A SEROLOGICAL SURVEY OF TOXOPLASMOSIS IN PIGS IN GHANA

A Thesis presented to
The Board of Graduate Studies,
University of Ghana, Legon
Ghana.

In part fulfilment of
the Requirements for the Degree of
Master of Philosophy (M. Phil.)
(Animal Science)

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JUNE, 1999

DECLARATION

I do hereby declare that except for other people's investigations cited which have been duly acknowledged, this exercise is a result of my own original research, and that this thesis, either in whole or in part, has not been presented elsewhere for another degree.

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To my mother *Grace Bansah*

for her Love and to *Matilda*

for her unflinching love and care.

ACKNOWLEDGEMENT

I wish to acknowledge with genuine appreciation my indebtedness to all individuals who have contributed in diverse ways to the successful completion of this thesis.

First of all, I gratefully acknowledge the Noguchi Memorial Institute for Medical Research (NMIMR), for providing such excellent facilities without which this research work will have been impossible. In lieu of this, I am thankful to Prof. F. K. Nkrumah, former Director of the NMIMR, the present Director, Prof. D. Ofori-Adjei, and the entire administrative personnel of the Institute for granting me permission to conduct this study.

I am greatly indebted to my supervisor Dr. B. D. Akanmori, Head, Immunology Unit, NMIMR, and Co-supervisors Dr. K. M. Bosompem of the Parasitology Unit, NMIMR and Dr. E. A. Canacoo, Lecturer and Farm Manager, Animal Research Station (ARS), Nungua, University of Ghana, Legon for their co-operation, encouragement, constructive criticism and invaluable assistance.

I am extremely thankful to Dr. Akanmori for not only securing the needed funds for such a big project and his promptness in furnishing me with the needed reagents but also for his close supervision of the laboratory investigations without which this work would not have been possible.

I am obliged to Dr. Bosompem for his continued enthusiastic interest, extensive and stimulating discussions and advice and also for thoroughly reading and scrutinizing this thesis. But for his close supervision of the laboratory work, this achievement would not have been possible. The effort and dedication is greatly appreciated.

This study benefited from grants offered by the National Agricultural Research Project (NARP) of the Council for Scientific and Industrial Research (CSIR) and the World Bank Research Fund for Universities (RFU), for which I am grateful.

It is a pleasure to acknowledge the excellent assistance offered by the staff members of the Immunology Unit and for their warm reception and co-operation throughout my stay at the NMIMR. In this respect, I am particularly thankful to Dr. Jorgen Kurtzhals, a visiting Danish researcher, Mr. Ben Gyan, Mr. Abdulrahman Saban Hammond, Mr. Michael Fokuo Ofori, Miss Beatrice Afua Nelson-Impraim, Miss Ruth Senyefia, Mr. John K. Amissah Tetteh, Mrs. Enid Owusu and Mrs. Judith Antwi for their technical advice, encouragement and help.

I owe a special gratitude to Mrs. Irene Ayi, Messrs W. K. Anyan and D. Akakpo of the Parasitology Unit for their assistance and help. I also wish to express my sincere thanks to the Transport Officer and all the drivers at NMIMR especially Mr. Eric Opare whose invaluable assistance during my sample collection is duly appreciated.

I sincerely thank my friend and course-mate Mr. William N. A. Vanderpuije of the Animal Science Department, University of Ghana, Legon whose companionship, encouragement, criticisms and fruitful discussions with me was so invaluable.

I owe a special gratitude to SHELL Ghana Limited for awarding me a graduate fellowship without which my two years stay at the university would probably have been impossible.

Finally, I wish also to thank my mother, brothers and sisters, colleagues and friends for their concern which did not only help me to endure the strenuous demands of graduate school but also contributed selflessly to the successful completion of this thesis. In this regard, I wish to mention in particular Messrs Eric Osei Asare, Prince Kweku Hodo, Grant

Ntrakwa, Dr. Kodjo Aryeetey, Isaac Ntsiful, Justice Agyei, Philip Bleboo, Augustus Mills-Aidoo, Isaac Agyemang, Bernard Kwame Asante, Bernard Taylor for their invaluable assistance and most especially Miss Matilda Taylor for her co-operation, understanding, encouragement, financial support and prayers for me throughout the execution of this thesis.

Above all, Glory be to God for his guidance, mercy, protection and love I have enjoyed all these years.

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ABBREVIATIONS

DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetracetate
ELISA	Enzyme Linked-Immunosorbent Assay
FITC	Fluorescein isothiocynate
gm	Gramme
hr	Hour
HRPO	Horseradish peroxidase
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILCA	International Livestock Centre for Africa
ILRAD	International Laboratory for Research on Animal Diseases
IFAT	Indirect Immunufluorescent Antibody Test
l	Litre
min	Minutes
ml	Millilitre
mM	Millimolar
mW	Molecular weight
N C	Nitrocellulose
NMIMR	Noguchi Memorial Institute for Medical Research
O. D.	Optical density
PBS	Phosphate buffered saline
pH	Negative logarithm base of hydrogen ion concentration
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
TEMED	N, N, N^{I}-N^{I}-tetremethylenediamine
µ	Microns
µg	Micrograms
WHO	World Health Organization

SUMMARY

Detection of *Toxoplasma gondii* infection by the direct demonstration of parasites is difficult due to the intracellular development of the organism, whilst the tissue culture technique is cumbersome and slow. Several workers have, therefore, relied on the demonstration of specific anti-*T. gondii* antibodies in serum and body fluids of both human and animal populations to obtain epidemiological data on toxoplasmosis. The applicability of this approach is based on the ability to differentiate acute infection, which is of major concern to the clinician from chronic or asymptomatic infection using the class of specific anti-*Toxoplasma* antibodies.

The work reported in this thesis was carried out, firstly, to obtain reliable information on the prevalence of anti-*T. gondii* antibodies in pigs in Ghana. Secondly, the study sought to find out the distribution of *T. gondii* infection among pig breeds in the three defined ecological zones of the country, namely, the Coastal Savannah, the Forest Belt and the Guinea Savannah. Thirdly, the present investigation sought to determine whether there is a relationship between antibody prevalence and reported abortions/stillbirths in pigs on particular farms in Ghana. To achieve these objectives the IFAT, which is a highly sensitive and specific test with high correlation with the standard Dye test was used to validate a microplate-ELISA as an immunoassay for mass screening of sera for anti-*T. gondii* antibodies. 100 sera from pigs randomly selected from the three defined ecological zones were examined for anti-*T. gondii* antibodies using IFAT, followed by the microplate-ELISA. Fifty-five (55) sera tested positive in both IFAT and microplate-ELISA whiles thirty-six (36) sera were negative. Six (6) sera, which were positive in IFAT, tested negative by microplate-ELISA whereas three (3) sera, negative with IFAT, were positive by

microplate-ELISA. Compared to the IFAT, the microplate-ELISA had a sensitivity and specificity of 90.2% and 92.3% respectively. There was also a high correlation (r = 0.82) between IFAT and microplate-ELISA.

The specificity of anti-*T. gondii* antibodies in pig sera detected by the IFAT and microplate-ELISA was confirmed by their reactivity with *T. gondii* antigens in the Western immunoblot assay. This was achieved by comparing antigen bands recognized by specific antibodies in a WHO *T. gondii* positive human reference serum to that of positive pig sera. Anti-*T. gondii* antibodies in both the human reference serum and pig sera recognized antigens located in similar bands with molecular weights ranging from 12 to 74 kDa. Anti-*T. gondii* antibodies in pig sera were dominated by antibodies that recognized antigens located in the 26, 30, 32 and 60 kDa band regions, including the major surface antigen of the tachyzoite, the p30.

In all, 614 pigs were sampled and examined for anti-*T. gondii* antibodies using the microplate-ELISA. A nationwide 39% *T. gondii* seroprevalence in pigs was obtained in the present study with prevalence rates of 43.9%, 30.5% and 42.5% in the three main ecological zones, namely, the Coastal Savannah, the Forest belt and the Guinea Savannah. Based on the result of the microplate-ELISA, it was found that pigs in the Forest belt had a significantly lower ($P<0.05$) antibody prevalence than those in both the Guinea and Coastal Savannah zones. Also, anti-*T. gondii* antibody prevalence was found to increase with the age of pigs. It was also found that pigs reared under the intensive management system generally had lower ($P<0.05$) *T. gondii* antibody prevalence than those under semi-intensive management. Again, female pigs were found to have a significantly higher ($P<0.01$) antibody prevalence than male pigs. The result of this work showed a difference ($P<0.05$) in *T. gondii* seroprevalence between Large White (38.8%) and crossbreed pigs (46.8%).

However, the differences in antibody prevalence between the exotic Large White (38.8%) and the indigenous Ashanti Black (42.5%) and also Ashanti Black (42.5%) and crossbreed (46.8%) pigs were not significant. Market pigs had a significantly lower ($P<0.05$) antibody prevalence than breeder pigs. Also, this study showed a high positive relationship between *T. gondii* antibody prevalence as determined by microplate-ELISA and abortions/stillbirths in 20 sows on a farm where abortions and stillbirths were reported to occur frequently. Of the 20 sows, 14 (70%) that had aborted before had specific antibodies against *T. gondii*. 2 (10%) of the pigs were pregnant and both had anti-*T. gondii* antibodies. Three other sows (15%) were seropositive and had farrowed one piglet each. The pig that had the highest antibody titre in this study was a sow that had lost all its seven piglets through stillbirth.

The high specificity and sensitivity (92.3% and 90.2%) obtained for the microplate-ELISA compared to IFAT in the present study suggest that the microplate-ELISA is an efficient serological assay for the mass screening of sera for anti-*T. gondii* antibodies in pig sera. The results obtained in this thesis also suggest a possible relationship between antibody prevalence and abortions/stillbirths in sows in Ghana. Thus, toxoplasmosis might probably be a possible cause of piglet losses through unexplained abortions/stillbirths reported by some pig farmers during the present survey. The 39% *T. gondii* antibody prevalence in pigs obtained nationwide also suggests that significant proportions of the pig population in Ghana have been exposed to *T. gondii* infection. Pork containing viable *T. gondii* tissue cysts could thus be a potential source of *T. gondii* infection in humans.

CHAPTER 1

INTRODUCTION

1.1 General introduction

The livestock industry is vital to economic development in sub-Saharan Africa (Jahnke, 1982). It was reported by Hrabouszky (1981) that the industry can contribute to the achievement of a wide range of objectives among which is the provision of meat products, which are important in improving the human diet because of the high quality of protein they contain. Ghana's average daily per capita of animal protein stood at 13.7g against 59.5g in the developed countries (FAO, 1992) even though the minimum daily requirement for normal growth and healthy mental development is 34g. This deficiency is often blamed on low productivity of the country's livestock industry and attributed to a host of factors, significant among which are animal diseases (Assoku, 1986). In tropical Africa, parasitic diseases tend to be widespread due largely to ideal climatic conditions, which favour the survival of many parasites and their vectors (ILRAD Reports, 1991).

Toxoplasmosis is a zoonotic disease caused by the parasite *Toxoplasma gondii*. The organism is an ubiquitous parasitic protozoan distributed worldwide and has the capacity to invade and multiply in nucleated cells of all homeotherms, including humans and the common farm animals, pigs, cattle, sheep and goats (Jones, Yeh and Hirsch, 1972; Werk, 1985; Morisaki, Heuser and Sibley, 1995). *T. gondii* belongs to the class *Sporozoa* and the superorder *Eucoccidea* (Mehlhorn and Walldorf, 1988). The organism exists in two distinct cycles: a sexual cycle in domestic cats and other feline species which are the principal or definitive hosts, and an asexual cycle which occurs in man and all susceptible warm-blooded vertebrates (Uggla and Buxton, 1990). *T. gondii* exists in three forms: the oocyst (resistant form in the external environment) the tachyzoite (proliferating form in nucleated cells especially phagocytes) and bradyzoite (inactive form found in intra-tissue cysts).

The sexual life cycle proceeds only in the intestinal epithelium of felids, which are infected by oral uptake of sporulated oocysts from the environment or bradyzoites in animal tissue. The sporozoites from the oocysts invade the epithelial cells of the gut, undergo asexual multiplication for a few generations followed by a sexual process, which produces oocysts. Sporulation of oocysts shed in cat faeces occurs between 2-4 days leading to the formation of sporocysts containing sporozoites. Following the ingestion of sporozoites by an intermediate host, including man, they transform to a rapidly proliferating form, the tachyzoites, which finally encysts in the tissues. The tissue cysts and oocysts are the principal forms of the parasite involved in transmission (Krahenbuhl and Remington, 1982). Dubey and Beattie (1988) reported that transmission of *T. gondii* to humans is principally through ingestion of tissue cysts in undercooked infected meat and the ingestion of food, fruits, vegetables and water contaminated with oocysts from cat faeces.

Toxoplasma gondii infection in swine was first reported on a farm in Ohio in the United States (Farell *et al.*, 1952). Ever since, various workers have reported swine toxoplasmosis and its involvement in infertility, abortions, mummified fetuses, stillbirths and premature births in sows (Cole *et al.*, 1953; Cole *et al.*, 1954; Koestner and Cole, 1960; WHO, 1969; Jolly, 1969; Work *et al.* 1970; Durfree *et al.*, 1974; Moriwaki *et al.* 1976; Koh *et al.* 1978; Beverley, Henry and Hunter, 1978). Post-natal mortality and wasting of piglets has also been documented (Farell *et al.*, 1952; Jolly, 1969; and Durfree *et al.*, 1974). The involvement of toxoplasmosis in abortions during pregnancy and related complications such as stillbirths, mummified fetuses and neonatal mortality in pigs has also been demonstrated experimentally by several workers (Moller *et al.*, 1970; Work *et al.*, 1970; Dubey and Urban, 1990) through inoculation of pregnant sows with the parasite. The loss of piglets

through wastage of foetuses in utero, death of piglets immediately after farrowing and gradual wasting of piglets as a consequence of *T. gondii* infection are, indeed, important causes of reduced productivity and economic loss to the pig farmer. Cole *et al.* (1953) reported a typical case on a farm in the United States where toxoplasmosis was found to be associated with 50% mortality of piglets and 138 piglets were lost, some through stillbirths.

Toxoplasmosis is usually a benign disease in human beings. Infection with *T. gondii* may range from inapparrent, through subclinical, chronic, and to acute which sometimes results in death of the animal. Chronic *Toxoplasma* infections may persist throughout the life of the affected individual (Marquardt and Demaree, 1985). In humans, the effect of the disease is most serious in non-immune pregnant women with no specific anti-*Toxoplasma* antibodies. In such circumstances, severe disease manifests in the foetus (Feldman, 1982; Marquardt and Demaree, 1985) and the results of such infection can range from mild to fatal. In some cases the central nervous system and eye may be involved and this may lead to severe neurological disease, blindness, mental retardation or death (Marquardt and Demaree, 1985; Frenkel, 1988). In adults, the manifestations of toxoplasmosis are variable and include symptomatic and asymptomatic lymphadenopathy, myocarditis, pericarditis, hepatitis, encephalitis, myalagia and maculopapular rash (Remington and Desmonts, 1976). In immunocompromised patients including those infected with the human immunodifficiency virus (HIV) and those receiving suppressive chemotherapy, severe complication may occur (Luft *et al.*, 1984).

The public health ramifications of swine toxoplasmosis is of major concern as pork products containing viable tissue cysts of *Toxoplasma* (Weinman and Chandler, 1956;

Feldman, 1982) are an important source of infection to humans. Pigs were reported to be the major meat source of *T. gondii* contamination for humans in the United States (Dubey, 1986b). It was estimated that pork products especially undercooked pork accounted for 50-70% of all cases of human toxoplasmosis. In a national seroepidemiological survey of pigs between 1983 and 1984, *T. gondii* specific antibodies were found in 23% of 11,229 market hogs and 42% of 613 adult hogs that were slaughtered for food in the United States. This indicated that over 23% of pigs consumed had been exposed to *T. gondii* (Dubey *et al.*, 1991).

Today, *T. gondii* is regarded as one of the most important obligate intracellular parasites of livestock and man, by veterinarians, physicians, research scientists as well as economists (Krahenbuhl and Remington, 1982). Almost 500 million people worldwide are reported to show serological evidence of *T. gondii* infection (Fayer, 1981; Dubey, 1986a). The significance of toxoplasmosis in public health is being increasingly recognized in tropical and sub-tropical areas where high anti-*T. gondii* antibody levels have been recorded in humans (Feldman, 1974). Indeed, various workers have reported higher prevalence rates of *T. gondii* infection in warm and moist areas than in dry and cold areas (Gibson and Coleman, 1958; Rodriquez-ponce, Molina and Hernandez, 1995).

In toxoplasmosis, laboratory diagnosis is necessary since the symptoms are not pathognomonic. This is particularly important in pregnant women and immunocompromised patients where the disease is more serious and requires early diagnosis and treatment. The most widely used laboratory tests for diagnosing active toxoplasmosis are demonstration of specific antibody and/or antigens in serum and body fluids or by the direct detection of the parasite in tissue culture. Direct demonstration of parasites by the tissue culture techniques is,

however, not very useful for diagnosis of active infection and clinical management because they are less sensitive and slow, requiring several weeks to obtain results. Its greatest value is in retrospective confirmation of infection. For these reasons diagnosis of toxoplasmosis by detection of anti-parasite antibody or antigen is very important.

