TOXOPLASMA GONDII: CULTIVATION, DETECTION AND PREVALENCE IN PERU AND THE UNITED STATES OF AMERICA

by

GERALDINE MIRIAM SAAVEDRA

(UNDER THE DIRECTION OF YNES R. ORTEGA)

ABSTRACT

This study investigated the cultivation of *T. gondii* in ten cell lines using different initial concentration of cells and tachyzoites, detection of infection by three methods, including histology, western blot and nested–PCR, and the prevalence of *Toxoplasma gondii* in pigs and cattle from USA and Peru.

Toxoplasma gondii tachyzoites were cultivated in different cell lines at initial concentrations of 10^3, 10^4, and 10^5 tachyzoites, per 10^4, and 10^5 cells individually. There was a significant difference ($p<0.05$) among cell lines regarding the propagation of tachyzoites. Most of the cell lines had an aggressive overgrowth that did not allow the propagation of tachyzoites. MRC-5 was the best cell line in which *T. gondii* grew providing a regular supply of viable tachyzoites.

The detection of *T. gondii* was determined by histology, western blot and nested-PCR in mice experimentally inoculated intraperitoneally and orally with 10, 10^2, 10^3, and $2x10^5$ tachyzoites. No significant difference ($p>0.05$) was observed among these three techniques in detecting *T. gondii* in mice intraperitoneally infected. A significant

difference ($p<0.05$) was observed between different methods of inoculation and among the initial concentration of tachyzoites used. Infection was found in 50% of the mice inoculated i.p. and in 30% of the mice inoculated orally among the three techniques.

The prevalence of antibodies to *T. gondii* in pigs and cattle from Peru and USA was determined by western blot. Sera of 137 pigs and 253 cattle were collected at a slaughterhouse in Lima, Peru. Sera of 152 pigs and 23 cattle were collected from a slaughterhouse in Georgia, USA. Immunoglobulin G (IgG) antibodies to *T. gondii* were detected in 27.7% of pigs and in 51.4% of cattle from Peru; in 16.4% of pigs and in 26% of cattle from USA.

INDEX WORDS: *Toxoplasma gondii*, Tachyzoites, MRC-5, Cell lines, Cell culture, Mice, IgG, IgM, Antibodies, Western blot, Nested-PCR, Histology, Prevalence, Toxoplasmosis, Pigs, Cattle, Peru, USA.

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DEDICATION

I would like to dedicate this thesis to my parents, Ricardo Saavedra and Frida Ebner, for their love and encouragement though out my entire life, thank you.

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Toxoplasma gondii is an obligate intracellular protozoan parasite, affecting a wide range of warm-blooded animals, including humans. It is one of the most important pathogens in immunocompromised individuals, causing necrotizing encephalitis, pneumonitis, and myocarditis. Toxoplasmosis in AIDS patients usually represents reactivation of latent infection, and this occurs when CD4 levels drop below 100 cells per cubic millimeter (Gagne, 2001). Ocular toxoplasmosis is one of the main clinical manifestations of human infection by the protozoan parasite *T. gondii*.

Immunocompetent individuals with a primary infection are generally asymptomatic, and these cases largely go unrecognized. It was believed to be harmless in immunocompetent individuals; however, new behavioral studies suggest that people with latent toxoplasmosis may find it more difficult to concentrate when compared to uninfected people (Flegr et al., 2002).

Congenital toxoplasmosis is one of the most serious problems and can result in a spontaneous abortion, a stillbirth, or a child that is seriously handicapped mentally and/or physically.

Toxoplasma can be transmitted to humans by: (1) ingestion of raw or undercooked meat infected with tissue cysts or uncooked foods that have been in contact with tissue cysts contaminated meat, (2) inadvertent ingestion of oocysts in cat feces through handling of unwashed fruits or vegetables, or (3) transplacentally (Gagne, 2001).

Milk of several species of animals, including sheep, goats, cows and mice have been found to be contaminate and could serve as a source of toxoplasmosis in humans (Powell et al, 2001). Infection of humans has been documented from ingestion of raw goat milk (Sacks et al., 1982; Skinner et al., 1990).

In developed countries, raw or undercooked meat containing *Toxoplasma* cysts probably serves as the main source of human toxoplasmosis (Dubey, 1986; Dubey, 1991; Dubey et al., 1990), however in developing countries, a main source of *Toxoplasma gondii* infection can be by food contaminated with cat feces, in addition to raw or undercooked meat.

Toxoplasmosis is probably the most widespread zoonotic disease in the world. It is estimated that more than 60 million people in the US carry the *Toxoplasma* parasite, with 30 to 50% of the US population having antibodies to *Toxoplasma gondii* and at least 30% of people in the world are infected with the organism (Aspinall et al., 2002), with a higher prevalence of infection in France, 60 to 90% of the population are serologically positive (Ajioka et al, 2001).

Within the United States, rates of *T. gondii* infection are low in the west central and mountain states and high in the east Atlantic and east central states because of the climates and condition of the soil (Pastorek, 1994).

In the United States, it seems that cattle do not carry *T. gondii*; however, pork is the main source of foodborne toxoplasmosis. It has been suggested that infected pork products cause 50-75% of all cases of human toxoplasmosis in this country (Gajadhar et al., 1998; Gagne, 2001), being the third leading cause of foodborne death in the United States (Gagne, 2001). However; modern meat production has reduced the prevalence of *T. gondii* in young pigs in Europe and North America (Dubey, 2000; Tenter et al., 2000).

In Canada, the seroprevalence of toxoplasmosis is 21-28% (Ffrench and Fish, 1961; Seah, 1973; Karim and Trust, 1977; Proctor and Banerjee, 1994), and it is estimated that there are 140-1400 cases of congenitally acquired toxoplasmosis per year, and between 70 and 280 infants are born severely affected every year (Carter and Frank, 1986).

In developing countries, such as Peru and Brazil, there is a high prevalence of specific antibodies (34% and 9%, respectively) to *T. gondii* in pigs.

The population with toxoplasmosis in Peru is calculated to be between 2 to 4 million, mainly poor women and their children.

Extensive studies have been undertaken for the detection of *T. gondii* in mice, pigs and sheep by different methods such as PCR, western blot, ELISA, IFAT, dye test, after been experimentally infected with *T. gondii*. Also, comparative studies of tissue culture and mouse inoculation have been done for demonstration of *T. gondii*, as well as use of different cell lines for *in vitro* propagation of *T. gondii* tachyzoites.

