
8. Adding 100 μl of the substrate solution to every well
9. Incubation 15 min ± 2 min at 21^0 C ($\pm 5^0$ C) in dark
10. Adding 100 μl of stop solution to each well
11. Reading and recording O.D. at 450 nm

Serological tests were done in serologic laboratory, PRTC, CVASU. For interpretation of the result S/P% was calculated as: S/P% = (OD 450 value of the sample - OD450 value of the negative control) / (mean OD 450 value of the positive control - OD450 value of the negative control) x 100. Any samples with S/P less than or equal to 40% were considered as negative, the samples with S/P between 40% to 70% were thought as suspected and samples with an S/P larger than or equal to 70% considered as positive.

3.6. Data management and analysis

Data generated from questionnaire survey and laboratory investigations were recorded and coded by Microsoft Excel spreadsheet (Microsoft Corporation) and using STATA version 14.0 for Windows (Stata Corp. College Station, USA). Univariable and multivariable logistical regression models used to know relation of the potential risk factors. Potential risk factors enclosed within the univariable models were selected based on the prevailing literature (Dubbey *et al.,* 1988; frenkel *et al.,* 1995). The seroprevalence was calculated as the number of seropositive samples divided by the total samples tested. The association of the potential risk factors (Age, sex, breed, presence of cat, access to outside, deworming, cooked meat, hunting practice, purpose of use) were analyzed by univariable logistical regression. Variables with P-value $\leq$ 0.25 in univariable logistical analysis were enclosed within the final multivariable logistical regression model (sex, presence of cats, deworming, cooked meat). Results were thought to be significant at P-value ≤ 0.05.

Chapter IV: Results

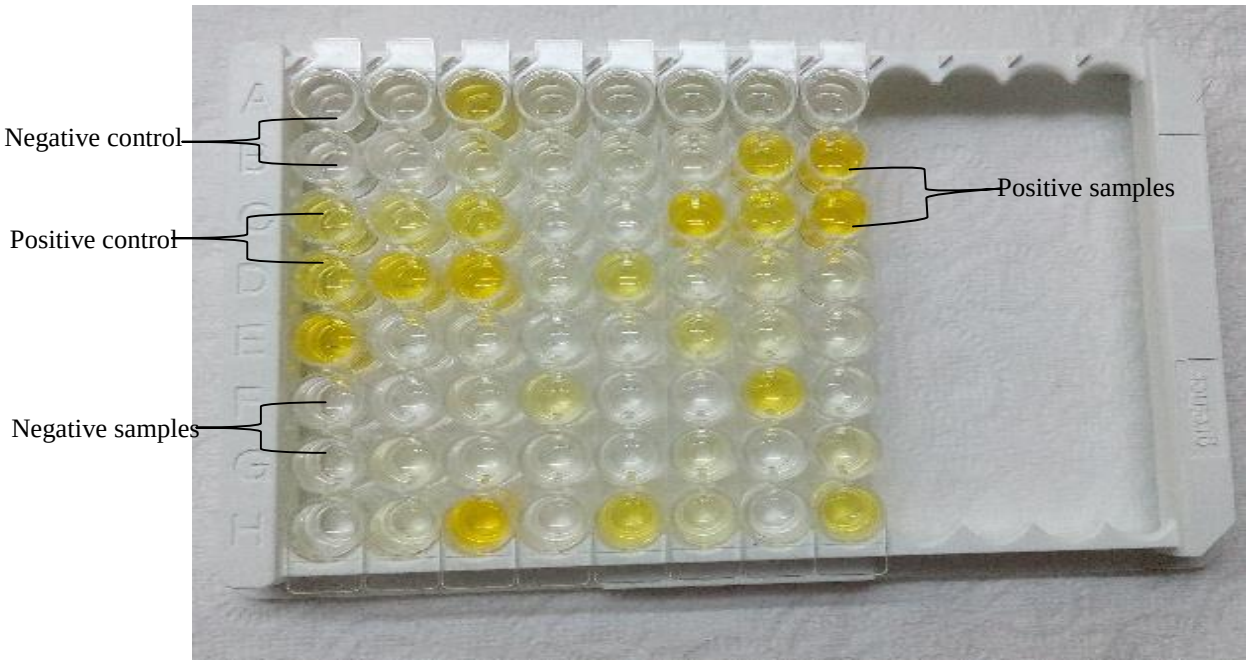
4.1. Seroprevalence of *T. gondii* infection