The methods available for demonstration of anti-*T. gondii* specific antibodies in serum include the Sabin-Fieldman Dye Test (SFDT), the Indirect Fluorescent Antibody Test (IFAT), the Complement Fixation Test (CF), the Indirect Haemagglutination Test (IHA) and the Enzyme-Linked Immunosorbent Assay (ELISA). With these serological methods, it is possible to differentiate between acute and chronic infection based on the class of immunoglobulin present. Immunoglobulin M (IgM) antibodies are usually demonstrated during acute toxoplasmosis whiles demonstration of immunoglobulin G (IgG) antibodies indicate a chronic infection (Weiland, 1982). In immunocompromised patients where the immune system is markedly altered, demonstration of specific antibodies may not be reliable (Burg, *et al.* 1989). In such cases, direct detection of *T. gondii* deoxyribonucleic acid (DNA) by the polymerase chain reaction (PCR) is a useful diagnosis. Also, demonstration of parasite DNA constitutes confirmation of infection in seropositive individuals.

The public health importance of toxoplasmosis especially in pregnant women and immunosuppressed individuals is therefore obvious. The occurrence of toxoplasmosis in West Africa has been documented. In Nigeria for example, antibodies to *T. gondii* have been detected in both livestock and man (Osiyemi *et al.*, 1985). However, very little data is available on both animal and human toxoplasmosis in parts of the West African sub-region. In Ghana presently, a lot of effort is being made to increase productivity in the livestock industry through improved breeding programmes and management practices. Nevertheless,

the epidemiological importance of toxoplasmosis and its economic impact on the Ghanaian livestock industry has not been determined, even though toxoplasmosis can be a cause of great economic loss to the livestock industry. There is an increase in recent years in the consumption of pork and pork products such as bacon and sausages which could be an important source of *T. gondii* infection for Ghanaians. However, the contribution of meat transmitted toxoplasmosis to humans in Ghana is unknown. It was for these reasons that a systematic study into the prevalence and distribution of *T. gondii* infection in pigs in different ecological zones of the country was conducted.

1.2 Objectives of the study

The objectives of the present study are as follows:

- To optimize and utilize two immunoassays (ELISA) and (IFAT) for the detection of anti-*T. gondii* IgG antibodies in swine sera.
- To establish the distribution and prevalence of anti-*T. gondii* antibodies in pigs in the three different ecological zones of Ghana.
- To determine whether there is a relationship between antibody prevalence and reported abortions/stillbirths in pigs on particular farms.

1.3 Justification

The establishment of a reliable laboratory diagnosis will serve as a basis for the definition of the epidemiology of the disease in livestock. The availability of data on the prevalence of *T. gondii* infection in pigs in Ghana would provide useful information on the possible role of the organism in unexplained abortions in pigs and its impact on pig production. Also, information on the incidence of *T. gondii* in pigs would outline the potential risk of pigs as a source of infection for humans. Furthermore, such data would provide the basis for further studies into human toxoplasmosis especially in immunodeficient individuals such as those infected with the human immunodificient virus (HIV) who usually suffer from "opportunistic infections" including toxoplasmosis.

CHAPTER 2

LITERATURE REVIEW

2.1 *T. gondii* and Toxoplasmosis

T. gondii was first described by Nicolle and Manceaux in 1908 who demonstrated meronts of the parasite in a gondi (*Ctendodactylus gondii)* a North African rodent. It has been the general consensus that there is a single species of *Toxoplasma* however Levine (1977) admitted seven species of importance including the controversial *Hammondia hammondi* (Frenkel and Dubey, 1975) which he called *Toxoplasma hammondi. T. gondii* is now generally accepted to be responsible for the disease called toxoplasmosis in both man and animals. Viable tissue cysts of *T. gondii* were first isolated from animal tissue in the United States (Jacobs, Remington and Melton, 1960; Remington, 1968).

2.2 Classification of *Toxoplasma*

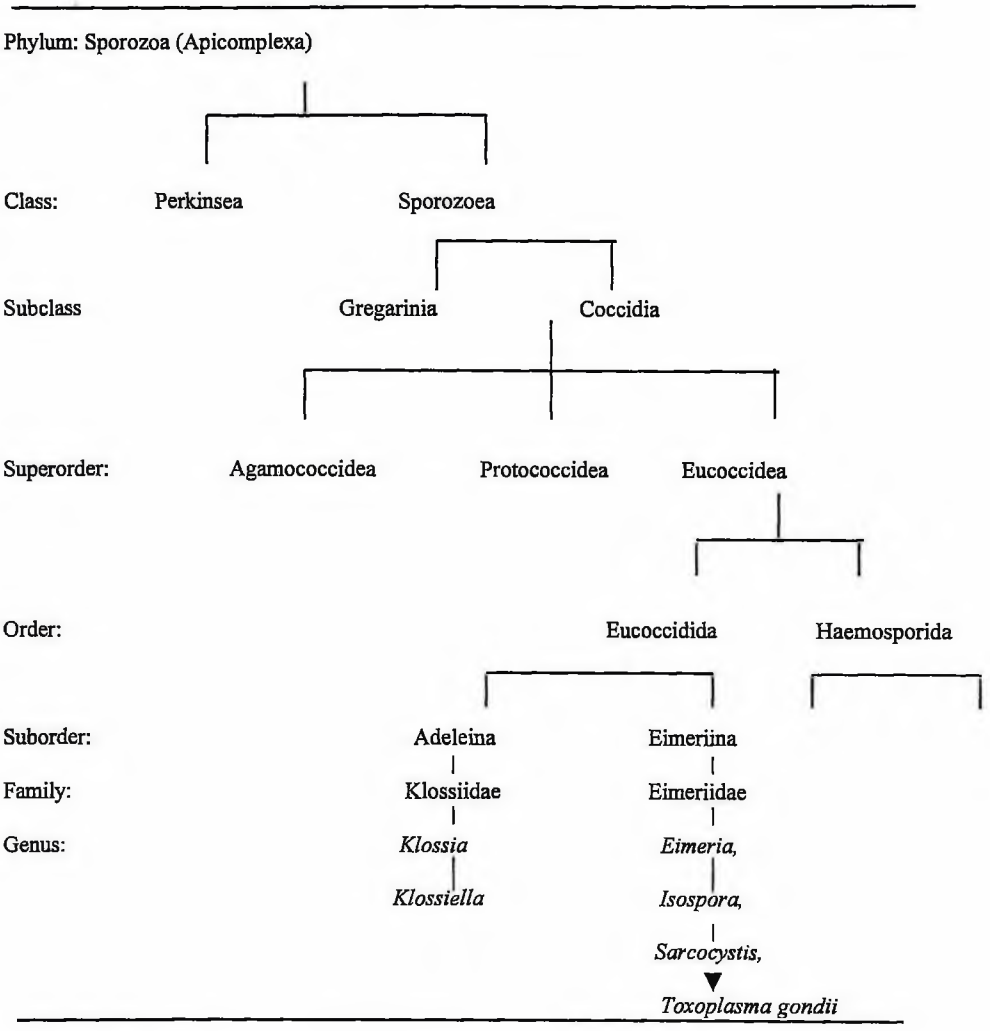
Members of the genus *Toxoplasma* belong to the kingdom *Protista,* subkingdom *Protozoa* and phylum *Sporozoa* or *Apicomplexa* (Levine *et al.,* 1980). They are further classified as belonging to the class *Sporozoea,* Subclass *Coccidea,* Superorder *Eucoccidia,* Order *Eucoccidida, Suborder Eimeriina* and Family *Eimeriidae* (Mehlhorn and Walldorf, 1988), as shown in Figure1. *Toxoplasma gondii* and other species of the Phylum *Sporozoa* such as *Plasmodium* spp. are characterized by the occurrence of the name-giving sporocysts (and/or oocysts) which produce the infectious sporozoites. In Table1 is listed the various tissue-forming coccidia including *T. gondii.* The life cycle of each of the listed species comprises a regular alternation of different sexual and/ or asexual generations.

Morphologically, the Sporozoa are a relatively uniform group with each possessing a typical apical complex. Due to the presence of this fine-structural feature, several groups of parasites were added to or excluded from the sporozoa. This led to the systematic concept of

Levine *et al.* (1980) which was however not generally accepted and has since been modified (Mehlhorn and Walldorf, 1988).

Apart from only a few species, the coccidia are intracellular parasites which have a life cycle consisting of three phases: *Schizogony* (asexual multiplication), *Gamegony* (sexual phase, which proceeds in general as oogamy with macrogametes and microgametes) and *Sporogony* in which the zygote initiates another asexual reproduction leading to the production of numerous infectious sporozoites. In species that are transmitted by the faeco-oral route, the sporozoites always include resistant stages (oocyst and/sporocysts).

Figure 1: Classification of *Toxoplasma gondii*



Adopted From Parasitology in Focus: Facts and Trends (Mehlhorn and Walldorf, 1988).

Table 1: Important tissue cyst-forming coccidia

Species	Intermediate Host	Final host
Toxoplasma gondii	**Humans and many animals**	**Cats**
Hammondia heydorni	**Cattle, sheep, goats, roe, Guinea pigs**	**Dogs, foxes and cayotes**
H. Hammondi	**Mice and other rodents**	**Cats**
Besnoitia wallacei	**Rodents**	**Cats**
B. Darlingi	**Opossums, lizards**	**Cats**
B. Besnoiti	**Cattle**	**Cats**
B. Jellisoni	**Mice, rodents**	**Unknown**
Frenkelia microti	**European voles (Microtus agretis)**	**European buzzards (Buteo buteo)**
F. Glareoli	**Bank voles (Clethrionomys glareolus)**	**European buzzards**

Adopted From Parasitology in Focus: Facts and Trends (Mehlhorn and Walldorf, 1988).

2.3 Morphology of *T. gondii*

The sporulated oocysts have a thin, flexible outer wall and two sporocysts each containing four sporozoites. The dimension of the oocysts was given by Marquardt and Demaree (1985) as 13μm by 12μm with a range of 12μm to 15μm by 10μm to 13μm. Sporocysts are 9μm by 6μm with a range of 8μm to 10μm by 6μm to 8μm. In the cat, five different merogonous generations have been described by Frenkel (1973). The meronts of these generations range from about 2μm to 17μm in length. These stages occur principally in the epithelial cells of the villi of the jejunum. In extra intestinal sites both in the cat and other hosts, there are two merogonous generations, (1) tachyzoite-forming groups and (2) bradyzoite-forming pseudocysts.

2.4 Forms of *T gondii*

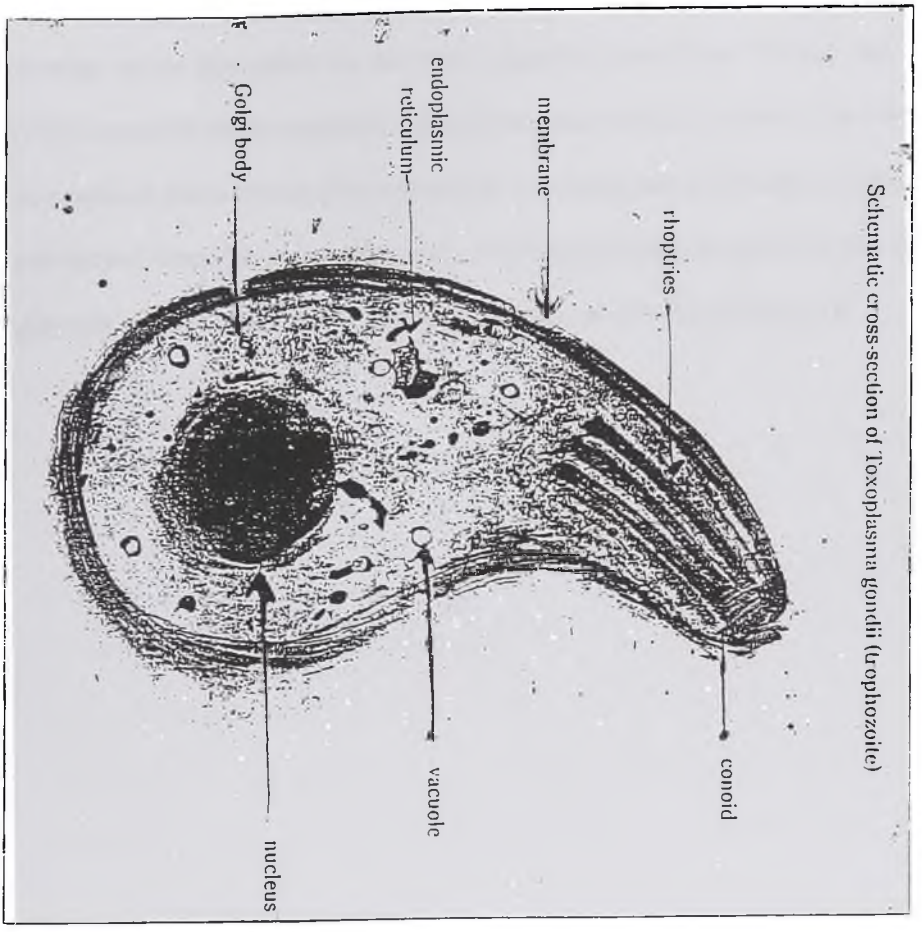
There are three forms of *T. gondii*: the tachyzoites, the tissue cysts and the oocysts. The tachyzoite is seen during the acute stage of infection and invades all types of mammalian cells except perhaps non-nucleated red blood cells (RBCs). The tissue cyst and the oocyst are the principal forms of the parasite involved in transmission.

2.4.1 *T. gondii* tachyzoite

As shown in Figure 2, the individual cell (trophozoite or tachyzoite) is usually crescentic or boat-shaped, thin walled with one end pointed and the other rounded. The typical *T. gondii* cell is made up of distinct cytoplasm and nuclear chromatin but there are no flagella or other visible external structures. The absence of inclusions, other than the

nucleus, differentiates this organism from the amastigote stage of *leishmania* spp. and *Trypanosoma cruzi* (Larsh, 1980). On the other hand, organisms like *Histoplasma* spp. are similar to *T. gondii. Toxoplasma* organisms may occur singly or in clusters (pseudocysts) within fixed host cells during acute infections. They may also be found at times within macrophages in exudates like the peritoneal, pleural or cerebral exudate and in circulating blood (Larsh, 1980). However, some may be extracellular. The tachyzoite has no particular tropism for any specific cell type, organ or tissue. Indeed it is capable of infecting both phagocytic and non-phagocytic cells. During the invasion of phagocytic cells by *T. gondii* tachyzoites, parasites are found within a modified endocystic vacoule known as the parasitophorous vacoule (PV) whose membrane lacks many host-cell derived components. Tachyzoites multiply in the PV with a generation time of 5-10 hours until the host cell is completely destroyed. The parasites within the PV are able to survive intracellularly because this vacoule (fusion-incompetent vacoule) neither fuses with lysosomes of the host cell nor does it become acidic (Joiner, 1993). Furthermore, the tachyzoite presents a group of structures located at its anterior pole that plays an important role in its development and survival in a host. These structures include rhoptries, (Figure 2) dense granules and micronemes (Dubremetz and Schwartzman, 1993; Joiner, 1993).

Figure 2: *Toxoplasma gondii* tachyzoite



The mode of entry of *T.gondii* tachyzoites into cells has however been the subject of considerable controversy. Vischer and Suter (1954) and Jones, Yeh and Hirsch (1972) have presented data suggesting that phagocytosis is the primary process by which the parasite enters host cells, whereas others (Lycke and Norrby, 1966) have communicated data showing active penetration as the most important mechanism. Ryning and Remington (1978) supported the phagocytosis school of thought when they reported that cytochalasin D, a compound which blocks phagocytosis by preventing microfilament formation, inhibited the entry of *Toxoplasma* into phagocytic cells (mononuclear phagocytes) and into cells not generally considered to be phagocytic (a line of mouse bladder tumour cells).

2.4.2 *T. gondii* tissue cyst

The second form of the parasite, the tissue cyst, is formed within the host cell cytoplasm and may contain only a few or several thousand organisms (Krahenbuhl and Remington, 1982). Cysts are demonstrable as early as the eighth day post-infection in animals and probably persist as viable parasites throughout the life of the host. Remington and Cavanaugh (1965) and Jacobs, Remington and Melton (1960) reported that although cysts may be found in virtually every organ, the brain, skeletal and heart muscles appear to be among the more common sites of latent infection. They however asserted that these sites have been more carefully examined than others.

2.4.3 *T. gondii* oocyst

A notable contribution was made to the understanding of the epidemiology of toxoplasmosis in 1970 independently by various workers (Frenkel, 1970; Frenkel, Dubey and Miller, 1970; Hutchison and Dunachie, 1971). These workers demonstrated that *T. gondii* has its sexual development occuring only in the epithelium of the small intestine of feline animals. As a result it is now known that the third form of the organism, the oocyst, is produced only in the felids. Schizogonic and gametogonic stages in the epithelium of the small intestine culminate in the development of oocysts. Using clones of *T. gondii* developed from a single tachyzoite, Pfefferkorn, Pfefferkorn and Colby (1977) showed that the genome of each tachyzoite contains the genetic potential for the production of both microgametocytes and macrogametocytes.