In this study, we have determined the best cell line for in vitro propagation of *T. gondii* using ten cell lines, two from mice and eight from humans, using adherent and non adherent cell lines. The immune response was determined by western blot in mice

experimentally inoculated intraperitoneally and orally with different concentrations of tachyzoites. Also we have determined by histology and nested-PCR, the infectivity of a low number of tachyzoites in mice. Finally, the prevalence of *T. gondii* in cattle and swine at slaughterhouses in Peru and the USA was determined.

LITERATURE REVIEW

Toxoplasmosis is caused by infection with *Toxoplasma gondii.* This coccidian protozoan is normally transmitted to humans by ingestion of either oocysts from cat feces, or by tissue cysts in raw or undercooked meat.

It is one of the most important problems in immunocompromised individuals. In immunocompetent individuals, toxoplasmosis is generally asymptomatic. Lymphadenopathy is the most common manifestation in the 10% - 20% of immunocompetent individuals whose primary *T. gondii* infection is symptomatic. Chorioretinitis, myocarditis and/or polymyositis are less frequently presented in these patients, but can occur (Montoya and Remington, 2000; Montoya, 2002).

During pregnancy, an infection with *Toxoplasma* can result in either fetal death or in a child with physiological, physical or neurological defefects. Fetal contamination is estimated to occur in 0.1 to 0.5% of infected pregnant women in the United Kingdom, 0.2 to 0.3% in Southern Finland, 1% in France, 0.2 to 0.8% in Canada, and 0.2 to 0.6% in the United States (Nguyen et al., 1996).

History

Toxoplasma gondii was first discovered in an African desert rodent, *Ctenodactylus gundi*, in 1908 by Nicolle and manceaux (Dubey and Beattie, 1988) and described the same year by Samuel T. Darling (De la Cruz, 1989). The name was derived from the Greek *toxon,* bow or arc, alluding to lunate shape (Frenkel, 1973). It was then found in many species of mammals and birds worldwide and also in humans. In 1923, Janku described *T. gondii* in the retina of a hydrocephalic child, but the role of the parasite as a human pathogen was not widely known until Wolf and Cowen reported congenital *T. gondii* infection in man. Their report stimulated considerable interest in human toxoplasmosis. Pinkerton and Weinman reported the first known cases of fatal toxoplasmosis in adult human patients. The development of the dye test by Sabin and Feldman was and still is the key to much of our present knowledge of toxoplasmosis (Dubey and Beattie, 1988).

In 1970, the life cycle of *T. gondii* was first described. Until then *Isospora* species were considered parasites of carnivores (dogs, cats) and birds and were thought not to be host specific. *T. gondii*, was first known to parasitize extraintestinal tissues of virtually all warm-blooded hosts, but then was found to be an intestinal coccidia of cats and to have in its life cycle an isosporan-like oocyst. Further life cycle studies indicated that some of the *Isospora* species that had been considered to have only an intestinal cycle also had stages in extraintestinal tissues (Dubey, 1993).The knowledge of *T. gondii* life cycle was completed by the finding of the sexual phase of the parasite in the small intestine of the cat. *Toxoplasma gondii* oocysts, the product of the merogony and gametogony, were

found in cat feces and characterized morphologically and biologically (Dubey et al., 1970a)

In 1977, an outbreak of toxoplasmosis involved patrons of a riding stable in Atlanta (Teutsch et al., 1979). Unlike other reported outbreaks that have been caused by eating raw or undercooked meat, this outbreak was caused by oocysts from cat feces (Dubey et al., 1981). An unusual high rate of clinical diseases was associated with this outbreak. Thirty five (95%) of the patrons of the stable who had laboratory evidence of infection had clinical illness characterized by fever, lymphadenopathy, and headache (Dubey et al., 1981).

In 1984, an epidemic of toxoplasmic encephalitis occurred in patients with AIDS (Luft et al., 1984; Wanke et al., 1987). In 1995, the first large outbreak of toxoplasmosis to be associated with municipal drinking water occurred in British Columbia, Canada. This was one of the largest outbreaks reported until that date. One hundred and ten acute *Toxoplasma* infections were identified. Of these, 42 infected women and 11 newborns were identified as well as 57 infections in non-pregnant individuals (Bowie et al., 1997; Bell et al., 1995).

In 2001, the largest outbreak of toxoplasmosis ever reported in the world occurred in Parana, Brazil, affecting 290 people, of which 176 became ill, including three pregnant women. The vehicle was again contaminated water (Promed, 2002).

Classification and *Toxoplasma gondii* strains

The life cycle of *Toxoplasma gondii* was proposed more 25 years ago. Since that time, despite attempts to make the genus polyspecific, there has been only one species,

Toxoplasma gondii, consistently recognized in the genus. Recent studies have been done to investigate the genetic diversity among strains in the species *T. gondii*. Results of this analyses confirm that the strain in the genus *Toxoplasma* comprise a limited number of clonal lineages, directly correlated with their virulence in mice (Johnson, 1998).

The genus *Toxoplasma* is classified in the Phylum *Apicomplexa* (Levine, 1977), class *Sporozoasida* (Leukart, 1879), subclass *Coccidiasina* (Leukart, 1879), order *Eimeriorina* (Leger, 1911), family *Toxoplasmatidae* (Dubey, 1993).

Only one species of the genus *Toxoplasma* has been observed to exist so far (Guo and Johnson, 1996), however, there are large differences in virulence among the various strains and isolates of *T. gondii*. This virulence diversity among isolates may have considerable impact on epidemiology, immunology, pathology and the parasite-host relationship. Some strains such as the extremely virulent RH strain have lost the ability to form oocysts in the cat, and as few as ten tachyzoites can be lethal for a mouse within a week when injected intraperitoneal (Guo and Johnson, 1996). The RH strain was isolated in 1939 from the brain of a 6 year-old boy. Other strains such as the avirulent S-1 strain form cysts in the brains of mice injected with 1000 oocysts (equivalent to 8000 tachyzoites) and these mice survive without ill effects (Dubey and Frenkel, 1973).

In human toxoplasmosis, the role of the infecting *Toxoplasma* strain is not easily assessed although it is clear that infections can have dramatically different outcomes. There is overwhelming evidence that the immunological status of the host plays a major part in the clinical presentation of this opportunistic infection (Darde, 1996).