An overall seroprevalence of *T. gondii* in the study area was 25% (95% CI: 14.7–37.8%). (Table-1)

The seroprevalence of *T. gondii* infection in females was higher (37.5%) than males (20.45%) (P = 0.178). The seroprevalence of *T. gondii* infection are varied in different age groups, ranging from 24% in <1-year-old, to 25.71% in more than 1-year-old, which increased with increasing of age (P = 0.88). Frequency of infection in dogs that had access to outdoors was (19.23%) compared to those did not access outside (29.41%). *T. gondii* in cooked meat consuming dogs (21.15%) had a lower seroprevalence than raw meat eating dogs (50%, P=0.079). No statistically differences were found in seroprevalence between pet and guard dogs (P =1). Dogs with history of deworming had a lower seroprevalence (19.44%) compared to dogs that had not been dewormed (33.33%). The seroprevalence was 50% in dogs with the presence of cats which was significantly (P=0.046) higher than those with the absence of cats (20%) in dog living area (Table 2).

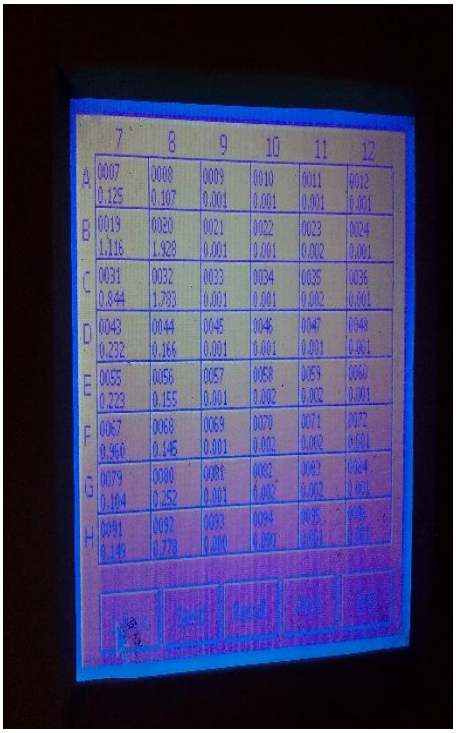
Toxoplasma gondii ELISA kits and various instruments (eg. Pipette and ELISA plate reader) used in this study are given in the following figures

(A) Instruments and ELISA kit



(B) ELISA Plate

(C) ELISA reader



(D) ELISA reader

Figure 7. (A). Instruments and ELISA kit (B). ELISA Plate (C), (D). Value of sample in ELISA reader

Table-1. The prevalence rate of total number of samples.

Total number of dogs	Positive	Prevalence (%)	P- value
60	15	25%	0.04

Table-2. Different risk factors of *Toxoplasmosis* in dogs

Factor	Category	Yes (%)	95% CI	P value
Age	1 year	6(24)	9.36 – 45.13	0.880
	More than 1 year	9(25.71)	12.49 – 43.25	
Breed	Pure	12(27.91)	15.33 – 43.67	0.408
	Local	3(17.65)	3.8 – 43.43	
Sex	Male	9(20.45)	9.8 – 35.3	0.178
	Female	6(37.50)	15.2 – 64.56	
Presence of Cat	Yes	5(50)	18.7 – 81.29	0.046
	No	10(20)	10.03 – 33.72	
Access of outside	Yes	5 (19.23)	6.55 – 39.35	0.367
	No	10(29.41)	15.1 – 47.48	
Deworming	Yes	7(19.44)	8.19 – 36.02	0.224
	No	8(33.33)	15.63 – 55.32	
Cooked meat	Yes	11(21.15)	11.06 – 34.7	0.079
	No	4(50)	15.7 – 84.3	
Hunting	Yes	6(31.58)	12.58 – 56.5	0.423
	No	9(21.95)	10.56 – 37.61	
Purpose	Pet	11(25)	13.19 – 40.34	1
	Guard	4(25)	7.27 – 52.38	