2.5 The life cycle and transmission of *T. gondii*

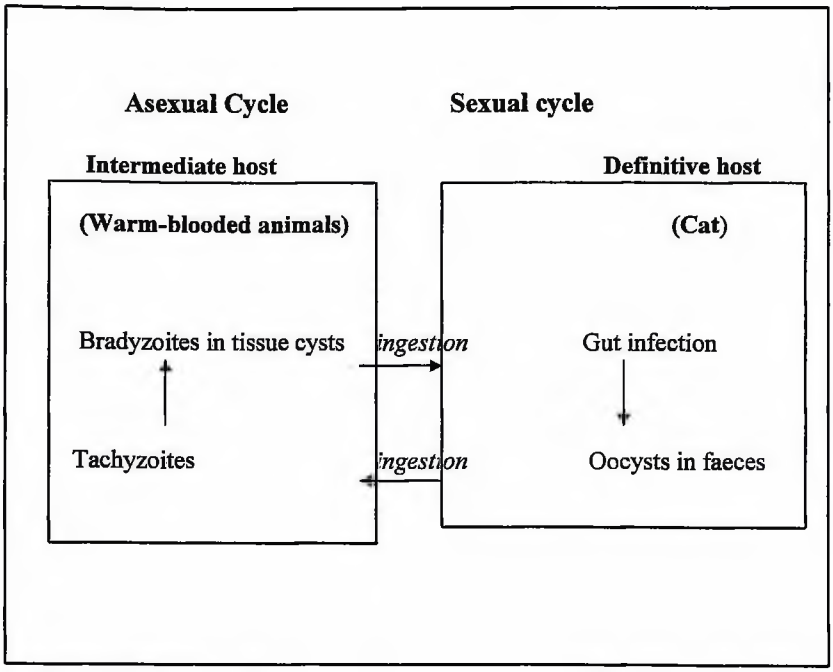
Although *T. gondii* has been found as a parasite in most tissues of nearly all

vetebrate species, sexual, development takes place only in the small intestines of domesticated and wild cats (Frenkel, Dubey and Miller, 1970; Hutchison *et al.*, 1970; Overdulve, 1970; Sheffield and Melton, 1970). In this respect a wide range of felids including ocelots, margays, bobcats, mountain lions, jaguarundis and Bengal tigers have been studied, but most information is available from the domestic cat. Cats are normally infected through any of three principal ways. Firstly, infection may follow the ingestion of animal flesh latently infected with tissue cysts that contain asexually produced organisms called cystozoites or bradyzoites. Secondly, they may be infected by the ingestion of acutely infected animals with asexual stages called trophozoites. Thirdly, infection may be acquired by the ingestion of oocysts from faeces of other cats. In an experiment involving oral infection of newborn kittens with *T. gondii* cysts in mouse brains, Dubey and Frenkel (1972) found trophozoites in the lamina propria of the small intestine and in the mesenteric lymph nodes, lungs and brain of the kittens soon after infection. The parasites persisted in these tissues and organs for many days thereafter.

Frenkel (1973) described five asexual (merogonous) stages in the epithelium of the small and large intestines of cats which differ from one another in size, number of organisms, method of multiplication, time of development and location in the intestine. The sexual stages (gametocytes) are found throughout the small intestine but most often are in the ileum 3-15 days after infection. Fertilized female gametes become surrounded by a protective wall and develop into oocysts, which enter the lumen of the gut and are shed in the faeces. A single cat may shed as many as 20 million oocysts per day in about 20g of faeces (Fayer, 1981). The oocyst shed in the faeces need to undergo internal development (sporulation) for about 48-72 hours in an aerobic environment before they become infective.

A sporulated oocyst contains two sporocysts, each with four sporozoites. These sporozoites are capable of infecting all homeotherms including man (Timofeev and Akulov, 1975). Figure 3 is a simplified graphical representation of the foeco-oral cycle of *T. gondii*.

Figure 3: The Life Cycle of *Toxoplasma gondii*



2.6 Dispersal and survival of *T. gondii* oocysts

The dispersal of oocysts in the environment has a direct bearing on the level of contamination to livestock. The site of deposit of cat faeces has been found to have a great influence on the subsequent dispersal of oocysts (Fayer, 1981). Oocysts of *Toxoplasma* are small and therefore easily dispersed by wind, rain, and fomites. In addition, oocysts in cat faeces deposited in fields, pastures or lawns may be dispersed by machinery, shoes, or feet of animals. In situations where oocysts in cat faeces are buried in gardens, sand boxes or play areas, they may further be dispersed by cultivation or digging. Oocysts deposited in barns or animal feed storage facilities (Penkert, 1973; Plant, Richardson and Moyle, 1974) may be distributed on the soiled bodies of rodents, birds, or other small animals or by the movement of feed. Oocysts present in metropolitan areas such as city streets may be dispersed through storm sewers into streams, lakes or bays. Biological dispersal may also occur when slugs, cockroaches, earthworms, flies, or isopods (sow bugs) ingest oocysts at one location and either deposit them at another location or are subsequently eaten by birds (Frenkel *et al*, 1975; Wallace, 1971)

Although oocysts of *Toxoplasma* have been reported to survive as long as 13 to 18 months in the environment, Hutchison (1967) and Frenkel (1975) have reported that climatic factors such as high humidity and moist conditions affect the period of survival and infectivity of all coccidial oocysts. In this case, unsporulated oocysts are more susceptible than sporulated oocysts to damage from extreme temperatures, low relative humidity and high direct sunlight. Unsporulated oocysts lose their infectivity after a day at -21°C, whereas sporulated oocysts survive at that temperature for up to 28 days. Furthermore, unsporulated oocysts are killed after a day at 37°C, whereas sporulated oocysts remain infectious for ten

months at that temperature (Frenkel, Ruiz and Chinchilla, 1975). It is important to note that oocysts have been found to be quite resistant to harsh chemicals such as chromic acid, sodium hydroxide, and sodium hypochloride. However, 5% ammonium hydroxite has been used successfuly to kill oocysts within 10 minutes (Dubey, 1977).

2.7 Seroprevalence of *T. gondii* in meat animals

Toxoplasmosis in livestock is often regarded seriously because of the major economic importance the disease has especially in sheep. Although transmission of *T. gondii* by tissue cysts is possible among carnivores, only transmission by oocysts can explain infection of herbivorous meat animals (Fayer, 1981). The seroprevalence of *Toxoplasma* in meat animals, therefore, is a reflection of the distribution of oocysts in the environment, which also depends primarily on the distribution of infected cats. Numerous surveys have been conducted on six continents to determine the prevalence of anti-*Toxoplasma* antibodies in meat animals and Dubey (1986a) has given a comprehensive data on the prevalence of toxoplasmosis in most counties of the world. The average rates of infection for cattle, sheep and pigs based on seroepidemiological survey in most countries were found to be 25%, 31% and 29%, respectively (Fayer, 1981; Dubey, 1986a). Although the prevalence of specific anti-*Toxoplasma* antibodies in cattle, sheep and pigs may be similar, marked differences in antibody levels have been observed in each of these hosts (Fayer, 1981). Given equal infective doses of *Toxoplasma*, the serum antibody titer becomes most highly elevated and remains elevated longest in sheep followed by pigs and then by cattle. Similarly, clinical illness and abortion from *Toxoplasma* are most often observed in sheep, somewhat less frequently in pigs, and rarely in cattle (Fayer, 1981).

Although *Toxoplasma* has been isolated from various organs of sheep, pigs and cattle, it is appropriate to note that parasites have been recovered from lamb and pork but not beef (Dubey and Streitel, 1976). Dubey (1988) reported that *T. gondii* can persist in edible tissues of pigs for as long as 875 days which is well beyond the lifespan of pigs raised for market and infective tissue cysts have been found repeatedly in commercial cuts of pork in both experimentally and naturally infected pigs. Viable *T. gondii* cysts were found in 24% of 50 pigs, 9.3% of 86 sheep, and none of 60 cattle in Maryland, USA (Jacobs, Remington and Melton, 1960). Ingestion of viable cysts in meat especially pork or pork products has been reported to be the major source by which humans become infected (Dubey, 1986b). In case of cattle, perhaps, the differences in host response and recovery of parasites have fostered the generally held view that cattle are innately resistant to *Toxoplasma* infection. Munday (1978) concluded that this resistance permits cattle to graze on heavily contaminated pastures that are unsafe for other livestock. *Toxoplasma* tachyzoites have been observed to be disseminated throughout the body during acute toxoplasmosis. They have been found in tears, nasal secretions, saliva, milk, vaginal secretions, semen, urine and faeces. Saari and Raisanen (1977) reported that tachyzoites in these secretions retain infectivity for days and have the ability to penetrate the mucous membranes of the host. *Toxoplasma* tachyzoites have been isolated from milk of cows, goats, sheep, pigs, dogs, cats, rabbits, guinea pigs and mice with natural or experimental infections (Jones, 1973). Reimann *et al.* (1975) documented the transmission of *T. gondii* to humans through unpasturized goat milk containing tachyzoites. Also important in the epidemiology of toxoplasmosis is the role of semen in transmission. In sheep, *T. gondii* in semen has been transmitted to ewes through vaginal inoculation (Spence *et al.*, 1978). However, actual

sexual transmission has not yet been documented.

2.8 Infection of pigs with *T. gondii*

The ingestion of oocysts from the environment appears to be the main source of *T. gondii* infection in pigs (Dubey, 1986a). Dubey and Beattie (1988) reported that swine may be infected directly through the ingestion of oocyst-contaminated feed, water, and soil or by consumption of infected rodents. The prevalence of *T. gondii* in two herds of pigs, 7 miles apart was compared using data collected in 1982-1983 by Lubroth, Dreesen and Ridenhour. Herd A was on an open range and herd B was maintained indoors under intensive management. The prevalence of anti-*T. gondii* antibodies was 28% in herd A and 0.8% in herd B. However, the seroprevalence of *T. gondii* in small mammals trapped from both farms were similar, suggesting that a rodent-pig cycle did not account for the differences in prevalence between the two farms. The authors attributed the high variability of the prevalence of *T. gondii* observed to the management systems practiced. Dubey (1986b) corroborated this in a similar study on pigs. The breed of pig was found to have no apparent relationship with prevalence of antibodies against *T. gondii* (Dubey, 1986a). Weinman and Chandler (1954, 1956) found a 48% *T. gondii* infection rate on a farm where rats were abundant in the swine houses and the pigs were fed uncooked garbage. Dubey (1988) reported that pork scrap including the viscera, tongue, brain fed to pigs need to be cooked to at least 70°C since these portions have been found to be potential sources of *T. gondii* infection. The practice in Ghana whereby most pig farmers' supplement feeding of their animals with kitchen leftovers such as cooked cassava from "chop-bars" frequented by cats may therefore be a source of infection to the animals.

Ikegami *et al.* (1970) in their work to determine the variability of infection rates with age, found a higher prevalence (40%) for pigs older than three years with more infection in boars (42.4%) than in sows (23.2%). Arambulo, Cabrera and Alge (1974) and Garcia, Ruppaner and Behymer (1979) also found higher infection rates in male pigs than in female pigs. However, Oshima, Tsubota and Hiroaka (1981) reported a 10-fold (11.4%) higher prevalence of *T. gondii* infection in breeding sows compared to market pigs, which had 0.7% prevalence.

2.9 Clinical toxoplasmosis in naturally infected pigs

Most *T. gondii* infections in pigs are subclinical. Swine toxoplasmosis was first reported from a farm in Ohio in the United States by Cole and his associates (Farell *et al.*, 1952; Cole *et al.*, 1953, 1954; Sanger and Cole, 1955; Koestner and Cole, 1960). The pigs on this farm had weakness, cough, incoordination, tremors and diarrhoea, and mortality was about 50%. Also, there were stillbirths, premature births and deaths of piglets soon after birth. Upon necropsy and histological examination, pneumonia, focal necrosis of the liver, hydrothorax, ascites, lymphadenitis, and enteritis were seen. However, it was then uncertain whether all these symptoms were as a result of *T. gondii* infection (Cole *et al.*, 1953).

2.10 Toxoplasmosis in man

In all mammalian species, *Toxoplasma* multiplies intracellularly at the site of invasion (normally the gastrointestinal tract). After host cell disruption the parasite invades adjacent cells and is spread throughout the body via the lymphatics and the blood stream (Hughes, 1985). The invasion of host cells and tissues induces immune responses, and with

the appearance of humoral and cell mediated immunity (CMI) the parasites are destroyed leaving only those protected by an intracellular habitat to multiply and cause disease (Krahenbuhl and Remington, 1982). In man, the symptoms of toxoplasmosis are generally similar to that of other diseases although the manifestations in adults are variable and include symptomatic and asymptomatic lymphadenopathy, myocarditis, pericarditis, hepatitis, encephalitis, myalagia and maculopapular rash (Remington and Desmonts, 1976).

The lymphadenopathic form of toxoplasmosis is recognized as the most common clinical manifestation of the disease in adults (Remington, 1974; Palmer, 1974). Some cases also mimic the haematological picture of infectious mononucleosis. The symptoms presented in either situation are however not pathognomonic. Accurate diagnosis of toxoplasmosis is, therefore, essential for differential diagnosis. *T. gondii* infection normally persist in the chronic state for the entire life of the host and recurrences of acute toxoplasmosis are rare. Recurrence of the disease involving fatal dissemination of encysted organism has been reported in immunocompromised individuals such as those with Acquired Immune Deficiency Syndromme (AIDS) (Luna and Lichtiger, 1971; Frenkel, Ruiz and Chinchilla, 1975; Remington, 1982; Krahenbuhl and Remington, 1982; Grandsen and Brown, 1983; Farkash *et al.*, 1986; Luft *et al.*, 1984). Fauci *et al* (1986) reported that in about 5% of AIDS cases, toxoplasmosis is the first indication of human immunodeficiency. In such cases rapid diagnosis of active *Toxoplasma* infection is essential for patient management and prognosis.

Since the first description of congenital toxoplasmosis, *T. gondii* has been recognized as an important pathogen during pregnancy and the perinatal period in women. Infection of non-immune women during pregnancy may lead to congenital infection

resulting in hydrocephaly, microcephaly, or intracerebral calcification in the foetus. Congenital complications are most severe if infection occurs during the first trimester of pregnancy. In such situations, prenatal diagnosis of the disease can permit a decision to terminate early pregnancy or initiate treatment of late-term fetuses in utero (Desmonts *et al.*, 1985). Recent seroepidemiological surveys in the United States indicate that the incidence of congenital toxoplasmosis is approximately one in every thousand births (Stray-Pederson and Lorentzen-Styr, 1979; Stray-Pederson, 1980). However, the annual cost for the aftercare of children with the sequelae of congenital toxoplasmosis runs into hundreds of millions of dollars (Krahenbuhl and Remington, 1982). In Ghana, as in many developing countries without reliable laboratory confirmation of clinical diagnosis, the number of cases of congenital toxoplasmosis is unknown.

2.11 Immunity to *T. gondii* infection

T. gondii infection is normally controlled by the host immune system leading mostly to an asymptomatic chronic infection. Immunity against the parasite is both humoral and cell mediated. The first immunoglobulin to be secreted during *toxoplasma* infection is IgM, followed by IgA and IgE, which can be detected during the acute phase of toxoplasmosis. On the other hand, IgG appears later and can be detected throughout the lifetime of the host, thereby providing a serological marker for detecting chronic infections.

Various workers in *Toxoplasma* seroepidemiological surveys have employed the measurement of specific IgG and the detection of specific IgM antibodies to identify and distinguish between both animals undergoing the acute phase of infection from chronic ones. However, the value of IgM as a diagnostic marker in elucidating the cause of

reproductive problems in sowherds is limited by the fact that often only a few concepted fetuses are transplancentally infected (Work *et al.,* 1970; Dubey and Urban, 1990).

A distinctive immunological factor of *T. gondii* infection is the persistent cell-mediated immune response that the parasite stimulates. This response protects the host against rapid tachyzoite replication and subsequent pathology (Gazinelli, Denkers and Sher, 1993a; Kasper and Boothroyd, 1993). The highly effective resistance that *T. gondii* induces against itself has been shown to involve $CD4^+$ and $CD8^+$ T lymphocytes displaying a type I cytokine expression profile (Gazinelli *et al.*, 1993b). In particular the production of gamma interferon (IFN-γ) by these lymphocyte populations has been demonstrated to play a major role in both acquired immunity to acute infection and the control of parasite growth in chronically infected hosts (Suzuki *et al.*, 1988; Suzuki, Conley and Remington, 1989).

The ability of *T. gondii* to trigger strong type I cell mediated immune response has been attributed to events that occur during the first few days after parasite entry into the host. In this respect, the interaction of tachyzoites with macrophages has been shown to lead to the production of a series of cytokines [interleukin-12 (IL-12), tumour necrosis factor alpha (TNF-α) and IL-Iβ] that drive the synthesis of IFN-γ by both T. lymphocytes and natural killer (NK) cells. These events appear to be necessary for control of acute infection (Gazinelli *et al.*, 1993b; Gazinelli *et al.*, 1994; Sher *et al.*, 1993). Recent studies on IFN-γ inducible genes suggest that nitric oxide (NO) is important in the acquired resistance to parasitic infections through the induction of IFN-γ production (Khan *et al.*, 1996). *In vitro* systems have also demonstrated that IFN-γ can exert indirect parasiticidal effect by inducing the production of (NO) in mouse macrophages (Adams *et al.*, 1990).