Structure and Life cycle

Toxoplasma gondii has three infectious stages: tachyzoites, the rapid multiplying form found in pseudocysts or clones; bradyzoites, latent form found in tissue cysts, and sporozoites present in oocysts found only in feces of the feline family (Smith, 1992).

Tachyzoites

Tachyzoites have a crescent shape and are approximately 2x6μm. Their anterior (conoidal) ends are pointed and their posterior ends are round. They have a pellicle (outer covering), polar ring, conoid, ropthries, Micronemes, mithocondria, subpellicular microtubules, endoplasmic retriculum, Golgi apparatus, ribosomes, rough surface endoplasmic reticulum, micropore, and a well defined nucleus. The nucleus is usually situated toward the posterior end or in the central area of the cell. Chromatin is distributed in clumps throughout the nucleus and the nucleolus is usually located centrally within the nucleus (Sheffield and Melton, 1968).

Tachyzoites multiply rapidly to destroy the host cell within 48 hours, they replicate with a generation time of 6 to 9 h in vitro until exiting the cell to infect neighboring cells, usually after accumulating 64 to 128 parasites per cell (Coppens and Joiner, 2001).

In the tachyzoite, the conoid defines the apical end of the parasite and is thought to be associated with the penetration of the host cell. Micronemes, rhoptries and dense granules are the three major secretory organelles, found predominantly at the apical end of the parasite. Microneme proteins are released very early in the invasion process, facilitating host-cell binding and gliding motility. Rhoptry proteins are also released

during invasion, and can be detected within the lumen and membrane of the newly generated parasitophorous vacuole (PV). Dense granule proteins are released during and after the formation of the PV, modifying the PV environment for intracellular survival and replication of the parasite (Ajioka et al, 2001)

Tachyzoites are not resistant to gastric secretions and are thus much less infectious via the oral route than either oocysts or bradyzoites (Weiss et al., 2000). Tachyzoite are associated with the acute disease phase.

Bradyzoite

This stage is present within tissue cysts; this stage is also called a cystozoite. Comparing to tachyzoites, this is a slowly replicating life stage which forms cysts during a chronic phase. Like tachyzoites, bradyzoites remain intracellular and divide by a unique binary fission termed endodyogeny (Weiss et al., 2000).

Bradyzoites differ only slightly structurally from tachyzoites. They are slender, smaller in size, and usually have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located. They contain several glycogen granules which stain red with periodic acid-schiff (PAS) reagent; these are either indiscrete particles or absent in tachyzoites. Biologically, bradyzoites are less susceptible to destruction by proteolytic enzymes than tachyzoites (Dubey and Beattie, 1988). The size of tissue cysts is variable, but on average a mature cyst is 50 to 70 µm and contains from 1000-2000 crescent-shaped 7 by 1.5µm bradyzoites. Tissue cyst depends on cyst age, the host cell parasitized, the strain of *T. gondii* and the cytological method used for measurement. Young and old cysts can be distinguished readily by their ultra structural

features. Degenerating cysts are often seen in the brains of mice with chronic toxoplasmosis (Weiss et al., 2000).

Bradyzoites develop in cysts within host cells in a variety of tissues, but they are common in neural and muscular tissues such as brain, heart, skeletal muscle and retina. Cysts are not static structures; they regularly break down or rupture host cells and reinvade others (Weiss et al., 2000). When tissue cysts rupture, however, they elicit a strong inflammatory response resulting in the formation of glial nodules in the brains of chronically infected hosts.

The tissue cyst wall is elastic, thin (<0.5µm), and argyrophylic and encloses hundreds of crescent-shaped slender bradyzoites. The bradyzoites are about 7x1.5µm (Mehlhorn and Frenkel, 1980). Initially, the tissue cyst develops in the host cell cytoplasm and its wall is intimately associated with the host cell endoplasmic reticulum and mithocondria and the cyst wall is partly of host origin. Some bradyzoites may degenerate in tissue cysts, especially in older cysts (Pavesio et al., 1992). The bradyzoite has a nucleus situated toward the posterior end. It contains electron dense rhoptries, and several glycogen granules, which are either in discrete particles or absent in tachyzoites. The prepatent period in cats following infection by bradyzoites is shorter than that following infection with tachyzoites (Dubey and Fenkel, 1976). Tissue cysts are more numerous in animals in the chronic stage of infection after the host has acquired immunity than in animals in the acute stage of infection. However, tissue cysts have been observed in mice infected for only 3 days (Dubey and Frenkel, 1976) and in cells in culture systems devoid of known immune factors (Hoff et al., 1977). Dubey (1993)

reported that it is possible that development of functional immunity and the formation of tissue cysts are coincidental.

Oocysts

Oocysts are the developed zygotes surrounded by a two-layered wall. The unsporulated oocyst contains a "sporont", which is a mass of cytoplasm and nucleopasm within the oocyst. Upon sporulation the sporont divides into two round masses called "sporoblasts". They later elongate and differentiate to form sporocysts. Within each sporocyst four sporozoites develop. The oocyst develops as a result of sexual processes in the feline intestine (Dubey, 1993).

Life cycle

The only definitive host is the cat or members of the feline family. The life cycle of *T. gondii* presents both sexual and asexual stages. After ingestion of tissue cysts from infected animals by the cat, the tissue cyst is lysed by the digestive enzymes and bradyzoites are released. These then penetrate the epithelial cells of the small intestine where they undergo initial asexual multiplication. This is followed by the sexual cycle in which male and female gametes migrate into the gut and combine to form a zygote which then develops into a immature oocyst. Final sporulation of the oocyst usually takes place approximately 1 to 5 days after shedding in the cat feces. Final sporulation depends on environmental conditions and does not usually occur below 4°C or above 37°C (Anonymous, 1997). The mature oocyst measures approximately 12 x 11μm and has two sporocysts with each containing four sporozoites. Sporulated oocysts are potentially

infective to any warm-blooded animal that might ingest it. Cats often shed oocysts over a period of 2-3 weeks with levels of shedding peaking at over a million per day. Water, vegetables, or animal feed can become contaminated and if they are ingested by an intermediate host, the host becomes infected, and can infect other intermediate hosts or the definitive host by tissue cysts in raw or undercooked meat. Bradyzoites released from tissue cysts or sporozoites released from oocysts penetrate intestinal cells and multiply as tachyzoites and eventually spread via lymph and blood to other sites. As immunity develops, replication of tachyzoites decreases and tissue cysts develop which do not normally produce a host reaction. Each cyst may contain 1000 bradyzoites which are found in brain, muscle, heart and visceral organs of both cats and intermediate hosts and persist for life. The encysted organisms are protected from circulating antibodies. Cysts may occasionally rupture but the released bradyzoites are inactivated by the immunocompetent host and as long as the immune system is intact, the encysted bradyzoites are inactive. Oocysts are never shed by intermediate hosts (Smith, 1992).