Note: **CI= Confidence interval**

Table 3. Univariate logistic regression analysis on the predictors of *T. gondii* infection in dogs of CMA

Factor	Category	Odds Ratio	95% CI	P value
Age	1 year	Ref		0.880
	More than 1 year	1.09	0.33 – 3.6	
Breed	Local	Ref		0.412
	Pure	1.8	0.44 – 7.43	
Sex	Male	Ref		0.184
	Female	2.33	0.67 – 8.14	
Presence of Cat	No	Ref		0.05
	Yes	4	0.97 – 16.5	
Access of outside	Yes	Ref		0.37
	No	1.75	0.51 – 5.94	
Deworming	Yes	Ref		0.228
	No	2.07	0.63 – 6.77	
Cooked meat	Yes	Ref		0.093
	No	3.73	0.8 – 17.34	
Hunting	No	Ref		0.425
	Yes	1.64	0.48 – 5.54	
Purpose	Guard	Ref		1
	Pet	1	0.27 – 3.75	

Note: **CI= Confidence interval, P ≤ 0.05**

The potential risk factors for seropositivity of dogs age, sex, breed, presence of cats, access to outsides, deworming, cooked food, hunting practice, purpose of use were analyzed by univariate logistic regression (Table 3). Univariable logistic regression analysis revealed that, the prevalence of occurring *T. gondii* infection was significantly varied with presence of cats p=0.05, which was (95% CI, 0.97 – 16.5) 4 times higher than absence of cats. No significant effect was observed for age, sex, breed, access to outsides, deworming, cooked food, hunting practice, purpose of use.

Table 4. Final multivariate logistic regression model to evaluate the effect of predictor variables on *T. gondii* infection in dogs in the study area

Factor	Category	Odds Ratio	95% CI	P value
Sex	Male	Ref		0.046
	Female	4.34	1.03 – 18.36	
Presence of Cat	No	Ref		0.098
	Yes	3.99	0.78 – 20.49	
Deworming	Yes	Ref		0.325
	No	1.99	0.50 – 7.89	
Cooked meat	Yes	Ref		0.325
	No	2.45	0.41 – 14.69	

Note: P ≤ 0.05

For multivariate logistic regression non-collinear variables with univariate P-value ≤ 0.25 were considered. Age, breed, access to outside, hunting and purpose of uses were excluded from final model due to univariable P- value > 0.25. Finally, sex, presence of cats, deworming, and cooked meat were entered into multivariable logistic regression model and the results are depicted in Table 4. In multivariate logistic regression analysis sex was the only risk factor significantly associated P= 0.04. Final multivariate logistic regression model showed that the odd ratio of determining *T. gondii* infection (Female) was 4.34 times higher than male.

Chapter V: Discussion

There is a severe lack of awareness about the extension and influence of toxoplasmosis infection and its consequences are in Pakistan because of parasitology laboratories that are not developed in Peshawar. This was the first study on the subject in Peshawar and even in Khyber Pakhtunkhwa. The study was conducted to investigate the seroprevalence of *T. gondii* and to find out the risk factors linked with this protozoan parasite in dogs.