2.12 Surface and intracellular *T. gondii* antigens of diagnostic importance

Handman, Goding and Remington (1980), Kasper, Crabb and Pfefferkorn (1982, 1983) and Couvreur *et al.* (1988) pioneered the characterization of tachyzoite surface proteins by generating monoclonal antibodies that identified five major surface proteins of moleculae weights (22, 23, 30, 35 and 43 kDa) designated P22, P23, P30, P35 and P43 according to their mobility in SDS-polyacrylamide gel electrophoresis. Kasper *et al.* (1983) showed that P30 or SAG-1 represents 5% of the total amount of tachyzoite proteins by generating a monoclonal antibody specific for P30. Handman, Goding and Remington (1980) radioiodinated surface antigens of RH strain *T. gondii* tachyzoites by the lactoperoxidase technique (Marchalonis, Cone and Santer, 1971) and analysed the solubilized membrane proteins using two-dimensional polyacrylamide gel electrophoresis. Four major labelled proteins of molecular weights of 43, 35, 27 and 14 kDa were identified. 2-Dimensional gel analysis of labelled extracts of the C 37, C 56 and RH strains of *Toxoplasma* tachyzoites revealed similar patterns of radioiodinated proteins. The four major proteins were detected consistently in all experiments and were immunoprecipitated by antibodies from mice chronically infected with *T. gondii* as well as sera from acutely infected humans. Kasper, Crabb and Pfefferkorn (1982, 1983) described two *T. gondii* precipitable antigens of molecular weights 22 kDa (P22) and 30 kDa (P30). The 30 kDa antigen also designated SAG-1 was found to be a membrane glycoprotein specific to tachyzoites and conserved in other strains of *T. gondii* (Mcleod, Mack and Brown, 1991). SAG-1 generates a strong humoral immune response and is implicated in stimulating the production of cytotoxic lymphocytes and IFN-γ (Khan, Smith and Kasper, 1988).

Analysis of *Toxoplasma* polypeptides using antigen extracts from sonicated

organisms by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and ^{25}I-concanavalin A (Con-A) binding studies detected 9 intracellular polypeptides of diagnostic potential (Johnson, McDonald and Neoh, 1981). The molecular weight range of the identified polypeptides was reported to be between 20 and 98 kDa and none was glycosylated.

2.13 Characterization of antigens by Western immunoblot

Western blotting is an electrophoretic technique that allows one to determine the reactivity of protein antigens (peptides) resolved on polyacrylamide gels. With the technique, peptides located in protein bands are probed with antibody raised against specific antigens. Immunoblotting, therefore, combines the resolution of gel electrophoresis with the specificity of immunochemical detection (Towbin, Staehelin and Gordon, 1979), and can be used to determine a number of important characteristics of complex protein antigens. The assay is also suitable for determining sample purity and the relative amounts and molecular weights or sometimes isoelectric point of proteins of interest (Grzych, Roussel-Velge and Capron, 1989).

2.13.1 SDS-denaturing discontinuous method

Polyacrylamide is the most commonly used polymer for gel electrophoresis of proteins. The gels are formed following polymerization of monomeric acrylamide into polymeric acrylamide chains and the cross-linking of these chains by N, N'-methylene-bisacrylamide (Hames and Rickwood, 1981). The polymerisation reaction is initiated by the addition of ammonium persulphate and it is accelerated by N, N, N^I-,N^I-

tetramethylethylenediamine (TEMED) which catalyzes the formation of free radicals from ammonium persulphate. It is advisable to degas the gel solution before the polyacrylamide catalysts are added since dissolved oxygen inhibits the polymerization process.

The most widely used polyacrylamide gel electrophoresis system is the denaturing SDS discontinuous method described by Laemli (1970). Separation of proteins by SDS-PAGE depends on the pore size of the polymeric gel (Copeland, 1994). This in turn is a function of the percentage of acrylamide used in the preparation of the gel, which invariably depends on the molecular weight range over which one wants to fractionate. In this technique buffers of different pH and composition are used to generate discontinuous pH and voltage and are used to concentrate and sharpen sample constituents before they enter the resolving gel (Copeland, 1994). Since the discontinuous system concentrates the protein in each sample into narrow bands, the applied sample may be more dilute than that used for continuous electrophoresis.

2.13.2 The SDS-PAGE electrophoresis principle

The basis of the SDS-PAGE principle is that a charged protein migrates in an electric field at a rate that is determined by its size and charge. The electric field is normally applied across a sheet of a polymer (polyacrylamide) whose pores act as a partial barrier to protein motion (Copeland, 1994). Before entering the electric field, the protein is denatured (stretched into linear peptides) by exposure to harsh conditions involving treatment with heat, denaturing detergents, reducing agents, and sometimes chaotropic agents such as urea. The peptides become coated with the anionic detergent, SDS. Under these conditions protein subunits are dissociated and their biological activities lost. In the denatured state, most

proteins bind SDS in a constant weight ratio so that they end up possessing similar charge densities and their rates of migration in the electric field is no longer dependent on the peptides inherent charge (Copeland, 1994). Instead, the rate is influenced solely by the molecular size. Larger proteins are therefore more strongly retarded in the polymeric gel than smaller proteins. It is a common practice to include a mixture of proteins, of very well defined molecular weights, in one or more lanes of the gel to serve as molecular weight standards (or markers). The molecular weight of the target protein can then be determined by comparison with the relative mobilities of these standards.

2.13.3 Characteristics of sample and gel

Most proteins are resolved on polyacrylamide gel containing 5 to 20% acrylamide and 0.2 to 0.5% bisacrylamide. It is recommended that for discontinuous polyacrylamide gel electrophoresis under denaturing conditions, 5% gels should be used for SDS-denatured proteins in the molecular size range of 60 kDa to 200 kDa and 10%, 15% gels for proteins in the range of 18 kDa to 75 k Da and 12 kDa to 45 kDa, respectively (Copeland, 1994). Over these ranges the relationship between the relative protein mobility and $\log_{10}$ molecular weight of the protein is linear. The true estimate of the molecular size of an unknown protein (its subunits) may therefore be made by comparing the relative mobility of the unknown protein to protein in a calibration mixture. It is important to note, however, that if two or more proteins have identical molecular size they will not be resolved regardless of charge differences and possibly constitutional differences between them. Purified protein complexes or multimeric proteins consisting of subunits of different molecular size will nevertheless be resolved into different bands.

2.13.4 Common problems encountered in polyacrylamide gel electrophoresis

If an electrophoretically separated protein will be electroeluted or electroblotted, then the highest purity reagents available should be used. After a separating gel is poured, it may be stored with an overlay of the same buffer used in preparing the gel. The stacking gel should be poured immediately prior to electrophoresis, otherwise there will be a gradual diffusion-drive mixing of buffers between the stacking and separating gels, which will cause a loss of resolution. It is recommended that the protein of interest should be present in 0.2 to 10μg/lane in a complex mixture of protein if the gel is to be stained by Coomasie Brilliant Blue. However, up to approximately 100μg of a complex antigenic mixture can be loaded onto a slab gel. Below is an account of some of the most important problems likely to be encountered in the electrophoretic analysis of proteins by SDS-PAGE.

In situations when there are band distortions (protein bands curve upwards at both sides of the gel called "smiling"). Copeland (1994) recommended that the running current be reduced, and the gel cooled with a circulating water and/or by increasing heat transfer from the gel by adding more buffer to the lower buffer chamber up to the level of the sample wells. Another problem involves the spread of protein bands laterally from gel lanes. This spreading which is due to diffusion of sample out of the wells may be corrected by reducing the time interval between sample application and running of the gel. Alternatively, the acrylamide percentage in the stacking gel should be increased from 4% to 4.5% or 5%, or the operating current in the stacking gel should be increased by 25%. On the other hand, the protein bands may be uneven because the stacking gel is not adequately polymerized or the bands may be distorted because of the presence of salt in the protein sample. This problem may be corrected by degassing the stacking gel solution thoroughly or by increasing the

ammonium sulphate and TEMED concentrations by 25%. Also, dialysis, gel filtration or precipitation may remove excess salt in the sample. Another cause of skewed protein bands is an uneven interface between the stacking and the separating gels which can be prevented by not disturbing the separating gel surface when overlaying it with buffer. When the protein bands are diffuse, it is advisable to increase the operating current by 25 to 50% or use a higher percentage of acrylamide. Double protein bands are observed when the protein is partially oxidized. Such oxidation can be reduced by increasing the concentration of 2-mercaptoethanol in the sample buffer or by preparing fresh protein samples.

2.14 Laboratory diagnosis of Toxoplasmosis

There are various laboratory procedures used for the diagnosis of toxoplasmosis. Some techniques are direct through demonstration of the parasites in tissues, whiles others are indirect and detect either circulating antigens or anti-parasite antibodies.

2.14.1 Diagnosis by direct demonstration of parasites or cysts in tissues

One of the most definitive ways to diagnose toxoplasmosis is to demonstrate parasites or cysts in tissues using conventional, histological or immunocytochemical techniques following biopsy. Usually, the indirect fluorescent antibody test (Terragna, 1971) or the sensitive peroxidase anti-peroxidase technique (Conlay and Jenkins, 1981) are used. These methods can utilize both formalin-fixed and unfixed *T. gondii* parasites as well as organisms in paraffin-embedded tissue sections. However, the techniques are subjective and have limitations in interpretation of results (Frenkel and Piekarski, 1978).

2.14.2 Diagnosis by direct demonstration of parasite DNA in infected animal

The polymerase chain reaction (PCR) which is based on the principle of gene amplification of DNA (Mullis and Faloona, 1987) is a powerful diagnostic tool complementing tissue culture and serology in the diagnosis of a plethora of diseases (Saiki *et al.* 1985). Recombinant DNA procedures have enabled the development of DNA probes for a number of human and animal pathogens especially human immunodeficiency viral types Ou *et al.* (1988). DNA gene amplification by the PCR is a highly sensitive and specific diagnostic tool Burg *et al.* (1989) and has been used by many workers in the diagnosis of toxoplasmosis in both veterinary medicine (Burg *et al.*, 1989; Turner and Savva, 1991; Wastling, Nicoll and Buxton, 1993) and human medicine (Saiki *et al*, 1985). Saiki *et al.* (1985) reported that the PCR is superior to the other direct methods, such as the tissue culture technique and is capable of detecting just one organism in an infected individual. The PCR however has the disadvantage of being highly technical, expensive and cannot be used readily for mass screening of specimen.

2.14.3 Diagnosis by subinoculation of infected tissues into experimental animals

In this approach biopsy specimen or postmortem material are macerated and injected into suitable experimental animals such as laboratory mice. The principle underlining the method is that bradyzoites of *T. gondii* within tissue cysts are able to cause infection when released and introduced intraperitoneally into susceptible animals. The injected brabyzoites are transformed into invading tachyzoites, which initiate an infection that induces host immune response. Diagnosis can then be made by demonstration of *T. gondii* tachyzoites in peritoneal fluid or anti-*T. gondii* antibodies in serum (Hughes *et al*, 1982).

2.14.4 Diagnosis by demonstration of circulating *T. gondii* antigens

The microplate-ELISA has been used to demonstrate circulating *T. gondii* antigens in the blood or serum of humans suffering from acute toxoplasmosis (Araujo and Remington, 1980). Araujo, Handman and Remington (1980) used mouse monoclonal antibodies as well as polyvalent antibodies raised against *T. gondii* tachyzoite to detect parasite antigens in the blood of infected hosts. However, based on a comparison made between monoclonal and polyvalent antibodies, they concluded that the polyvalent sera were more satisfactory for the detection of antigenaemia than the monoclonal antibodies. Nevertheless, the use of monoclonal antibodies has been found to have several advantages including ease of standardization and good specificity. The use of antigen detection for diagnosis of toxoplasmosis circumvented the shortcomings of the antibody detection method since the demonstration of specific *T. gondii* antigens in the serum of an animal is evidence of a current infection. This is because some parasites get destroyed by the host's immune responses leading to the release of soluble antigens in the blood, and in the absence of parasites the released antigens disappear rapidly from circulation. This observation has been made in the diagnosis of other parasitic diseases such as trypanosomiasis and schistosomiasis (De Jonge *et al.*, 1989).

2.14.5 Diagnosis by demonstration of antibodies in serum and body fluids

Since the beginning of the 20th century, many attempts have been made to detect antibodies to parasite antigens using various serological methods (Weiland, 1982). In this direction, the complement fixation test (CFT), gel precipitation, direct agglutination and the

indirect haemagglutination tests (IHA) were the first techniques to be developed. Subsequently, electrophoric techniques like immunoelectrophoresis and antigen carrier methods like the latex or bentonite antigen carrier systems were introduced. The direct and indirect immunofluorescence techniques have been in use since the 1940s and newer techniques including enzyme immunoassays and radioimmunoassays were established in the 1970s (Weiland, 1982).

Similar to most parasitic diseases not every pig with a positive antibody titre habours *Toxoplasma* tissue cysts. Nevertheless, in toxoplasmosis the presence of anti-*Toxoplasma* antibodies has been found to be normally associated with the presence of viable *T. gondii* organisms in the tissues of affected swine (Hirvela-Koski, 1990). It is therefore not surprising that several workers have used antibody detection serological methods for the diagnosis of *T. gondii* infections in both humans and swine. The most promising sero-immunological techniques used so far include the Sabin-Feldman Dye Test (SFDT) (Sabin and Feldman, 1948; Feldman and Lamb, 1966), the indirect fluorescent antibody test (IFAT) (Yamoaka and Matsumoto, 1970; Shirahata and Shimuzu, 1974; Mahajan *et al.*, 1977; Walls and Barnhart, 1978; Gordon, Duncan and Kingsley, 1981), and the enzyme-linked immunosorbent assay ELISA (Waltman *et al.* 1984; Dubey *et al.* 1995; Lind *et al.* 1997). These assays are therefore generally accepted in most laboratories for *T. gondii* seroepidemiological surveys. Other serological assays utilized in diagnosis of toxoplasmosis are the indirect haemagglutination test (IHA) (Jacobs and Lunde, 1957; Hagiwara and Katsube, 1976), latex agglutination test (Lunde and Jacobs, 1967; Ohshima, Tsubota and Hiroaka, 1981) and complement-fixation test (Catar, Bergendi and Holkova, 1969). Recently, the polymerase chain reaction (PCR) which is based on the detection of parasite

DNA in blood and body fluids has been used by various workers (Burg *et al.* 1989; Turner and Savva, 1991;Wastling, Nicoll and Buxton, 1993) as a confirmatory test for demonstration of the parasite in both humans and animals.

Even though the release of parasite antigens and the production of anti-parasite antibodies always follow infection of susceptible hosts by parasites, the situation in immunocompromised individuals could be different. Indeed, detection of anti-*T. gondii* antibodies in individuals with severe immune system dysfunction has been found to be unreliable due to the absence of specific immunoglobulins. Results obtained by different serological methods for the detection of *T. gondii* antibodies have been found to be influenced by the stage of infection and also to be dependent on the antigen preparation used in the assays (Lovgren, Uggla and Morein, 1987). This in part explains the need for confirmatory diagnosis following demonstration of anti-*Toxoplasma* antibodies.

2.14.5.1 *The Dye Test*

Since its description by Sabin and Feldman (1948), the dye test has been standardized (Beverley and Beattie, 1958), adapted to a microscale (Waldeland 1976; Balfour *et al.*, 1982) and remains the definitive test for toxoplasmosis. The dye test is based on the observation that *T. gondii* tachyzoites are lysed when incubated in the presence of immune serum and an accessory factor or complement obtained from normal serum from unexposed individual (Schreiber and Feldman, 1980). Immune lysis occurs and the dead parasites are unable to actively exclude the vital stain methylene blue from their cytoplasm. However, in the absence of specific antibodies in the test serum, parasites are not killed and are able to actively exclude the dye and thus are not stained. The dye test, therefore, is

essentially a neutralization assay, mainly for demonstration of *T. gondii* specific IgG antibodies. The dye test is however not used routinely because it is potentially hazardous to laboratory workers and requires a high degree of technical expertise both for its safe execution and accurate interpretation.

2.14.5.2 *Indirect fluorescent antibody test* (IFAT)

IFAT is a method for localizing antigen by reacting it with fluorochrome-labelled antibodies. The resulting antigen-antibody complex can then be visualised using a fluorescent microscope. The most commonly used fluorochrome is fluorescien isothiocynate (FITC), which fluoresces with a visible greenish colour when excited by ultra-violet light. Fluorescent antibody tests may be either direct or indirect (Benjamini and Leskowitz, 1988). The direct method is primarily for antigen detection and involves identifying the target antigen with fluorescent-labelled specific antibodies. The indirect method (IFAT), involves first probing the target antigens with specific antibodies, referred to as primary antibody, followed by reaction with a fluorochrome-labelled anti-immunoglobulin raised against the host species from which the primary antibody originated. The *T. gondii* IFAT has been reported to be highly specific (Hirvela-koski, 1990). Furthermore, a high correlation has been found between the IFAT and the dye test (Walton, Benchoff and Brooks, 1966; Sulzer and Hall, 1967; Kramer, Chalupsky and Hubner, 1970) which is the confirmatory test for toxoplasmosis. In addition, similar titres are obtained with the same sera in the two tests. In most laboratories, IFAT titres greater than 1:8 or 1:16 are considered to indicate chronic infection, and titers greater than or equal, to 1:1000 suggest the possibility of acute infection (Krahenbuhl and Remington, 1982). Although most workers consider the IFAT and dye tests

to have similar specificities, sera that contain anti-nuclear antibodies normally give false-positive reactions by the IFAT (Araujo *et al.*, 1971). Furthermore, binding of naturally occurring IgM antibodies present in test sera frequently lead to the polar staining phenomenon that may be misinterpreted as positive reactions (Hobbs, Sole and Bettelheim, 1977; Sulzer, Wilson and Hall, 1971), and thus anti-IgG fluorescein-tagged conjugates are preferred. Although it has high sensitivity, IFAT suffers from the disadvantage of subjective interpretation, the use of specialized expensive microscopes, and difficulty to automate which limits the number of samples that can be tested in a given time.