Propagation of *Toxoplasma gondii*

Propagation of *Toxoplasma gondii* tachyzoites is essential for all experimental models, genetic studies (Chatterton et al., 2002; Sibley and Boothroyd, 1992; Roos et al., 1994), biochemical pathway and drug studies (Chatterton et al., 2002; Fichera and Roos, 1997; Fichera et al., 1995) and the development of serological tests (Chatterton et al., 2002; Verhofstede et al., 1988; Ashburn et al., 2000).

Toxoplasma gondii can be propagated either in the peritoneal cavities of mice or in tissue culture. *Toxoplasma* multiplies intracellularly in host cells, followed by the

destruction of those cells and invasion of adjacent cells spreading throughout the body via lymphatic and the blood stream. Animal inoculation is the most effective method for propagating and isolating *T. gondii* from tissues or body fluids (Hughes, 1986). Mice can be readily infected by intraperitoneal injections of bradyzoites or tachyzoites. Depending on the virulence of the strain, mice develop either an acute infection with parasite-rich ascites or a chronic infection characterized by the presence of cysts in the brain (Derouin, 1987).

Tachyzoites can be produced by serial passage in the peritoneal cavity of mice or cotton rats. Although convenient and reliable, animal culture is ethically undesirable, and the maintenance of an animal facility is expensive (Ashburn, 2000, Hughes et al., 1986). The risk of human infection during animal inoculation is avoided (Hughes et al., 1986). Also, even when large numbers of parasites can be harvested from mice; they are often difficult to separate from host cells and other debris in the peritoneal fluid (Chamberland and Current, 1991).

Cell culture provides large yields of *Toxoplasma* tachyzoites without the use of animals, with little cell contamination, high yields of secretory antigen and parasites which are suitable for serodiagnosis of toxoplasmosis (Harmer, 1996). Protein profiles of whole tachyzoites isolated from mice or from cell lines do not have any major differences (Chamberland and Current, 1991).

Toxoplasma gondii tachyzoites are not extensively modified by *in vitro* cultivation (Chamberland and Current, 1991; Noriega et al, 1988), hencet it would be appropriate to use such a method of culture to produce large quantities of tachyzoites

required for biochemical characterization of specific cellular components (Chamberland et al., 1991).

In vitro culture of *T. gondii* can be performed with a broad range of cell lines (Bunetel et al., 1995), including adherent and nonadherent cells (Ribeiro et al., 2000).

In vitro assays are very important to evaluate the effects of new drugs against intracellular microorganisms, particularly when there are associated in vivo factors such as the host immune system, which can interfere at the intracellular level (Ribeiro et al., 2000).

Tachyzoite and bradyzoite specific antigens

At least five major tachyzoite membrane antigens have been identified by surface radioiodination, ranging in molecular weight from 6 to 95 kDa (Warren, 1993). Gross (1996) identified in tachyzoites five major surface proteins of 22, 23, 30, 35 and 43 kDa (designated P22, P23, P30 and P43), by generating monoclonal antibodies according to their mobilities in SDS-polyacrylamide gel electrophoresis. The predicted molecular weight of these antigens shows variability among the different laboratories. This may be due in part to different methods of iodination, parasite strain variability, or perhaps technical differences in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedures (Warren, 1993).

Sera from humans that have been naturally infected with *Toxoplasma* bind a broad range of antigens in western blots. These antigens, ranging in molecular weight from 22 to 67 kDa, are recognized by the IgG fraction from acutely or chronically infected patients and may differ from antigens recognized by the IgM fraction. It was

observed that IgM was directed primarily against a 6 kDa antigen, whereas the IgG fraction bound this antigen as well as a variety of others of higher molecular weight (Warren, 1993).

P30 (SAG1) represents 5% of the total amount of tachyzoite proteins. It appears that most of the surface proteins electrophorese more slowly under reduced gel conditions than under non reduced conditions. In addition, P35 and P30 proteins co-migrate under unreduced conditions (Gross, 1996). P30 or SAG1 is a stage-specific antigen, because it can be detected only in rapidly dividing and invasive tachyzoites and not in the bradyzoites of tissue cysts or sporozoites from oocysts, and it appears to have an important role in both immune and pathogenic mechanisms of the parasite (Gross, 1996).

The majority of *T. gondii* excretory proteins are components of the dense granules within electron-dense secretory organelles. During intracellular development of the parasite, the dense granules appear to continuously release their contents into the parasitophorous vacuole (PV). Dense granule antigens (GRA) then associate differentially with either the PV membrane and/or the tubulovesicular network (Fischer et al., 1998). These antigens may represent a group of proteins that may perhaps be of importance in the development of a protective vaccine against *T. gondii* (Warren, 1993).

Transmission

Infection with *T. gondii* can occur through four routes of transmission, including (1) congenitally by transmission of tachyzoites during primary infection of the mother; (2) by ingestion of food or water contaminated with oocysts; (3) by ingestion of raw or

undercooked meat containing the bradyzoite form in tissue cysts (Dubey, 1996), or (4) by receiving blood or tissues with tachyzoites or bradyzoites.

The human infective dose for *T. gondii* is not established but extrapolation from animal studies suggests a dose of less than 10^4 organisms (Remington et al., 1995).

The relative importance of these different sources of infection is not defined and may vary from one region to another, depending on diet, culinary methods, prevalence of infected cats (Remington et al., 1995), farming techniques (Bustamante and Suarez. 2000; Remington et al., 1995), climatic conditions such as temperature, rainfall and humidity (Mensah et al., 2000).