Researchers have used completely different laboratory tests to observe seroprevalence of *T. gondii* infection in dogs in several countries. Microscopic agglutination test (MAT) is most generally used (Dubey *et al*., 2007; Lopes *et al*., 2011). MAT is currently indicated to offer false positives because of cross reactivity. Latex agglutination test (LAT) has additionally been used (Jittapalapong *et al*., 2007; Ahmad *et al*., 2001). However, Ahmad *et al* (2001) indicated that skeletal muscle would possibly provide false positive because of meddling issues (rheumatoid factor and immunoglobulin antibodies). Immunofluorescent antibody test has been employed by others (Sedlak and Bartova, 2006; Oncel *et al*., 2007; Hosseininejad *et al*., 2011). Indirect haemagglutination antibody test (IHA) has additionally been used (Li *et al*., 2012). Enzyme-linked-immunosorbent assay has recently been employed by several researchers (Meireles *et al*., 2004; Hosseininejad *et al*., 2011; Shadfar *et al*., 2012). Different studies of comparison of merits and demerits of serologic tests of *T. gondii* are available (Miereles *et al*., 2004). In comparison of ELISA with IHA, superiority recommend of ELISA. ELISA was utilized in this study, with ID screen infection indirect multispecies kit. Within the study, the seropositivity of *T. gondii* in dogs was 25% (95% CI: 14.7-37.8%). Similar result found by (Sharma *et al*., 2014) in Greneda, West Indies and by (Kamani *et al*., 2010) in Yerwa-Maiduguri, Nigerian.

Study conducted in Czech Republic (Oncel *et al*., 2007) indicated 25.9% *T. gondii* infection in dog. During a previous study (Pakistan) the seroprevalence of *T. gondii* in dogs was 25.4% (Ahmad *et al.,* 2014). Moreover, the results of the study discovered that seroprevalence rates were extremely related to gender and presence of cats. However, no statistically association was found in purpose of uses (as pet and guard). It may be due to less sample size.

The result showed that seroprevalence was higher in female dogs (37.50%) than males (20.45%), that is in accordance with findings (Ahmad *et al.*, 2014) in Pakistan and (Sharma *et al*., 2014) in West Indies. Higher risk of infection in females are often because of lower immune system in female dogs during and after pregnancies and lactation. Significant difference was discovered between the proportion of seropositive male and female dogs (Table 4). It is observed that the epidemiology of the disease does not appear to put either of the sex at a disadvantage as far as acquisition of infection is concerned. Statistical analysis showed that there was significant difference in gender (P=0.04) which was also reported by other researchers (Sharma *et al*., 2014; Zarra *et al*., 2017).

Higher prevalence found in dogs older than one year could also be attributed to extend within the risk of the contact with parasite with age (Cabral *et al*., 1998). Higher seroprevalence in older dogs is reported by (Pena *et al*., 2006 and Chinese *et al*., 2011). Results of this study indicate that the possibility of getting *T. gondii* antibodies will increase with age > one year (25.71%,95% Cl; 12.49-43.25%) and has been ascribed to higher risk for exposure to those protozoan parasites over time, rising the exposure in older dogs (Hosseininejad *et al*., 2011). In previous studies in dogs of North America, Korea and China, the seroprevalence of *T. gondii* infection didn't increase with age whereas in surveys in dogs of Trinidad and Tobago and Taiwan, sero-prevalence of *T. gondii*increase in accordance with age (Ali *et al*., 2003; Alvarado – Esquivel *et al*., 2014; Duan *et al*., 2012; Nguyen *et al*., 2012).

Considering the breed relationship, native breeds were 17.65 % seropositive, wherever as pure breeds were found to be 27.91 % seropositive (Table 2.). During this study there have been distinction found between pure and native breeds (P=0.408). No statistically significant variations were found in pure and local breed which is similar with finding of Zarra – Nezhad *et al.,* 2017. It may be due to less sample size and people prefer pure breed than local breed in Bangladesh

Since cat is the primary host, seropositivity of *T. gondii* in cats bears a relationship with seropositivity in dogs (Sedlak and Bartova, 2006; Oncel *et a*l., 2007). This study found a big seroprevalence (P=0.05) between dog who contact with cats and absence of cats. The seropositivity rate was found to be 50% within the dogs that have close

association with cats. On contrary, it attenuates considerably to 20% on the dogs, where house owners don't let their dogs to contact with cats (Table 2). This may be due to dogs living in association with cats eat food contaminated with oocysts of *T. gondii* shed by cats.