2.14.5.3 *Microplate enzyme-linked immunosorbent assay (ELISA)*

The micro-plate ELISA is a solid-phase assay which employs the property of various plastics (polyvinyl, polyacrylic or polysterene) to adsorb monomolecular layers of proteins onto the surface (Benjamini and Leskowitz, 1988). The binding is non-specific but saturable and is essentially irreversible (Goding and Handman, 1984). The ELISA was first developed by Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971) and later adapted for the diagnosis of protozoan diseases (Voller *et al.*, 1976). The IgG and the double sandwich IgM-ELISA have been used by numerous workers to detect *Toxoplasma* antibodies in blood and body fluids in humans (Naot, Desmonts and Remington, 1981; Hughes *et al.* 1982) and in swine (Waltman *et al.* 1984; Dubey *et al.* 1995; Lind *et al.* 1997). Microplate-ELISA is rapid, requires relatively little technical expertise, can be assessed objectively and lends itself to automation. Waltman *et al.* (1984) found comparable sensitivity and specificity between ELISA and the Dye-Test and asserted that ELISA may be useful as a screening test for toxoplasmosis. Also, a good correlation between ELISA and IFAT has been reported

(Walls, Bullock and English, 1977; Balsari *et al.*, 1980; Violand, Mitchell and Kleeman, 1982; Felice *et al.* 1983; Hirvera-koski, 1990). Generally, IFAT detects antibodies which appear at the early stage of the infection and are directed against membrane surface antigens of intact trophozoites (Karim and Ludlam, 1975), whereas most antibodies detected by ELISA appear later in the course of infection and are directed against internal parasite antigens (Oliver, Dreeson and Prickett, 1983). According to Hirvela-koski (1990) it is difficult to find a satisfactory cut-off which will produce as few false-negative and false-positive results as possible. For this reason, various laboratories use different methods of determining the cut-off, and it is therefore useful to confirm antibody detection ELISA results with a test which has a good specificity like IFAT. The microplate-ELISA is however more suitable for screening large numbers of samples (Waltman *et al.*, 1984; Uggla, 1986).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study areas

Pigs were sampled from the three main ecological zones of Ghana, namely the Coastal Savannah, the Forest Belt and the Guinea Savannah.

3.1.1 The Coastal Savannah

This Zone stretches along the coast of Ghana and has the least amount of rainfall of between 740 and 890mm per year (Dickson and Benneh, 1988). It has two rainfall maxima and mean monthly temperatures similar to the forest belt. The vegetation of this zone is characterized by grass with isolated patches of shrub and few trees. The Coastal Savannah plains have more than 18% of the national cattle population (ILCA, 1992). Sheep, goats and swine are found throughout this Zone.

3.1.2 The Forest Belt

The Forest Belt lies in the southern part of Ghana. It is composed of moist - semidecidious forest which has an annual rainfall of 1,250-1,750mm and evergreen rain forest with over 1,900 mm rainfall per year (Dickson and Benneh, 1988). The belt has two rainfall maxima occurring around May-June and September-October. The mean monthly temperatures range from 26°C to 30°C. This Zone accounted for less than 8% of the national cattle herd with sheep and goats raised in small flocks on farms and households primarily on free range. Exotic swine (mostly large white) are kept under intensive and semi-intensive management whiles local pigs (Ashanti Black) are mainly on free range. The swine population in Southern Ghana was estimated to be approximately 54% (Ghana Livestock Census, Animal Health Veterinary Records, 1995).

3.1.3 The Guinea Savannah

The Guinea Savannah zone lies in the northern part of Ghana. This Zone has a single rainy season from May to October followed by a prolonged dry season (Dickson and Benneh, 1988). The mean annual rainfall is between 1,100mm and 1,150 mm whilst the mean monthly temperatures vary from about 36°C in March to about 27°C in August. The vegetation is typically grassland with few, widely scattered trees. The major livestock present include cattle, sheep, goats and swine. The cattle population in Ghana was estimated to be a little over a million and this Zone alone accounted for over 72% as reported by (Ghana Livestock Census, Animal Health Veterinary Records, 1995). The Guinea Savannah also accounted for 37% and 41% of the national sheep and goat populations, which were about 2 million each respectively. The swine population in this Zone was also estimated to be 169,387 or 46% of the national total.

3.2 Animals sampled, areas sampled from and system of management

A total of 641 apparently healthy pigs were randomly sampled from selected farms within the three ecological zones described previously. Both Ashanti Black (local)) and Large White (exotic) pigs as well as crosses between these two distinctly different breeds (crossbreeds) were sampled. Sample collection was done between October 1997 and May 1998. Data recorded on each animal sampled included the age, sex and identification number. Also, the physiological state of sows (pregnancy and lactation) as well as information on association with other livestock and presence of cats was recorded. On each farm visited, information on management practices such as the type of farming (intensive, semi-intensive or free-range) as well as the availability and source of water was recorded.

The feeding practices including the kind of feed and frequency of feeding were also recorded. Farms were categorized as intensive when pigs were housed all year round in sty with well drained concrete floors and fed with premix concentrate, maize bran and wheat bran supplemented with fishmeal. Farms were categorized as semi-intensive when pigs were supplemented with kitchen leftovers and sometimes watered with pool, river or stream water and also animals occasionally left out in the open. Farms were further grouped into urban and rural farms. This categorization was based not on the system of management but rather on whether farm was sited within a community (cities/towns) or outside including farms in the various villages. Human population was taken into account as well as presence of domestic cats. This was primarily to show whether association with humans and human activity has any influence on dissemination of parasite.

3.3 Blood collection and separation of sera

Blood was collected from the punctured ear vein into sterile 6ml tubes (Falcon,[R] USA). Piglets were restrained by holding them firmly to the floor whiles matured pigs were restrained by passing a rope behind the tusks and round the upper jaw. Such restrained animals became motionless when the rope was pulled and the tension sustained by tying it to a rigid post. An ear was then massaged to enhance the blood flow and then disinfected with 70% ethanol. Sterile needles (21G, Termo Europe N. V), were used to puncture the ear vein and blood allowed to flow freely into labelled collection tubes. Approximately 0.5-3ml of blood was collected depending on how freely the blood flowed. The tubes were then capped and blood allowed to clot in the shade. Clotted blood was centrifuged at 1000g for 10min and a micropipette used to transfer the serum into properly labelled 1.5ml eppendorf tubes

and stored at 4°C (on ice). In the laboratory, sera were stored at -40°C until required for immunoassay.

3.4 Parasites (*T. gondii* tachyzoites)

Dr. Jonathan Wastling of Institute of Biomedical and Life Sciences, University of Glasgow, Scotland provided Tachyzoites of *Toxoplasma gondii* (RH-strain). The Parasites were transported on ice in PBS.

3.5 Maintenance of *T. gondii in vivo*

Inbred 4-8 weeks old female BALB/c mice (colony of the laboratory Animals Research Unit) of the NMIMR were injected with 0.1ml of 1.0 x 10^7 *T. gondii* tachyzoites intraperitoneally. Infected mice usually cuddled together 48 hours post inoculation and had a rough coat. These mice normally had high parasitaemia and were therefore sacrificed after 72 hours. Infected mice were anaesthetized using vaporized diethyl ether in an airtight chamber and then transferred into a sterile flow hood (SterilGARD hood, Baker Company Inc, USA) where they were secured firmly in dorsal recumbency onto a sterilized dissecting board with 26G hypodermic needles. The fur at the mid-line was wet with 70% ethanol to disinfect the area and reduce the possibility of contamination.

A mid-line incision was then made with an iris scissors and the skin retracted at both flanks, stretched and secured firmly with needles onto the board, without opening the peritoneal cavity. Peritoneal exudate containing tachyzoites were sucked using 26G sterile hypodermic syringes with needles and dispensed into sterile PBS, pH 7.4 in the ratio (1:200). The fluids were mixed by inversion of the tube and about 8µl of the suspension

transferred with a micropipette into an improved Neubauer counting chamber to determine the concentration of parasites. The parasite suspension was then washed three times by centrifugation, each at 500g for 10 min. This was done so as to reduce cellular contamination to levels between 1-2% of parasite concentration. For each passage of the parasites in mice, $1x10^6$ tachyzoites/ml was injected intraperitoneally and the harvest done as described.

3.6 Storage of *Toxoplasma* tachyzoites (Cryopreservation)

Peritoneal exudate containing *T. gondii* tachyzoites were suspended in an equal volume of cell-wash medium consisting of PBS with 10% foetal calf serum (FCS) and cellular debris removed by three times centrifugation at 500g for 10min each. The final tachyzoite pellet containing about $1x10^8$ parasites was re-suspended in cell wash medium containing 10% Dimethylsulphoxide (DMSO) and 20% FCS. The suspension was then left at room temperature for 1 hour to equilibrate and 0.5ml aliquots pipetted into 2ml cryopreservation vials (Nunc, Denmark), which were immediately insulated by wrapping in tissue paper and stored at -80°C overnight. The frozen vials were transferred into liquid nitrogen at -196°C.

3.7 Re-suscitation of cryopreserved tachyzoites

A vial of cryopreserved tachyzoites was retrieved from liquid nitrogen at -196°C and thawed rapidly in a water bath at 37°C. Some of the specimen was used to assess the viability of the parasites either on the basis of their motility as observed under the light microscope at x400 magnification or by their ability to infect mice following intraperitoneal

inoculation. Re-suscitated organisms were used within 1hr of thawing.

3.8 The indirect microplate-enzyme-linked immunosorbent assay (ELISA)

The microplate-ELISA was used for the mass screening of pig sera for anti-*T. gondii* IgG antibodies. The assay consisted of the preparation of antigen, standardization, coating and actual ELISA as follows:

3.8.1 Preparation of *T. gondii* tachyzoite antigen lysates

Crude antigen lysates were prepared from tachyzoites of *T. gondii* (RH-strain) propagated *in vitro* and prepared as described earlier. A volume of 1.5ml of sterile distilled water was added to a frozen parasite pellet containing approximately $2x10^9$ *T. gondii* tachyzoites and thawed at room temperature to rupture the organisms. The resulting suspension was immediately frozen again at –80°C for 30min and thawed on ice. This was repeated twice to ensure an appreciable level of parasite rapture. Partially disrupted tachyzoites were subsequently sonicated on ice, using a Tomy Ultrasonic Disrupter U.D – 201, Japan, for 0.1 sec and 0.9sec rest continuously for 5min. This was done at a frequency output of 50%. The crude antigen lysate was aliquoted into eppendorf tubes and centrifuged in a refrigerated centrifuge (Sakuma RSL-05A, Japan) for 30min at 3,500*g*. The supernatant containing soluble antigens was then aliquoted into sterile eppendorf tubes and stored at -80°C until use.

3.8.2 Determination of protein concentration of antigen lysates

Each prepared *T. gondii* tachyzoites antigen (crude) lysate was diluted serially in

distilled water alongside a standard protein, bovine serum albumen (BSA) of known concentration. A 1:4 dilution of Bio-Rad protein assay reagent (Biorad, USA) was also prepared in distilled water and dispensed 100µl/well into a 96-well microtitre plate. Ten microlitre volumes of two-fold dilutions (1:2 to 1:128) of test and standard proteins were then transferred separately into different wells each containing 100µl of the Bio-Rad reagent. Well A1 that contained only the Bio-Rad reagent served as a blank. The contents of the wells were mixed briefly on a plate shaker set to a low speed to avoid foaming and the optical densities read in an ELISA plate reader at an absorbance of 415nm with reference 492nm. A graph of optical density (O.D) versus concentration of standard BSA protein was plotted and the curve used to estimate the concentration of test sample (antigen) by extrapolation. The protein concentration of the *T. gondii* tachyzoites antigen (crude) used for analyzing anti-*T. gondii* antibodies by microplate-ELISA were estimated to be 5.7µg/µl.

3.8.3 Establishment of a microplate-ELISA for detection of anti-*Toxoplasma* antibodies in swine sera

The micro-plate ELISA technique by Voller *et al.* (1976) were adopted with some modifications and used for screening pig sera for specific anti-*T. gondii* IgG antibodies. The optimum working dilutions for the antigen lysates, the test sera and the anti-pig conjugate were determined by checkerboard titration (Voller, Bidwell and Bartlett, 1980). The optimal antigen coating concentration was found to be 1:1000 when diluted in coating buffer (34.5mM $NaHCO_3$ and 15.1mM Na_2CO_3, pH 9.6) and applied 100µl/well. The test sera were used at an optimal concentration of 1:500 and the optimal conjugate concentration was also

1:500.

3.8.4 Antibody detection by microplate-ELISA

Soluble *Toxoplasma* tachyzoites antigen lysate was diluted in coating buffer and dispensed 100µl/well into each of the wells of flat-bottomed 96-well microtitre plates (Dynatech, USA). The plates were agitated for 1min. and the antigen coated by incubation at 4°C overnight.

The antigen coated plates were washed 3-cycles with washing buffer (0.136mM NaCl, 9.7mM Na_2HPO_4, 1.4mM $KHPO_4$, 2.7mM KCl and 0.05% Tween-20, pH 7.4) using an automatic ELISA plate washer (Microplate autowasher, EL 404, Bio-Tek Instruments, Japan). This was to remove excess unbound antigen. The washed plates were flipped empty and incubated with test sera (100µl/well) at 37°C for 1hr. Test sera were diluted 1:1000 in washing buffer containing 1% BSA and dispensed in duplicates. The plates were then washed three times as before and incubated for 1hr at 37°C with rabbit anti-pig IgG conjugated to horseradish peroxidase (HRPO) diluted 1:500 in washing buffer containing 1% BSA (100µl/well). The plates were washed 3-cycles to remove excess unbound conjugate. The presence of bound anti-*Toxoplasma* antibody and enzyme conjugate was then revealed by adding a substrate solution made up of 0.1M $C_6H_8O_7$ and 0.2M Na_2HPO_4, pH 5.0 (phosphate/citrate buffer), 5µl of 70% hydrogen peroxide (H_2O_2) and one tablet (10mg) of O-phenylenedihydrochloride (OPD). Colour was allowed to develop for 30 min. at room temperature in the dark. The substrate solution changed to yellow in positive wells with bound anti-*T. gondii* antibody-enzyme-conjugate complexes. The optical densities were read

with an ELISA-plate reader (MTP-32 Microplate Reader, Corona Electric, Japan) at an absorbance of 492 nm. Well A1 was coated with antigen but not incubated with test serum and thus served as control for blanking. Also, known positive and negative control sera were included on each plate to eliminate or reduce inter-plate variation. A cut-off value of OD≥0.5 was chosen based on the OD ranges obtained in this test and also on the ODs of the positive and negative control sera. Test sera with OD values below the cut-off value were considered to be seronegative.

3.9 The indirect immunofluoresent antibody test (IFAT)

The indirect-fluorescent antibody test described by Benjamini and Leskowitz (1988) was used to determine the reactivity of *T. gondii* tachyzoite surface antigen with anti-*T. gondii* IgG specific antibodies in swine sera. In this assay, the tachyzoites were first coated onto multi-well immunofluorescent slides and the surface antigens probed with primary specific antibodies in test sera. Bound primary antibodies were detected by reaction with a fluorochrome-labelled anti-pig antibody.

3.9.1 Coating of microslides with formaldehyde killed *T. gondii* tachyzoites

Toxoplasma gondii tachyzoites suspension containing $1x10^7$ parasites/ml was used to coat multi-well (12-well) autoclaveable microslide (Cell Line LTD, USA). Parasites were first killed with 0.2% (v/v) fomaldehyde by incubation for 10min and 10µl of the suspension delivered using a micropipette into each well on the microslide. The slides were then air dried at room temperature in a hood (SterilGARD hood, Baker Company, USA) overnight and the specimen fixed by complete immersion in absolute methanol for 10min. The fixed

slides were wrapped in tissue paper in batches of five and stored at -40° C in airtight polyethelene bags containing silicon as a drying agent.

3.9.2 IFA assay

Microslides previously coated with *T. gondii* tachyzoites were retrieved from storage at -40°C and allowed to equilibrate at room temperature on a clean bench for 30min before use. The slides were washed three times by immersion at different periods (1min, 3min and 5min) in PBS, pH 7.4, each with a buffer change. The washing was performed with gentle rocking on a plate shaker. The slides were then tapped gently on a tissue to remove large droplets of buffer and the wells incubated with 20µl of test sera previously titrated two-fold in washing buffer from 1:4 to 1:4096. The samples were tested in duplicates and incubated in a moist chamber at 37°C for 30min. The slides were washed three times as described before and then incubated for 30min with conjugate [rabbit anti-pig fluorescein isothiocyanate (FITC) whole molecule] (Sigma) diluted 1:500 in PBS containing 0.1% trypan blue as counter stain. The slides were washed to remove excess unbound conjugate and mounted using 50% (v/v) glycerol in PBS. The slides were viewed under a fluorescent microscope (Olympus BX 60 F-3, Olympus optical Co. LTD, Japan) with lens (Olympus UP lan F1, 40 X / 0.75 Ph 2, ∞/0.17) at x400 magnification attached to an Olympus BH2-RFL-T3 power supply unit and fitted with a 100watt high pressure mercury burner.