Transmission by Oocysts.- Cats and all felidaes are fundamental for the transmission of *T. gondii*, because they are the only species capable of shedding oocysts in their feces. Felidae excrete *T. gondii* oocysts in feces 3 to 10 days after ingesting bradyzoites, ≥ 18 days after ingesting of sporulated oocysts, and ≥ 13 days after ingesting tachyzoites (Dubey, 1998a). Those must sporulate outside the body of the host. The sporulation process which usually takes from 1 to 5 days depends on temperature, moisture, and other environmental conditions. As a rule, the duration of excretion is from 1 to 3 weeks and is rarely repeated, and may be re-stimulated by malnutrition by *Isospora felis*, or by administration of cortisone (Dubey and Beattie, 1988). Transmission can occur by consumption of water, fruit, or vegetables contaminated with oocysts shed in the feces of infected cats.

Transmission by tissue cysts. – Tissue cysts are usually by consumption of raw or undercooked meat. Viable tissue cysts have been detected in tissues of naturally infected animals, particularly in pigs and sheep, and also in wild animals. Serological studies have revealed evidence of widespread *T. gondii* infection in meat-producing animals, particularly pigs, sheep and goats (Tenter et al., 2000). Tissue cysts are extremely rare in cattle; however, there are several studies associating *Toxoplasma* infection with bovine (Huong et al., 1998; Gondim et al., 1999a; Dubey, 1986; Harkins et al., 1998; Gondim et al., 1999b; Dubey, 1990; Montoya et al., 1981; Samad et al., 1993; Esteban-Redondo, 1999).

Pork was previously regarded as the most important source of *T. gondii* infection in humans (Dubey, 1994), although modern meat production has reduced the prevalence of *T. gondii* in young pigs in Europe and North America (Dubey, 2000; Tenter et al., 2000). However, older animals found to have higher prevalence of the organism (Mensah et al., 2000) are routinely used in the production of sausages, salami and cured meats (Dubey, 2000). Moreover, such products often contain meat from multiple animals in a single serving. Together these factors result in a higher potential risk of infection after consumption unless these foodstuffs are very well cooked (Aspinall et al., 2002). Cured meat has been associated with acute toxoplasmosis in pregnancy (Warnekulasuriya et al., 1998).

Transmission by tachyzoites.- Tachyzoites were thought to be unable to survive outside the body of its host and be destroyed by gastric secretion (Dubey and Beattie, 1988; Weiss et al., 2000; Tenter et al., 2000); however, new studies have revealed that

tachyzoites can be infectious orally to cats and mice (Dubey, 1998), therefore can survive in gastric secretions.

Tachyzoites can infect by transplacental transmission from a mother to her fetus, by transfusion of packed leukocytes, and by all too frequent laboratory acquired infections. Although *T. gondii* has been found in the semen of goats, sheep and man, there is practically no risk of venereal transmission. Also, it has been detected in saliva (Dubey and Beattie, 1988).

Tachyzoites *T. gondii* have been detected in the milk of sheep, goats, cows and mice, and infection by ingestion of raw goat milk has been documented in humans (Powell et al., 2001). Milk can be a source of human toxoplasmosis (Dubey and Beattie, 1988; Powell et al., 2001); however, Dubey (1986; 1994) reported that milk from infected cows is regarded as being of negligible importance because cattle are resistant to *T. gondii* infection (Huong et al., 1998). However, Skinner et al. (1990) suggested that more attention should be given to milk as well as meat from goats as a potential source for human toxoplasmosis because of their greater susceptibility to infection and their higher rate of seropositivity than cattle (Gondim et al .,1999b).

The risk to the fetus by transplacental transmission is determined by gestational age at the time of infection and not by maternal symptoms. Premature infants are at particular risk and tachyzoites are often widely distributed in fetal tissue. Involvement of the CNS often leads to severe impairments, including mental retardation, spasticity, ocular disease, and deafness (Morris, 1996).

Diagnosis of *Toxoplasma* infection

Toxoplasma infection can be diagnosed by serologic tests, amplification of specific nucleic acid sequences, histologic demonstration of the parasite and/or its antigens, or by isolation of the organism (Remington et al., 2001).

Serologic Tests

Serological assays are the primary methods for detecting *Toxoplasma* infections. A combination of serological tests are usually required to establish whether an individual has been infected in the past or recently. Tests such as the Sabin Feldman dye test (DT), double sandwich Immunoglobulin M (IgM) enzyme linked immunosorbent assay (ELISA), Immunoglobulin A (IgA), and Immunoglobulin E (IgE) ELISA are usually used to determine if the infection has been acquired recently or in the more distant past (Montoya, 2000). The DT is essentially a complement-mediated neutralizing type of antigen-antibody reaction. This test once was considered one of the most reliable tests for toxoplasmosis in humans; however, is now considered too insensitive for the diagnosis of toxoplasmosis in cattle (Dubey, 1986).

Immunoglobulin G (IgG) antibodies. - –IgG antibodies are usually measured by an ELISA, DT, Immunofluorescence assay (IFA), or modified direct agglutination test (MAT). IgG antibodies usually appear within 1 to 2 weeks of acquisition of infection, peak within 1-2 months, decline at various rates, and usually persist for life (Montoya, 2002). The IgG test was the first method developed to differentiate between recent and distant infection in a single serum sample if the sample is obtained in the first trimester of

pregnancy. The presence of high avidity IgG antibodies can be used to rule out the occurrence of an acute infection within the past 3 to 5 months (Liesenfeld et al., 2001). It is useful in helping to differentiate acute from chronic infections (Dannemann et al., 1990).

IgM antibodies. – IgM antibodies are usually measured by double sandwich or capture IgM ELISA, IFA or immunosorbent agglutination assay (IgM-ISAGA). They usually appear earlier during infection but decline more rapidly than IgG antibodies. IgM antibodies can be detected as long as 12 years after acute infection (Bobic et al, 1991).

IgA antibodies. – IgA antibodies can be detected in sera of acutely infected adults and congenitally infected infants by an ELISA or ISAGA. They can persist for several months or more than a year. Therefore, they are not very useful for diagnosis of acute infection in adults. However, they are useful for diagnosis of congenital toxoplasmosis (Montoya, 2002).

PCR

PCR for detection of *T. gondii* DNA in body fluids and tissues has successfully been used to diagnose congenital, ocular, cerebral and disseminated toxoplasmosis (Montoya, 2002). B1, P30 and 18rDNA genes have been used extensively to detect *T. gondii* DNA in a variety of tissues, in both immunocompromised and immunocompetent individuals. However, B1 genes have been determined to be highly conserved in the *T. gondii* strains tested to date (Jones et al., 2000).