Dogs that had access to outside sometimes showed high seroprevalence, as a result of accesses to the outside atmosphere was prompt as vital factors for dog's infection (Lucas *et al*., 1999). Generally, dogs attend outside for exercise or hunting and have the chance of *T. gondii* infection. However, during this study, the seroprevalence was high (29.41%) in those dogs kept in inside (table 2). It may be due to poor management. Dogs with history of deworming had a lower seroprevalence (19.44%) compared to dogs that had not been dewormed (33.33%). Similar study conducted in Islamic Republic of Iran (Zarra – Nezhad *et al*., 2017) found higher (63%) *T. gondii* infection in non-dewormed dogs than dewormed (25%).

Humans and different animals can become infected by ingesting tissue cysts from raw or uncooked meat. Humans jointly become infected by eating undercooked dog meat where dogs used for food (Li, *et al*., 2012). Association between raw meat consumption and *T. gondii* seropositivity was reported by different researcher (Mengesha, 1984; Walle *et al*., 2013). This result relating to consumption of raw meat, was in line with previous findings, suggesting that feeding dogs raw meat are thought of a risk issue for *T. gondii* infection but wasn't statistically significant (P=0.079) (Shadfar *et al*., 2012). The seroprevalence was higher (50%) in dogs consuming raw meat than dogs consuming boiled meat.

T. gondii may be transmitted to carnivorous mammals by ingesting infected prey like birds and rodents. However, this study did not notice any association ($P>0.05$, $\chi2=0.423$) of the infection with hunting practice in dogs. Lack of association of hunting with *T. gondii* infection in this study might even be attributed to low prevalence of infection in prey animals like rodents, birds etc. *T. gondii* in pet dogs had a lower seroprevalence than guard dogs which similar with findings of Zarra – Nezhad *et al*., 2017.

Finally, no statistically significant association was found between the infection rate and age, breed, deworming, access of outside, diet, hunting and purpose of use which is in line with some previous studies (Alvarado *et al*., 2014; Hosseininejad *et al.*, 2011). Consequently, the results of this study visible that *T. gondii* infection may possibly cause a threat toward safety and public health in city of Chattogram. However, plenty of research is needed to find out the causes of environmental infection with *T. gondii* and prevent it in dogs and humans living throughout this area. If people, mainly those with immunodeficiency, are exposed to cats, dogs and soil, applying the sanitary principles is very important to prevent them from being infected.

Chapter VI: Conclusions, Recommendations and Future Directions

6.1. Conclusions

The overall seroprevalence of infection in CMA was 25% (95% CI: 14.7–37.8). The seroprevalence is related to presence of cats and gender. The findings of this study may be helpful for future studies and extend public awareness of the epidemiology of *T. gondii* infection in dogs in Chattogram. Early detection and management of infection in dogs that live in the studied areas will scale back the incidence of the infection in humans and different intermediate hosts.

6.2. Recommendations

The following recommendations points are forwarded.

1. Strategies to stop exposure of dogs of *T. gondii* ought to target improvement of management of dogs, feed hygiene (cooked meat) should be practiced, hunting control.
2. Presence of cats in the house or dogs living area should be avoided. If cat's excretory product found close to around in dogs living space, it should be clean.
3. Deworming of pet dogs and cats frequently.
4. Creating public awareness regarding infection should be increase.

6.3. Future Directions

Further large-scale studies ought to be conducted so as to spot the genotype and population structure of *T. gondii* strains.

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Appendix-I

A Questionnaire survey to investigate for *Toxoplasma gondii* infection in dogs

General Information

1. Age: a) More than 01 years ☐ b) Less equal 01 year ☐
2. Breed: a) Local ☐ b) Pure ☐
3. Sex: a) Male ☐ b) Female ☐

Environment

1. Does your dog have access to outdoors? Yes ☐ No ☐
2. If presence of cats? Yes ☐ No ☐

Diet

1. What types of meat have you fed your dog? a) Cooked meat ☐
   b) Raw meat ☐
2. Does your dog hunt? Yes ☐ No ☐

Others

1. The purpose of use? a) Pet ☐ b) Guard ☐
2. Deworming status? Yes ☐ No ☐