3.10 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of Toxoplasma proteins was performed with the ATTO Corporation Slab Gel Apparatus (Bunkyo-Ku, Tokyo, Japan), following the SDS-Tris-glycine

discontinuous buffer system (Laemli, 1970).

3.10.1 Assembly of slab apparatus and preparation of resolution and stacking gels

The gel casting apparatus had the following components: a pair of glass plates previously wiped clean with absolute alcohol, a U-shaped rubber gasket also wiped with absolute alcohol and four spring clamps. The rubber gasket was used to seal the gap between the pair of cleaned glass plates, one rectangular, and the other notched to form a mould. The glass plates were mounted upright by the clamps.

Acrylamide resolution and stacking gels were prepared as follows and used to prepare (12-15%) gradient gels:

Solution A, 12% resolution gel (one gel)

29.2% (w/v) acrylamide	
1.6% (w/v) N, N'-methylene bis-acrylamide	6ml
1.5M (Tris-aminomethane)-HCL, pH 8.8	3.75ml
1% (w/v) sodium dodecyl sulphate (SDS)	
10% (w/v) Ammonium persulphate (APS)	0.05ml
N, N, N', N'-tetramethylenediamine (TEMED)	0.01ml
Distilled water	5.25ml

Solution B, 15% resolution gel (one gel)

29.2% (w/v) acrylamide	
1.6% (w/v) N, N'-methylene bis-acrylamide	7.5ml
1.5M (Tris-aminomethane)-HCL, pH 8.8	3.75ml

1% (w/v) sodium dodecyl sulphate (SDS)	
10% (w/v) Ammonium persulphate (APS)	0.05ml
N, N, N', N'-tetramethylenediamine (TEMED)	0.01ml
Distilled water	3.75ml

Solution C. stacking gel (one gel)

29.2% (w/v) acrylamide	
1.6% (w/v) N, N'-methylene bis-acrylamide	1ml
1.5M (Tris-aminomethane)-HCL, pH 6.8	1.5ml
1% (w/v) sodium dodecyl sulphate (SDS)	
10% (w/v) Ammonium persulphate (APS)	0.018ml
N, N, N', N'-tetramethylenediamine (TEMED)	0.006ml
Distilled water	3.6ml

Separate resolution gels (solution A, 15%) and (solution B, 12%) were prepared in clean 50ml beakers and degassed in a vacuum chamber for 15min. 12-15% resolution acrylamide gradient gels were then prepared by mixing and pouring the two solutions with the aid of an ATTO chromatographic peristaltic pump (ATTO, Kunkyo-ku, Tokyo, Japan). Equal volumes (11ml) of the gels were poured into the two separate chambers of a gradient gel former. The higher concentrated gel (15%) was poured into the first chamber closer to the outlet and the lower concentrated gel (12%) poured into the adjacent chamber. A stirrer in the higher concentrated gel was started and the tap joining the two chambers opened before dispensing the mixture into the space between the assembled glass plates via a rubber tube. The gel former was immediately rinsed with distilled water to prevent polymerization

of gel in the delivery tubes. The poured gel was overlaid with ethanol and left at room temperature to polymerize.

After polymerization, the overlaid ethanol was poured off and the gel surface rinsed with distilled water. The stacking gel (solution C) was poured on top of the polymerized resolution gel and an appropriate comb fitted carefully to cast the wells for sample application. Care was taken to avoid creating air bubbles. The comb was removed carefully after the stacking gel had polymerized and the gel unit assembled into an ATTO Corporation Cell Electrophoresis apparatus and running buffer (24.8mM Tris, 191.8mM Glycine and 3.47mM SDS) poured into the top (cathode) and lower (anode) chambers.

3.10.2 Preparation of samples and electrophoretic run

Antigen lysates of *T. gondii* tachyzoites (RH strain) prepared as described previously (Section 8.4.1) was diluted with sample buffer (5% SDS, 5% 2-mercaptoethanol, 30% glycerol in 0.5M TBS pH 6.8) at a ratio of 2:1 (sample to buffer). The samples were loaded 25µl/well of protein per lane. Standard molecular weight markers, α-lactalbumen (bovine milk) 14 kDa, Carbonic anhydrase 29 kDa and Albumin (egg ovalbumin) 45 kDa (Sigma, USA) were prepared as directed by the manufacturer and used. The antigen lysates and molecular weight markers were denatured by boiling for 5 minutes and 1 minute, respectively. Denatured sample and markers were centrifuged to remove particulate matter and the sample loaded. Electrophoresis was done at a constant current of 15mA supplied by an electrophorectic power supply (ATTO Corporation, Japan) and maintained until the tracer dye (bromophenol blue) reached the interface of the stacking and resolution gels. The current was then increased to 25mA and kept constant till the tracer dye had barely run out

sandwiched gel and electrophoretic transfer conducted at 30mA constant current overnight. The blotted PVDF membrane was removed, cut into strips and immunoassayed.

3.10.4 Analysis of transferred *T. gondii* antigens by ELISA

Antigen blotted PVDF strips were blocked by incubation in blocking solution [5% skimmed milk in Tris-buffer (8.0g NaCl, 0.2g KCl and 3g Trisma base (pH 8.0) for 30min. The strips were then incubated in test sera that were ELISA and/or IFAT positive and a reference positive serum (WHO 3rd International Standard anti-Toxoplasma serum, Human, Statensserum Institut, Dk-2300, Copenhagen). Sera were diluted 1:125 in blocking buffer and incubated at room temperature for one hr. on a gentle rocker. After incubation the strips were washed three times with Tris-buffer, pH 8.0 to remove excess unbound antibodies. The membrane strips were then incubated in Rabbit anti-pig IgG antibody (whole molecule) diluted 1:500 in blocking buffer and incubated for 1hr. Membrane strips were washed as described above and incubated in a substrate 3,3-diaminobenzidine (DAB) substrate solution (6mg DAB, 10µl of 35% H_2O_2 in 10ml TBS) until the positive bands were clearly stained brown. Membrane strips were then washed in excess distilled water and air dried on filter paper. The molecular weights of the antigen bands were determined by extrapolation on a curve plotted with the standard molecular weight markers.

CHAPTER 4

RESULTS

4.1 Validation of microplate-ELISA for the detection of anti-*Toxoplasma* antibodies in swine sera using IFAT

One hundred sera collected from pigs randomly selected from the three different ecological zones in Ghana (Coastal Savannah, Forest belt and Guinea Savannah) were analyzed by microplate-ELISA, and then by IFAT so as to demonstrate the presence of anti-*T. gondii* antibodies to surface antigens of the parasites. Table 2 summarizes the anti-porcine IgG detection rates obtained by the two assays. Fifty-five (55) sera tested positive in both IFAT and microplate-ELISA as judged by either a complete green fluorescence extending around the entire periphery of the parasites in IFAT or based on a cut-off Optical Density (OD) value greater or equal to 0.5 (≥ 0.5). Thirty-six (36) sera tested negative in both IFAT and microplate-ELISA. As shown in Figure 4, six (6) sera which tested positive in IFAT, were negative by microplate-ELISA whiles three (3) sera that tested negative with IFAT were positive by microplate-ELISA.

From Table 2, the sensitivity and specificity of microplate-ELISA as compared to IFAT as a gold standard assay were:

Sensitivity=55/61 x 100%

=90.2%

Specificity=36/39 x 100%

=92.3%

The Optical density measurements obtained by detection of anti-*T. gondii* antibodies using the microplate-ELISA were converted to enzyme immunoassay units (EIU) and used to illustrate the differences between IFAT and ELISA results:

$$\text{EIU} = \left| \frac{\text{(O.D of test serum) – (O.D of negative control serum)}}{\text{(O.D of positive control serum) – (O.D of negative control serum)}} \right| \text{ X 100\%}$$

Figure 4 shows the levels of agreement between microplate-ELISA and IFAT based on EIU. 30 sera with EIU less or equal to 10.0 that tested negative with IFAT (titre ≤ 1/4) were also negative by microplate-ELISA. On the other hand, 39 sera with EIU values of 50.1 or more were all positive in the IFAT. Out of 31 sera with EIU values ranging between 10.1and 50.0, 6(19.4%) were positive in IFAT but negative by microplate-ELISA. However, 3(9.7%) negative by IFAT tested positive with microplate-ELISA (Table 3). Generally, there was a high correlation between IFAT and ELISA (r=0.82)

Table 2: Comparison of IFAT and microplate-ELISA results in detection of anti-*T. gondii* antibodies using one hundred randomly selected pig sera

		IFAT		
		+	-	
ELISA	+	55	3	58
	-	6	36	42
Total		61	39	100

Optical density expressed in Enzyme Immuno-assay units (EIU)	<1/4	1/4	1/16	1/64	1/256	1/1024
130.1-140.0					1	
120.1-130.0			1			1
110.1-120.0					3	1
100.1-110.0					2	
90.1-100.0			1	2	5	1
80.1-90.0				1	7	
70.1-80.0			3	1	1	
60.1-70.0			1	2		
50.1-60.0			2	1	2	
40.1-50.0	1		2	1	1	
30.1-40.0	2		6	1		
20.1-30	5		4	1		
10.1-20.0	5	1	1			
0.1-10.0	9	3				
<0	17	1				

IFAT titre (fourfold)

Figure 4: **Agreement between IFAT and microplate-ELISA in the detection of anti-*T. gondii* antibodies with respect to 100 swine sera examined.**

Dotted lines represent cut-off points for IFAT and Microplate-ELISA.

4.2 The specificity of anti-*T. gondii* antibodies of diagnostic importance as demonstrated by their reactivity with *T. gondii* tachyzoite antigens using the Western immunoblot assay

The aim of the work reported here was to identify anti-*T. gondii* antibodies of diagnostic importance as revealed by their reactivity with *T. gondii* tachyzoite antigens in the western immunoblot assay. This was achieved by comparing reactive antigen band patterns identified by anti-*T. gondii* antibodies in pig sera to a WHO *T. gondii* positive human reference serum (Figure 5). The molecular weights of antigens identified by antibodies in both the human reference serum and pig positive sera ranged from 12 to 74 kDa. Antibodies identified in the reference serum were located in 12 different bands, 74, 61, 52, 47, 36, 32, 30, 26, 24, 21, 19 and 12 kDa. On the other hand, antibodies in pig serum I, which was IFAT/ELISA positive, identified antigens located in protein bands with molecular weights 74, 61, 52, 47, 36, 30, 26, 19 kDa compared to 74, 61, 32, 30, 26, 24 kDa by pig serum II (Table 3). The ELISA, IFAT seronegative pig serum (serum III) showed no visible band patterns. Except for a 21 and 12 kDa antigen bands, both the human reference serum and the positive pig sera identified similar antigens. All the positive test sera showed strong reactivity with the 30 kDa antigen.

Table 3: Estimated molecular weights (kDa) of *T. gondii* tachyzoite antigens recognized by sera from pigs and a human reference serum in the western immunoblot assay

Reference Serum*	ELISA, IFAT positive pig sera		ELISA, IFAT negative pig serum
	Serum I	Serum II	
74	74	74	-
61	61	61	-
52	52	-	-
47	47	-	-
36	36	-	-
32	-	32	-
30	30	30	-
26	26	26	-
24	-	24	-
21	-	-	-
18	19	-	-
12	-	-	-

Key:

***Human reference serum: WHO** *T. gondii* infected human standard reference serum.
kDa: kiloDalton.

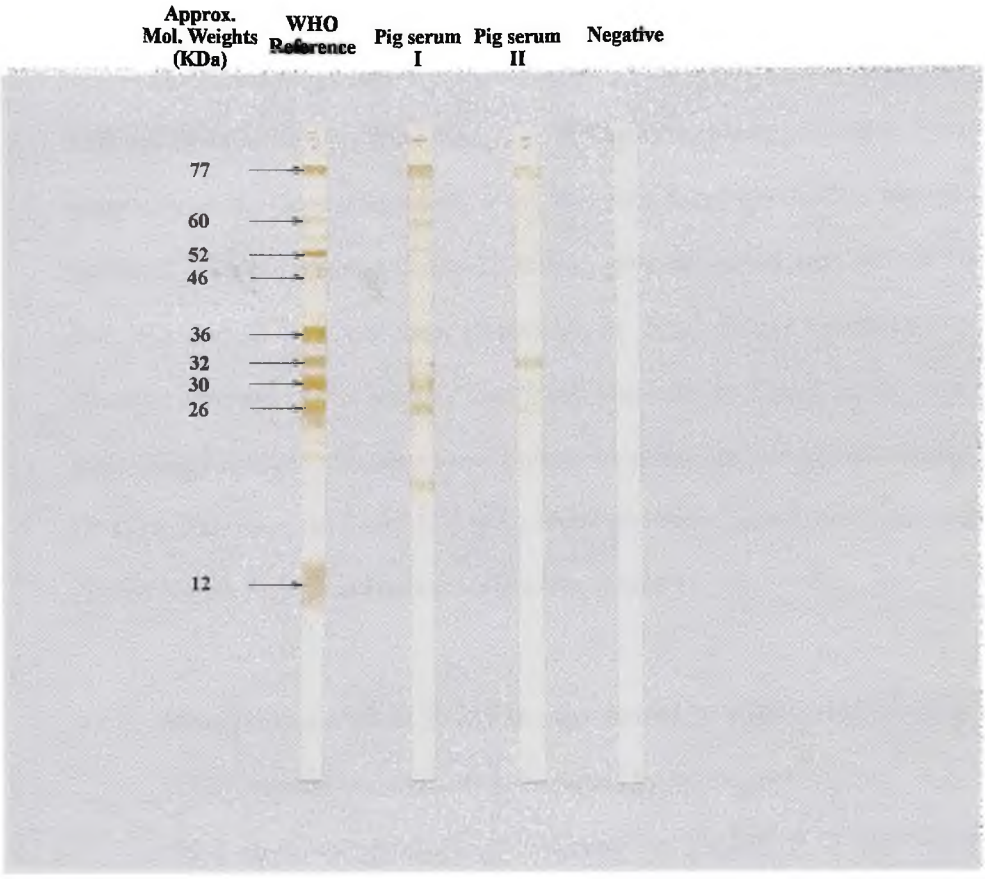


Figure 5: Western blots of sonicated *T. gondii* tachyzoite (RH strain) proteins separated by SDS-PAGE and probed with pig test sera and WHO *Toxoplasma* positive human reference serum

Pig sera I and II are Microplate ELISA and IFAT positive,
Negative serum is ELISA and IFAT negative

4.3 The prevalence of anti-*T. gondii* antibodies in pigs in different ecological zones of Ghana as determined by microplate-ELISA

The prevalence of anti-*T. gondii* antibodies obtained for pigs sampled randomly from different farms in the three ecological zones of Ghana are shown in Table 4. Out of 214 pigs sampled from the Coastal Savannah, 94(43.9%) were found to have IgG specific antibodies against *T. gondii* compared to 39/128(30.5%) from the Forest Belt and 127/299(42.5%) from the Guinea Savannah zone (Figure 6). A 39% national seroprevalence rate was therefore obtained. As shown in Table 4 and illustrated in Figure 5, the prevalence rates were similar in the two Savannah zones where the difference was not statistically significant ($P>0.05$). However, the Forest Belt had a lower prevalence, which was statistically different ($P<0.05$) from each of the two Savannah zones (Table 4).

4.4 Seroprevalence of *T. gondii* in pigs reared in urban and rural areas in the different ecological zones as determined by microplate-ELISA

The *T. gondii* seropositivity rates obtained for pigs on urban and rural farms in the different ecological zones of Ghana are shown in Table 5 and presented graphically in Figure 6. In the Coastal Savannah zone the prevalence of toxoplasmosis 58(45.7%) was higher in pigs reared on urban farms as compared to those on rural farms 36(41.4%), Table 5, but the difference was not significant ($P>0.05$). Likewise, the toxoplasmosis prevalence in the Forest Belt was higher in urban pigs (32.5%) than in the rural animals ($P>0.05$). However, urban pigs in the Guinea Savannah had a lower prevalence rate ($P<0.05$) of *T. gondii* infection than the pigs from the rural farms (Figure 6). The toxoplasmosis prevalence rates for the urban pigs was significantly higher ($P<0.05$) in the Coastal Savannah area

compared to the Guinea Savannah, whereas the converse was true for the rural pigs in the two zones (Table 5). Also, the toxoplasmosis prevalence rate for rural pigs in the Forest belt (26.7%) was statistically lower ($P<0.05$) than that of the Guinea Savannah zone (46.8%).

Table 4: Prevalence of anti-*T. gondii* antibodies in pigs from different ecological zones of Ghana as determined by microplate-ELISA

Zone	No. Sampled	No. Positive	Prevalence (%)*
Coastal Savannah	**214**	**94**	**43.9[a]**
Forest Belt	**128**	**39**	**30.5[b]**
Guinea Savannah	**299**	**127**	**42.5[a]**

Key:

*percentages followed by different alphabets are significantly different ($P<0.05$).