The B1 gene is the most sensitive and specific gene of *Toxoplasma gondii*. It has the advantage of being repeated 35 times in the genome (Burg et al., 1989), which increases the sensitivity of detection of low numbers of *Toxoplasma* by PCR (Wastling et al., 1993). Also, they do not amplify DNA from a variety of bacterial and fungal species and the reaction sensitivity remains unchanged in the presence of increasing amounts of human DNA. Jones et al. (2000) compared these sequences with all other sequenced DNA available from current databases and determined there was no complementary to any other known sequence (Jones et al., 2000).

Histologic Diagnosis

Toxoplasma gondii infection in different animals can be confirmed by definitive histological examination (Suarez-Aranda, 2000; Esteban-Redondo, 1999). The parasite has been detected in soft tissues such as brain, cardiac muscle, skeletal muscle, small intestine, liver and diaphragm. The brain is the most frequently infected tissue in either aborted fetuses or live animals, and has been the organ of choice for isolating *T. gondii* for diagnostic purposes (Uggla et al., 1987). However, in tissues of food animals such as pigs, it is estimated that the number of tissue cysts is less than 1 tissue cyst per 50 g of tissue. Therefore, it is not useful to demonstrate the presence of the parasite by histological examination of tissues (Dubey et al., 1996)

Detection of tachyzoites in tissue sections or smears of body fluid establishes the diagnosis of an acute infection (Remington et al., 2001; Montoya, 2002). It is often difficult to detect tachyzoites in conventionally stained tissue sections. Multiple tissue

cysts near an inflammatory necrotic lesion are indicatives of an acute infection or reactivation of a latent infection (Montoya, 2002).

With the induction of immunity, the parasites are reduced in number, making histological identification of the tachyzoite difficult. At this stage, only the bradyzoite containing tissue cyst can be observed (Warren, 1993).

Viability

Oocysts

Oocysts are very resistant to adverse environmental conditions and may retain infectivity for several months at -5°C. In tap water, oocysts can remain viable for up to 17 months (Anonymous, 1997). *T. gondii* oocysts can survive for 24 h when they are treated with 75% or 95% ethanol, 100% methanol, or 10% formalin (Kutièiæ and Wikerhauser, 1993). Also oocysts can survive a 24 h treatment with 1% and 2% water solution of Vikon S (potassium peroxisulphate) and for at least seven weeks when dried and stored at room temperature in a relative humidity of between 30 and 68%, but were inactivated after three weeks at -20°C, and after 15 minutes at 58°C.

Sporulated oocysts are killed by low dose gamma irradiation, being incapable of multiplication when treated with a dose of 0.3 kGy; however, viable sporulated oocysts could occasionally survive at a dose of 0.35 kGy (Dubey et al., 1996). Therefore, a dosage of 0.5 kGy is recommended for killing oocysts, which also kills tissue cysts of *T. gondii* (Dubey and Thayer, 1994). However, mice administered sporulated oocysts irradiated at 0.5 kGy develop antibodies to *T. gondii* (Dubey, 1994).

Tissue cysts

Tissue cysts are more resistant to digestive enzymes (i.e. pepsin and trypsin) than tachyzoites. Although they are less resistant to environmental conditions than oocysts, they are relatively resistant to changes in temperature and remain infectious in refrigerated (1-4°C) carcasses or minced meat for up to 3 weeks, probably as long as the meat remains suitable for human consumption. Tissue cysts also survive freezing -1 to -8°C for longer than a week (Kotula et al., 1991). Most tissue cysts are killed at temperatures of -12°C or lower, but occasionally some tissue cysts may survive deep-freezing (Dubey, 2000). Storage of *Toxoplasma* cysts at -20°C results in morphological changes (Callaway et al., 1968) and loss of infectivity (Work, 1968; Dubey, 1974). Tissue cysts are killed by heating to 67°C (Dubey et al., 1990; Dubey, 2000). Survival of tissue cysts depends on the duration of cooking; they can survive at 50°C for 10 minutes or at 60°C for about 4 min. Microwave heating does not kill tissue cysts (Lunden and Uggla, 1992). Microwave cooking is regarded as being less efficient that conventional methods to kill microorganisms in food, even if the same temperature is measured at the end of cooking. The rapid heating by microwaves does not provide the cumulative time-temperature relation necessary for destruction of microorganisms, and reflection of microwaves at the surface of the food and by bone creates hot and cold spots (Knutson et al., 1987).

Curing and smoking is effective in killing encysted *T. gondii*. The parasitical effect of curing with salt and sugar is probably due to changes in osmotic pressure. Also smoking involves salting, which can explain the destruction of infective parasites, even when smoking is performed at a temperature normally insufficient to kill *T. gondii*

(Lunden and Uggla, 1992). However, viable *T. gondii* in ready to eat cured meat samples (Warnekulasuriya et al., 1998) or in 8% NaCl for 4 days have been observed (Sommer et al., 1965).

Irradiation at 0.5 kGy kills tissue cysts; and mice injected with those tissue cysts do not develop antibodies to *T. gondii*. This contrasts results observed with irradiated oocysts (Dubey and Thayer, 1994).

Tachyzoites

Tachyzoites are sensitive to environmental conditions and are usually killed rapidly outside the host. Therefore, it is generally believed that horizontal transmission of *T. gondii* infections via tachyzoites is not important epidemiologically. However, they may occur infrequently (Tenter et al., 2000).

Tachyzoites are sensitive to proteolytic enzymes and usually are destroyed by gastric digestion (Tenter et al., 2000). However, a recent study revealed that tachyzoites may occasionally survive for a short period of time (up to 2 hours) in acid pepsin solutions, and that oral application of high doses of tachyzoites may cause an infection in mice and cats (Dubey, 1998).

Tachyzoites are sensitive to pasteurization temperatures. In addition, tachyzoites are highly susceptible to both heating and salt concentration, and thus, even mild cooking will kill tachyzoites in eggs (Tenter et al., 2000).

In general, it is believed that most cases of horizontal transmissions of toxoplasmosis to humans are caused by ingestion of one of two of the persistent stages, i.e. tissue cysts in raw or undercooked meat or oocysts in food or water contaminated

with feline feces (Dubey and Beattie, 1988; Tenter et al, 2000). However, Paul (1998) indicated that drinking milk may be a potential risk factor for horizontal transmission of toxoplasmosis to humans due to a recent study assessing risk factors associated with primary *T. gondii* infections in women of childbearing age in Poland.