Table 5: **Seroprevalence of *T. gondii* in pigs reared in urban and rural environments in the different ecological zones of Ghana as determined by microplate-ELISA**

Zones	Area	No. Sampled	No. positive	Prevalence*
Coastal Savannah	Urban	127	58	45.7[a]
	Rural	87	36	41.4[a]
Forest Belt	Urban	83	27	32.5[a]
	Rural	45	12	26.7[a]
Guinea Savannah	Urban	83	26	31.3[b]
	Rural	216	101	46.8[c]

Key:

*Percentages followed by different alphabets are significantly different ($P<0.05$).

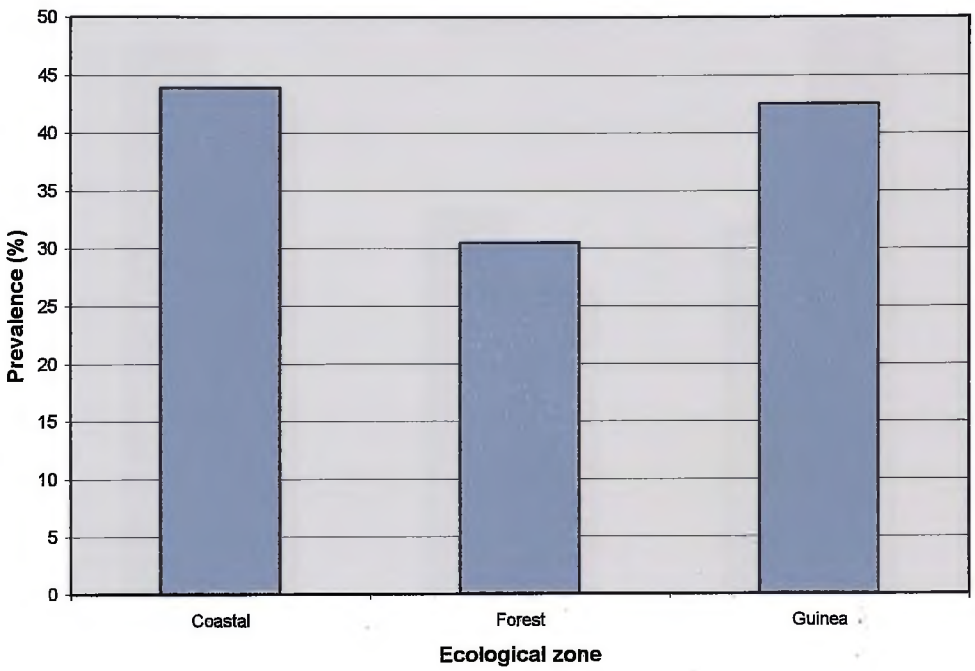


Figure 6: Prevalence of anti-*T. gondii* antibodies in pigs in the different ecological zones of Ghana as determined by microplate-ELISA.

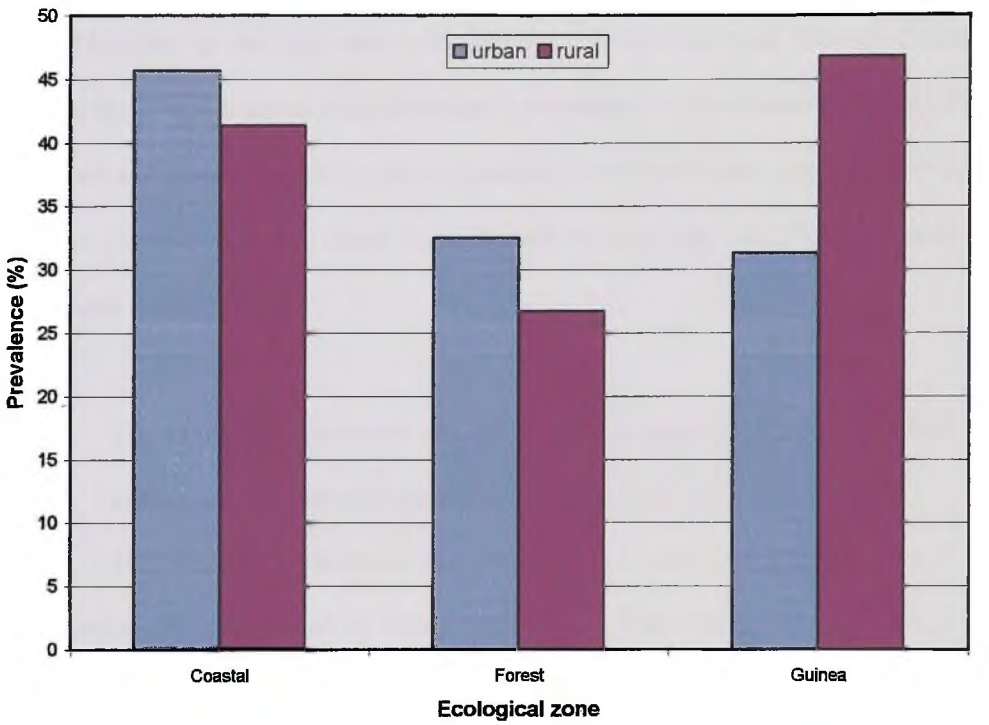


Figure 7: Seroprevalence of *T. gondii* in pigs reared in urban and rural areas in the different ecological zones of Ghana as determined by microplate-ELISA.

4.5 The relationship between age of pig and seroprevalence of *T. gondii* as determined by the microplate-ELISA

The relationship between age of pig and seroprevalence of *T. gondii* is shown in Table 6. As shown graphically (Figure 8), age appeared to have a significant effect on *T. gondii* seroprevalence in pigs, which increased progressively with age. As shown in Table 6, 65 out of the 135 older pigs (ages ≥13 months) sampled (48.1%) were positive, compared to 13/114 (11%) of the pigs aged 1-5 months. For the pigs aged between 6-12months, 67(36.4%) tested positive. The differences in prevalence of toxoplasmosis between the three defined age groups (1-5, 6-12 and ≥13 months) were statistically significant ($P<0.05$). *T. gondii* infection rate was found to be highest in older pigs (48.1%) and lowest in the youngest animals (11%).

4.6 The relationship between sex, breed of pigs and prevalence of anti-*T. gondii* antibodies infection as determined by microplate-ELISA

The relationship between sex, breed of pig and prevalence of anti-*T. gondii* antibodies are summarized in Tables 7 and 8. As illustrated in Figure 9, the *T. gondii* infection rate 201/438 (45.9%) was higher in female pigs than in male pigs 59/203 (29.1%). The difference in antibody prevalence between male and female pigs was statistically significant ($P<0.01$) (Table 7).

Out of a total of 464 Large White pigs sampled and tested for anti-*T. gondii* antibodies, 180(38.8%) were seropositive whereas 29/68(42.6%) of the Ashanti Black pigs tested had antibodies against *T. gondii.* 51(46.8%) out of 109 crossbreeds tested were seropositive (Table 8). As indicated in Table 8, Large White pigs had the lowest

toxoplasmosis infection rate (38.8%) and the crossbreeds had the highest (46.8%). The difference in prevalence rate between the Large White and Ashanti Black pigs, as well as that between Ashanti Blacks and the crossbreeds was not statistically significant ($P>0.05$). However, the prevalence difference between Large White and the crossbreeds was significant ($P<0.05$).

Table 6: The relationship between age of pigs and seroprevalence of *T. gondii* in pigs as determined by microplate-ELISA

Age	Pigs Sampled	No. Positive	Prevalence (%)*
1-5 months	**114**	**13**	**11[a]**
6-12 months	**184**	**67**	**36.4[b]**
≥13 months	**135**	**65**	**48.1[c]**

Key:

*Percentages followed by different superscripts are significantly different from each other ($P<0.05$).

Table 7: The relationship between sex of pig and the prevalence of anti-*T. gondii* antibodies as determined by microplate-ELISA

Sex	No. Sampled	No. Positive	Prevalence (%)*
Male	203	59	21.1[a]
Female	438	201	45.9[b]

Key:

*Percentages followed by different superscripts are significantly different from each other ($P<0.01$).

Table 8: The relationship between breed of pig and the prevalence of anti-*T. gondii* antibodies as determined by microplate-ELISA

Breed	No. Sampled	No. Positive	Prevalence (%)*
Large White	**464**	**180**	**38.8[a]**
Ashanti-Black	**68**	**29**	**42.6[a]**
Crossbreeds)	**109**	**51**	**46.8[b]**

Key:

*Percentages followed by different superscripts are significantly different ($P < 0.05$).

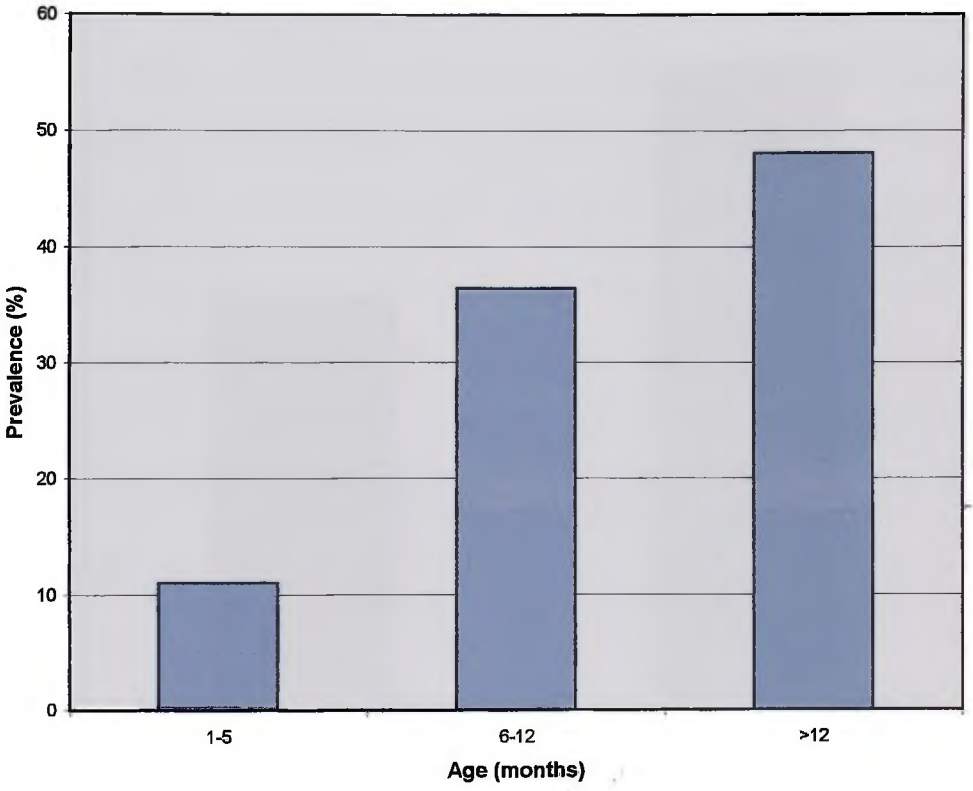


Figure 8: The relationship between age of pig and seroprevalence of *T. gondii* as determined by microplate-ELISA.

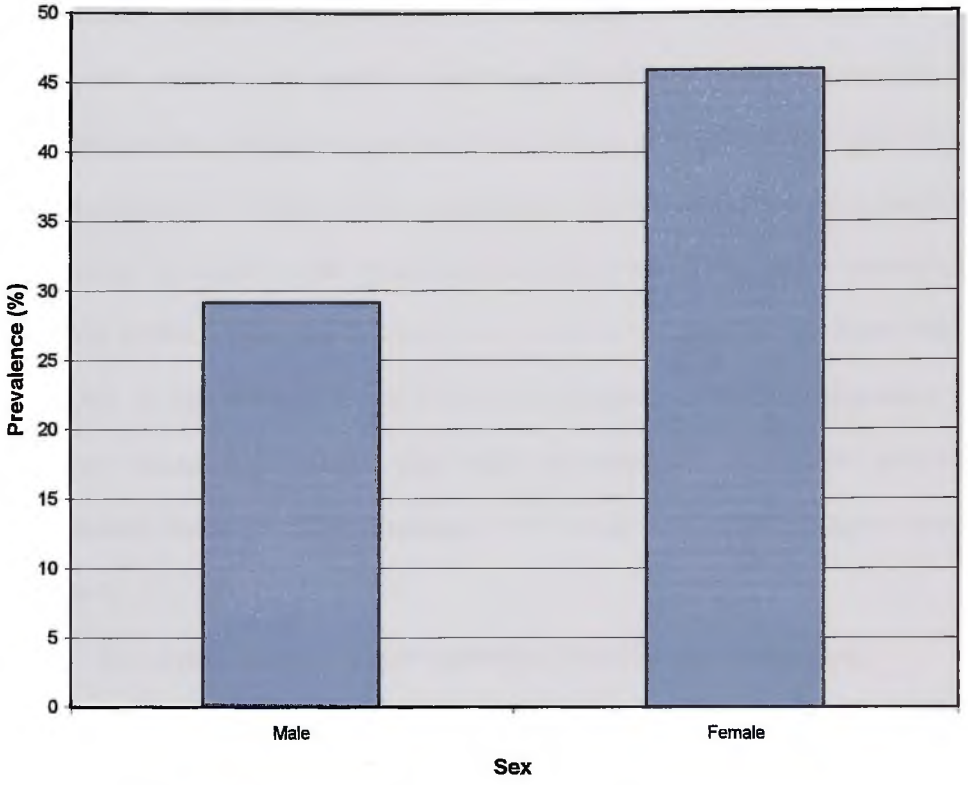


Figure 9: The relationship between sex of pig and seroprevalence of *T. gondii* as determined by microplate-ELISA.

4.7 Effect of system of management of pigs on the prevalence of *T. gondii* infection as determined by microplate-ELISA

A summary of the effect of the system of management on the seroprevalence of *T. gondii* in pigs sampled from the three ecological zones is presented in Table 9. In the intensive management system, pigs sampled from the Coastal Savannah showed a significantly higher ($P<0.05$) antibody prevalence than those from the Guinea Savannah. There was, however, no significant difference ($P>0.05$) in antibody prevalence in pigs sampled from the Coastal Savannah and Forest belt and also between the Forest belt and the Guinea Savannah (Table 9). In the semi-intensive management system, pigs sampled from the Coastal Savannah had the highest antibody prevalence (70%), which was significantly different ($P<0.05$) from that obtained in the Forest belt. Except for the Forest belt where there was no significant difference in antibody prevalence ($P>0.05$) in pigs under the two different management systems, pigs under semi-intensive management generally had significantly higher antibody prevalence ($P<0.05$) than those under intensive management (Table 9).

4.8 Prevalence of anti-*T. gondii* antibodies in market and breeder pigs

Table 10 summarizes the seroprevalence rates of *T. gondii* in market pigs, which were sampled from a commercial farm and breeder pigs from a livestock breeding station in the Coastal Savannah zone. Breeder pigs had a higher *T. gondii* infection rate than market pigs. Whilst 6/31 (19.4%) of the pigs sampled from the commercial farm had antibodies against *T. gondii*, as many as 18/27 (66.7%) of the breeder animals were seropositive. The difference in seropositivity rates between the commercial and breeder pigs was significantly different ($P<0.01$).

Table 9: Effect of system of management of pigs on the prevalence of anti-*T. gondii* antibodies in the three different ecological zones of Ghana as determined by microplate-ELISA

Systems of management	Ecological Zone	No. Sampled	No. Positive	Prevalence* (%)
Intensive	CS	31	6	19.4[a]
	FB	24	6	25.0[ab]
	GS	61	24	39.3[b]
Semi-intensive	CS	60	42	70[a]
	FB	19	8	42.1[b]
	GS	58	34	58.4[ab]

Key:

CS: Coastal Savannah
FB: Forest Belt
GS: Guinea Savannah

*Percentages followed by different superscripts are different from each other ($P<0.05$).

Table 10: Prevalence of antibodies against *T. gondii* in Market and Breeder pigs as determined by the microplate-ELISA

Farm	No. Sampled	No. Positive	Prevalence (%)*
Commercial	**25**	**6**	**16.1[a]**
Breeder	**27**	**18**	**66.7[b]**

Key:

*Percentages followed by different superscripts are significantly different ($P<0.01$).

4.9 Relationship between *T. gondii* seropositivity rates and abortions/stillbirths in sows on a pig breeding station

Table 11 shows the relationship between prevalence of anti-*T. gondii* antibodies as determined by microplate-ELISA and abortions/stillbirths in 20 sows on a farm where abortions and stillbirths were reported to occur frequently. Of the 20 sows, 14 (70%) that had aborted before had specific antibodies against *T. gondii*. Also, 2 (10%) of the pigs were pregnant and both had specific antibodies against *T. gondii*. Three other sows (15%) were seropositive and had farrowed one piglet each. The pig that had the highest antibody titre in this study was a sow that had lost all its seven piglets through stillbirth.