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CHAPTER 2

COMPARISON OF TEN CELL LINES FOR IN VITRO CULTIVATION OF *TOXOPLASMA GONDII.* [1]

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To be submitted to Journal of Parasitology.

COMPARISON OF TEN CELL LINES FOR IN VITRO CULTIVATION OF *TOXOPLASMA GONDII.*

ABSTRACT

The purpose of this study was to identify a sustainable cell line that could continuously provide a large number of *Toxoplasma gondii* tachyzoites. Infection and multiplication of tachyzoites were compared using different adherent cell lines, including MRC-5, HT-29, Hs738.St/Int, Caco-2, HCT-8, WEHI-265.1, Hs1.Int, and FHs74 Int, and non-adherent cell lines such as Sp2/0-Ag14 and J774A.1. MRC-5 was the most suitable cell line for cultivating tachyzoites, providing tachyzoite numbers of $9.73x10^6$/ml. No infection or multiplication was observed in any of the non adherent cell lines. Since MRC-5 can provide a large yield of pure tachyzoites in a short time, tachyzoites derived from MRC-5 can be used in any technique used to study *T. gondii*.

INTRODUCTION

Toxoplasma gondii can be cultured using tissue culture and mouse inoculation methods (Slifko et al., 2000). There are no differences in viability of tachyzoites when cultured *in vivo* or *in vitro* (Chamberland et al., 1991; Hughes et al., 1985; Suresh et al., 1991). However, the results of *in vivo* cultivation are often delayed because the encysted parasites may not be identified until at least 30 days after inoculation (Derouin et al., 1987). In contrast, results from cell culture can be identified within two days after inoculation, moreover cell cultures have several advantages compared to *in vivo* maintenance such a lower cost and less time. *Toxoplasma gondii* grows well in cell lines (Derouin, et al, 1987); however, the optimal cell line and culture conditions that could provide large quantities of *Toxoplasma gondii* tachyzoites need to be identified.

MATERIALS AND METHODS

Cell lines. Ten cell lines (MRC-5, HT-29, Hs738.St/Int, Caco-2, HCT-8, WEHI-265.1, Sp2/0-Ag14, J774A.1, Hs1.Int, and FHs74 Int) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown at 37°C with 5% CO_2 in 25 cm^2 sealed flasks (Corning Incorporated, Corning, NY) with 5 ml of culture medium. When a monolayer was formed, the cells treated with 1X trypsin and transferred to 6 well plates. An inverted microscope 20X was used to examine the cell growth.

Culture medium for the different cell lines included:

MRC-5. Grown in minimum essential medium Eagle with 2mM L-glutamine (BSS), 1.5g/L sodium bicarbonate, 0.1 mM non essential amino acids, 1.0 mM sodium piruvate, 10% FBS. Antibiotics were not used.

HT-29. Grown in McCoy's 5a medium with 1.5mM L- glutamine and 10% Fetal Bovine serum.

Hs738.St/Int. Grown in Dulbecco's modified Eagle's medium with 4mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5g/L glucose, 1.0mM sodium piruvate and 10% of FBS.

Caco-2. Grown in minimum essential medium Eagle (MEM Eagle) with 2mM L-glutamine, Earle's BSS, 1.5g/L sodium bicarbonate, 0.1 mM nonessential aminoacids, 1.0mM sodium piruvate, and 20% of FBS.

HCT-8. Grown in RPMI 1640 medium with 1mM sodium piruvate and 10% of FBS.

WEHI-265.1. Grown in Dulbecco's modified Eagle's medium with 4.5g/L glucose, 0.05mM 2-mercaptoethanol and 10% of FBS.

Sp2/0-Ag14. Grown in Dulbecco's modified Eagle's medium with 4mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5g/L glucose, 1.0mM sodium piruvate and 10% of FBS.

J774A.1. . Grown in Dulbecco's modified Eagle's medium with 4mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5g/L glucose, 1.0mM sodium piruvate and 10% of FBS.

Hs1.Int. Grown in minimum essential medium Eagle (MEM Eagle) with Earle's BSS, non-essential amino acids and 10% of FBS.

FHs74 Int. Grown in Dulbecco's modified Eagle's medium with non-essential amino acids, 0.05mM sodium pyruvate, 1mM oxaloacetic acid, 0.2 units/ml insulin and 10% of SFB.

Table 2.1. Source and characteristics of cell lines

	Origin		Cultural conditions	
Cell line	organism	tissue	growth properties	fluid renewal
MRC-5	human	lung fibroblast	adherent	1 to 2 times per week
HT-29	human	colorectal adeno-carcinoma colon	adherent	2 to 3 times per week
Caco-2	human	colorectal adeno-carninoma colon	adherent	1 to 2 times per week
HCT-8	human	ileocecal colorectal Adenocarcinoma., Colon	adherent	every 2 to 3 days
WEHI -256	mouse	abelson murine 1 Eukemia virus Induce tumor	suspension	every 2 to 3 days
J774.A.1	mouse	macrophage monocyte	suspension	2 to 3 times per week
FHs74 Int	human	normal small intestine	adherent	every 2 to 3 days
Hs1.Int	human	normal intestine	adherent	every 2 to 3 days
Hs738.St/Int	human	normal mixed stomach intestine	adherent	every 2 to 3 days
Sp2/0-Ag14	mouse	B lymphocyte, Hibridome	suspension	every 2 to 4 days

Source: ATTC

Growth of the cell lines. Ten cell lines were used to determine both the initial concentration of cells and the amount of time needed for optimum growth of cells (before the monolayer appears). Seven cell lines were from human sources and three from mice, their characteristics are described in Table 2.1. Cells were prepared in concentrations of 10^4 and 10^5/ml in duplicate. Over the course of 4 days, cells were counted daily using an hemocytometer (Hausser Scientific, 0.100 mm deep), and observed using a bright field microscope at 20X (Leica ATC 2000). Trypan blue solution 0.4% (Sigma Chemical Co., St. Loius, MO), at a proportion of 1:1 (trypan blue:cells) was used to determine viability of the cells.