Table 11: The relationship between *T. gondii* seropositive rates and abortions/stillbirths in sows on a pig breeding station as determined microplate-ELISA

Identification Number	ELISA reactivity	Abortions/stillbirths record
175	-ve	Farrowed twice (some stillbirths reported)
184	+ve*	Farrowed 7 piglets (all stillbirths)
166	+ve	Pregnant (not farrowd before)
S137	+ve	Farrowed 8 piglets (1 died)
199	+ve	Pregnant (not farrowed before)
177	+ve	Farrowed 9 piglets (4 died)
153	+ve	Farrowed 8 piglets (1 died)
186	+ve	Farrowed 1 piglet
002	+ve	Farrowed 9 (2 died)
S103	+ve	Farrowed 1 piglet
027	+ve	Farrowed 9 (4 dead)
01030	+ve	Farrowed 5 (1 died)
163	+ve	Pregnant (not farrowed before)
S114	+ve	Farrowed twice (some died)
144	+ve	Farrowed 9 piglets (all stillbirth)
167	+ve	Farrowed once 8 piglets (all died)
170	+ve	Aborted
S134	+ve	Farrowed once 8 piglets (all died)
200	+ve	Farrowed 9 (all died)
202	+ve	Farrowed 3 (all died)

Key:

+ve = seropositive; -ve = seronegative; *had highest antibody titre in study.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.0 Discussion

In order to plan effective control strategies or determine the impact on livestock productivity due to toxoplasmosis, there is the need to obtain accurate epidemiological data on aspects such as the disease prevalence, incidence and the extent of distribution in livestock. The aim of this study was, therefore, to carry out a nationwide survey to estimate the prevalence of *T. gondii* infection in pigs in the three defined ecological zones of Ghana namely, the Coastal Savannah, the Forest Belt and the Guinea Savannah.

ELISA has been reported to be a rapid, sensitive and an easily performed test for routine serology and for screening of anti-*Toxoplasma* antibodies (Walls, Bullock and English, 1977; Camargo *et al.*, 1978 ;Waltman *et al.* 1984; Dubey *et al.*, 1991; Dubey *et al.*, 1995; Lind *et al.*, 1997). However, it is difficult to find a satisfactory cut-off for an ELISA which will produce few false-negative and false-positive results as possible (Hirvela-koski, 1990). It was therefore suggested that when ELISA is used in screening anti-*Toxoplasma* antibodies in porcine sera, validity of the test should be confirmed with a test, which has a good specificity like IFAT or the Dye test (Hirvela-koski, 1990). Though the PCR is also highly sensitive and specific and directly detects parasite DNA, it has a drawback of being highly technical and cumbersome and therefore is not practicable to use for mass screening for toxoplasmosis.

Validation of the ELISA used in this study was achieved by comparing it with IFAT as a reference test. In earlier studies, several authors including Camargo *et al.* (1978), Mineo, Camargo and Ferreira (1980), Lovgren, Uggla and Morein (1987) and Verhofstede, Sabbe and van Renterghem (1987) reported that different serological tests for the diagnosis

of toxoplasmosis are influenced by the stage of infection and also by the antigen preparation used. Whilst IFAT employs cell surface antigens of whole parasites, the antigen in ELISA consist of the soluble fraction of disrupted trophozoites. In spite of these, ELISA has been reported to have a high correlation with IFAT for detecting anti-*Toxoplasma* IgG antibodies (Walls, Bullock and English, 1977; Balsari *et al.*, 1980; Violand, Mitchell and Kleeman, 1982; Felice *et al.* 1983). The results obtained with this study agreed with this finding as the level of agreement between ELISA and IFAT was high ($r=0.82$) and the difference in seropositivity rates was not statistically significant ($p>0.01$). Karim and Ludlum (1975) reported that the IFAT detect mostly antibodies, which appear at the early stage of infection against antigenic components of the membrane of intact trophozoites. On the other hand, the ELISA could, as well, detect antibodies that appear later in the course of infection (Oliver *et al.* 1983). This may partly explain the observation made in this study whereby some ELISA negative sera tested positive with IFAT, and also some sera with low OD values had relatively high IFAT titres. According to Hobbs, Sole and Bettelheim (1977) and Sulzer, Wilson and Hall (1971) fluorescence tagged antibody used in diagnosis of toxoplasmosis should be only anti-IgG. This is necessary to avoid misinterpretation of polar staining due to naturally occurring IgM antibodies. In the present study, though anti-pig IgG FITC was used, some few sera showed polar fluorescence but were considered as none-specific staining and thus negative. Notwithstanding, the sensitivity and specificity of the ELISA compared to IFAT were high, 90.2% and 92.3%, respectively.

All the routine toxoplasmosis diagnostic assays are based on the demonstration of anti-parasite antibodies. This is so even though it is generally accepted that the presence of anti-parasite antibodies does not necessarily indicate active infection. However, in

toxoplasmosis, Dubey *et al.* (1995) and Wingstrand *et al.* (1997) reported that tissue cysts as well as specific antibodies tend to persist for prolonged periods in most mammals (including swine). Hence, the demonstration of seropositivity usually correlates well with meat infectivity. Indeed, the measurement of specific antibodies to *Toxoplasma* in swine sera by ELISA was reported by Lind *et al.* (1997) to have important application in the determination of actual infection prevalence and also as a tool for the surveillance of toxoplasmosis infection in swine herds. The microplate-ELISA is ideally suited to test large numbers of animals basically due to the fact that it does not involve serial dilution of specimen as in other serological tests like the DT, IFAT and IHAT. Furthermore, the microplate-ELISA requires relatively little technical expertise and lends itself to automation.

On comparison of anti-*Toxoplasma* antibody detection assays, Dubey (1990) cautioned that care should be taken in any attempt to directly compare different assay results. This is because differences in mode of sample collection, number of animals examined, and the type of *T. gondii* serotypes used could influence the results. He however indicated that minimizing hemolysis of blood samples collected and avoiding repetitive freeze/ thaw could reduce such variability between assay results. In this study therefore, collecting the samples directly into siliconized tubes and allowing the serum to form in the shade minimized haemolysis of blood specimen. Also, the sera were aliquoted to avoid repetitive freeze and thaw so as to make the ELISA and IFAT results comparable.

In the present study, a national toxoplasmosis prevalence rate of 39% was obtained. Prevalence of anti-*T. gondii* antibodies on individual farms ranged from 16.1% to as high as 66.7%. In a similar nationwide serosurvey conducted to estimate the prevalence of *T. gondii* infection in swine in the United States, Dubey *et al.* (1991) found an average seroprevalence

rate of 24.0%. In an earlier epidemiological survey conducted in Georgia, United States by Dreesen and Prestwood (1980), 31.0% of 1,241 pigs were positive for anti-*T. gondii* antibodies. In France Guillo and Desmonts (1960) reported a 30.0% nationwide *T. gondii* seropositivity in pigs, whilst in a review Fayer (1981) estimated a 29.0% average *T. gondii* infection rate worldwide. The national seroprevalence rate of 40.6% obtained for Ghana in this study is therefore on the higher side. Nevertheless, Dubey *et al.* (1992) reported a prevalence of 48.5% in Hawaii State in the United States, and Dubey (1990) noted prevalence rates ranging between 5-69% in different county's in Illinois State also in the USA. The overall prevalence of toxoplasmosis differs from region to region influenced mainly by climate and the system of management.

The seroprevalence of *T. gondii* in pigs obtained for the Coastal Savannah, Guinea Savannah and Forest Belt in Ghana were 43.9%, 42.5% and 30.5%, respectively. Statistically, there was a significance difference ($P<0.05$) in the *T. gondii* seroprevalence rates between each of the two Savannah zones and the Forest Belt, which had a lower prevalence. Gibson and Coleman (1958), Fleck (1972) and Marquart and Demaree (1985) reported that *T. gondii* prevalence is higher in moist and warm environments than cold and dry environments. This they attributed to a longer survivability and infectivity period for oocysts in moist/ warm environments compared to cold/ dry areas. Interestingly, in this study, the Forest Belt had the lowest *T. gondii* seropositivity rate (30.5%) though it had the highest annual rainfall and relative humidity compared to the two Savannah zones. This contrasting observation may be explained by differences in the system of management. Whilst pigs on farms in the Forest Belt were housed in enclosed areas (sty's) all year round those in the Guinea Savannah, except those at the breeding stations, were occasionally

released into the fields and sometimes the town/village to feed and water. The animals are released because the Savannah's are characterized by long dry periods during which farmers find it difficult to obtain herbage and water for the confined animals. The roaming animals are exposed to oocysts deposited by felines and disseminated by machinery or feet of animals (Fayer, 1981) leading to higher *T. gondii* infection rates.

Weigel *et al.* (1995) reported that confinement housing could decrease or eliminate exposure of pigs to cats. Fayer (1981) had earlier asserted that the dispersal of oocysts in the environment has a direct bearing on the level of contamination to livestock. The shedding of infective oocysts in cat faeces is, therefore, generally considered to be a primary factor in the dissemination of toxoplasmosis (Frenkel, Ruiz and Chinchilla, 1975). In a study on the incidence of blood-borne parasites of livestock in southern Ghana, Assoku (1979) identified poor system of management as important. According to Brunner and Gillespie (1973) animals kept under the free-range system are usually exposed to severe environmental stress factors such as fatigue, undercurrent infections and frank bad management, which leads to their greater susceptibility to infection. This study showed a significantly lower *T. gondii* seroprevalence rate in pigs reared under intensive management compared to those kept semi-intensively. In their work to compare the *T. gondii* seroprevalence rates in pigs on two farms, Lubroth, Dreesen and Ridenhour. (1982-1983) found a 28% and 0.8% antibody prevalence for the extensive and intensively managed farms, respectively. However, *T. gondii* antibody prevalence in small mammals trapped from both farms was similar, suggesting that a rodent-pig cycle on the farm did not account for the differences in prevalence between the two farms. In this study the feeding of uncooked kitchen leftovers to pigs was found to be a common practice on most rural farms

especially in the Coastal Savannah. In an earlier study, Weinman and Chandler (1954, 1956) found a 48% *T. gondii* seroprevalence rate on an intensively managed farm where uncooked garbage was fed to pigs, and rats were also abundant in swine houses. They concluded that, contaminated garbage and probably a rodent-pig cycle could have accounted for the seemingly high anti-*T. gondii* antibody prevalence.

In a study on swine toxoplasmosis, Dubey *et al.* (1986a) found no difference in *T. gondii* seroprevalence in different breeds of pigs. However, in the present investigation, there was a significant difference ($P<0.05$) in *T. gondii* antibody prevalence between Large White (38.8%) and crossbreed pigs (46.8%). This difference in antibody prevalence did not appear to be due to difference susceptibility of the breeds as most crossbreed pigs were kept under extensive management where they had high association with cats and other livestock species. Furthermore, they were fed with kitchen leftovers that could easily be contaminated with *T gondii* oocysyts. On the other hand the exotic Large White pigs had minimal exposure to *T. gondii* oocysts under intensive management. The high antibody prevalence in the indigenous Ashanti Black pigs was not surprising as they were mostly kept under extensive management.

There were cats on all the farms visited (both urban and rural farms). Fayer (1981) reported that *Toxoplasma* oocysts deposited by felines in city and suburban streets may easily be dispersed by wind and rain and also through storm sewers and streams. In the Coastal Savannah, it was observed that most rural pig farmers used stream water and water from pools that were accessible to other livestock to water their pigs. Such water, which could easily be contaminated with viable *T. gondii* oocysts, is a potential source of infection to the pigs. The high anti-*T. gondii* antibody prevalence obtained in the Coastal Savannah

zone was therefore not surprising. Indeed, on a relatively large commercial pig farm in the Coastal Savannah, cats were kept at the feed storage facility to keep rodents away. This farm had the highest *T. gondii* seroprevalence rate (66.7%) in this survey. The contribution of cats to transmission of *T. gondii* under these circumstances was reported by Timofeev and Akulov (1975) who indicated that infective cats may shed as many as 20 million oocysts per day in about 20g of faeces to contaminate livestock feed.

The present investigation showed a significantly higher *T. gondii* infection rate ($P<0.01$) in female pigs than in male pigs. This was in agreement with Brunner and Gillespie (1973) that female animals are more susceptible to infections because of the stresses of gestation, parturition and lactation. Also, the present study showed a significantly higher *T. gondii* seroprevalence in breeder pigs compared to market pigs, with the two established pig breeding stations having the highest prevalence rates in the country. Dubey *et al* (1991) and Zimmerman *et al.* (1990) reported that breeding swine are more likely to have *T. gondii* antibodies than finishing or market pigs. This they explained to be a reflection of an increased probability of exposure to *T. gondii* with increased age. The present investigation showed a significantly higher *T. gondii* seroprevalence rate ($P<0.05$) with the oldest pigs having the highest antibody prevalence. Evidence has been presented to suggest that the increase in toxoplasmosis prevalence with age may be influenced by susceptibility to infection. In mice, Crandall (1975) reported decreased resistance to infection with age. Also, Gardner and Remington (1977) investigated the relationship between age of mice and resistance to *Toxoplasma* infection and found a decreasing resistance with age, which became more pronounced as the animals aged further.

In the present study, there was a positive relationship between prevalence of anti-*T.*

gondii antibodies and abortions and stillbirths on a farm where abortions/stillbirths were reported to occur frequently. 70% (14) out of 20 sows that had aborted before had specific antibodies against *T. gondii*. Also, the pig that had the highest antibody titre by ELISA had lost all its seven piglets through stillbirth. The high probability of involvement of toxoplasmosis in the recorded abortions/stillbirths is suggested by the results of earlier studies in pigs (WHO, 1969; Farell *et al.*, 1952; Cole *et al.*, 1953, 1954; Koestner and Cole, 1960), and sheep (Siim, Biering-Sorenson and Moller, 1963; Dubey and Beattie, 1988). These studies showed that toxoplasmosis is a worldwide zoonotic disease responsible for abortions and stillbirths in animals, and demonstrated the involvement of *T. gondii* in swine abortions, stillbirths, premature births and death of piglets soon after birth (Farell *et al.*, 1952; Cole *et al.*, 1953, 1954; Koestner and Cole, 1960). Also, toxoplasmosis is reported to be the single most important cause of infectious abortions in sheep especially in countries with temperate moist climate such as New Zealand and Norway (Siim, Biering-Sorenson and Moller, 1963; Dubey and Beattie, 1988).

The specificity of anti-*T. gondii* antibodies detected by the IFAT and microplate-ELISA was further demonstrated based on their reactivity with *T. gondii* tachyzoite antigens using the Western immunoblot assay. Though ELISA and IFAT demonstrate the presence of antibodies in sera, they do not identify the various *T. gondii* proteins recognized. In the present study, antibodies in both the WHO human reference serum and pig sera recognized antigens located in bands with molecular weight ranging from 12 to 74 kDa. Wastling, Harkins and Buxton (1994) reported similar molecular weight range (11 to 78 kDa) in a study involving the kinetics of antibody responses in sheep vaccinated with the S48 strain of *T. gondii*. In their study, they observed that the response was dominated by antibody

recognizing the 32, 30, 24 and 11 kDa antigens, which were found to be common to all positive sheep sera used. In the present study, the 30 kDa antigen was recognized by antibodies in both the human reference serum and pig sera. Various workers have demonstrated the reactive band patterns of *T. gondii* antigens using the SDS-PAGE. For example, Johnson, McDonald and Neoh (1981) analysed *Toxoplasma* polypeptides using antigen from sonicated *T. gondii* tachyzoites by SDS-PAGE and ^{25}I-concanavalin A (Con A) binding studies, and identified reactive *T. gondii* antigens with molecular weight range between 20 and 98 kDa. Several other workers including Handman, Goding and Remington (1980), Kasper, Crabb and Pfefferkorn (1982, 1983) and Couvreur *et al.* (1988) characterized *T. gondii* tachyzoite surface antigens by SDS-PAGE, and identified five major surface proteins with molecular weights 22, 23, 30, 35 and 43 kDa designated P22, P23, P30, P35 and P43. The identification of the 26, 30, 32, 36 and 60 kDa bands by both the pig and human reference serum in this work demonstrate the presence of these major antigens.

In conclusion, the present study has shown that the microplate-ELISA is a suitable serological assay, with sufficiently high specificity and sensitivity (92.3% and 90.2%) and a high correlation (0.82) with the IFAT. The micoplate-ELISA was found to be suitable for the mass screening of swine serum samples for antibodies against *T. gondii.* The results obtained showed a nationwide *T. gondii* seroprevalence in pigs in the three ecological zones of Ghana. The significantly high positive relationship between *T. gondii* seropositivity and abortions/stillbirths in pigs also suggested involvement of toxoplasmosis in low productivity of pigs in Ghana. Infected pigs might be an important reservoir of *T. gondii* tissue cysts which could be a source of infection for humans. There is, therefore, an urgent need for more work to demonstrate *T. gondii* tissue cysts in seropositive pigs in Ghana. Also, there is

the need to conduct further studies into the health impact of management practices in pig breeding as it appears to be important in the dissemination of oocysts.

Important Observations.

Based on the observations made in this study, the following prevention measures may be suggested to help control the transmission of ***T. gondi*** in Ghana.

Avoid contamination of feed or water with *T. gondii* oocysts in cat faeces by:

1. Using rodenticides and traps to prevent cats from hunting rodents.
2. Covering, closing or locking feed storage facilities to prevent cats from entering defaecating in them.
3. Promptly removing cat faeces from buildings, stores, or cages, and flushing, burning, or burying them to destroy oocysts.
4 Preventing cats from sharing facilities used by other animals.

1. In the case of humans, avoid contamination with oocysts from cat faeces by:

a. Feeding cats dry, canned or thoroughly cooked food.

b. Emptying litter boxes daily, before oocysts sporulate.

c Wearing gloves or thoroughly washing hands after working in the garden or soil and before eating or touching the face.

d. Covering childrens sandboxes when not in use.

e. Boiling drinking water from streams, ponds, or lakes frequented by cats.

2. Avoid ingestion of tissue cysts by cooking meat over 70°C and washing hands after handling raw meat.

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