Toxoplasma gondii tachyzoites. RH strain of *Toxoplasma gondii* tachyzoites were obtained from Dr. R. Gilman, A.B. Prisma, Lima, Peru. Tachyzoites were maintained by intraperitoneal passage in CD-1 mice. They were passed through a 5 μm filter (Gelman Laboratory, Ann Arbor, MI) to remove debris and cells, and washed four or five times with PBS, pH 7.4 (0.1M NaCl, 0.002M KH_2PO_4, 0.003M KCL, 0.01 Na_2HPO_4, dH_2O) by sedimentation of cells when centrifuged at 1300xg for 15 min. Tachyzoites were counted using an hemocytometer. Trypan blue was used to determine viability of the tachyzoites.

Infection of the cell lines with T. gondii tachyzoites. Cells at initial concentrations of 10^5 were prepared in 6 well flat bottom tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). These were made in duplicate. Before confluence, the medium was removed and the cells were inoculated with 10^3, 10^4, and 10^5 tachyzoites/ml. They were incubated for 15 minutes at 37°C with 5% of CO_2, and then 2.5 ml of medium was added. Tachyzoites were diluted with RPMI 1640 medium (ATTC). Growth curves were determined.

Probability of infection

$$\% \text{ probability} = \frac{\text{\# test repetition}}{\text{\# times cells were infected}} \times 100$$

Statistical analysis

Experiments were repeated seven times. The statistical analysis system (SAS) was used to analyze the data. The level of significance α=0.05, was used in all cases. Mean was achieved using Duncan's multiple range test.

RESULTS

Determination of time and concentration of cells to obtain optimum growth. The best concentration of cells for optimum cell growth was 10^5 for all cell lines used in this study. Cell lines MRC-5, Hs1.Int, Hs738.St/Int, Sp2/0-Ag14, J77A4.1 and FHs74Int achieved confluence within one day; HCT- 8, Caco-2, and HT-29 required two days and WEHI-265.1 three days.

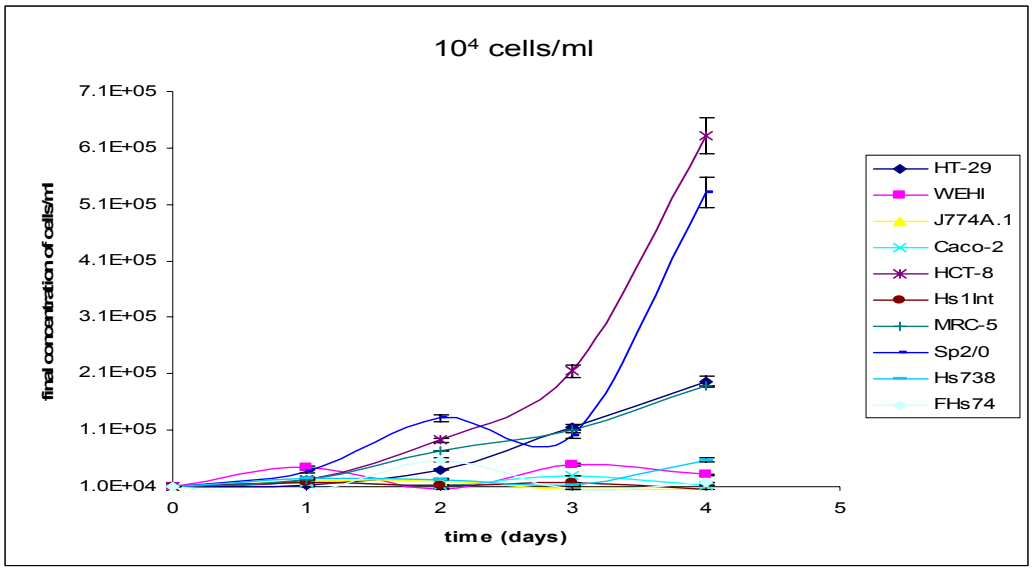

Fig.2.1. Growth of 10^4 cell lines/ml at different times

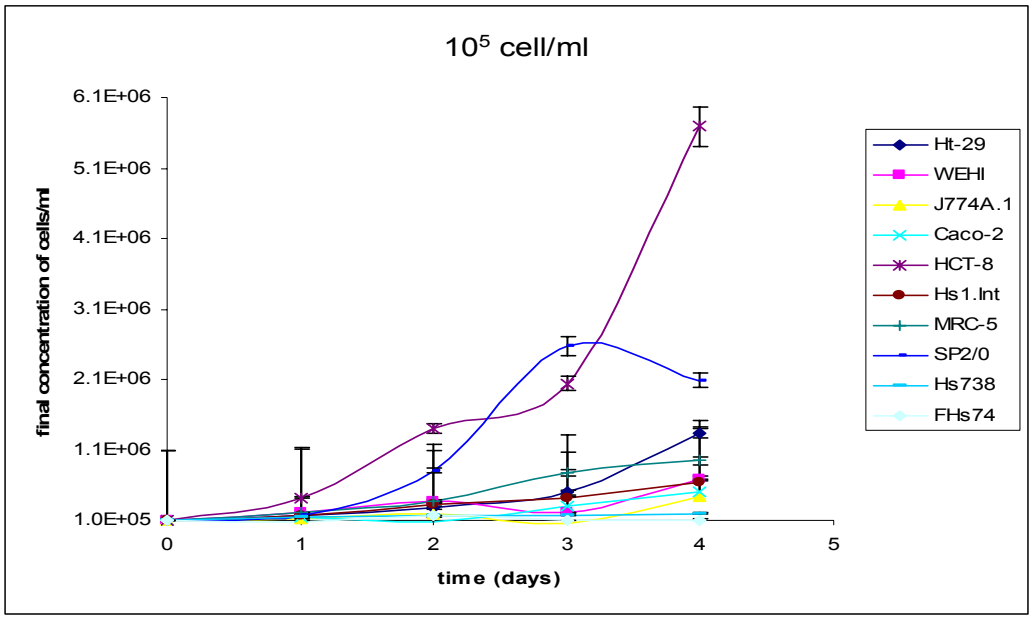

Fig.2.2. Growth of 10^5 cell lines/ml at different times.

Optimal cell line for the cultivation of Toxoplasma gondii. The best cell line in which tachyzoites infect and replicate was MRC-5, with an initial concentration of 10^5cells/ml. Parasitic vacuoles (rosettes) were clearly observed after 2 or 3 days post inoculation (P.I)., and the optimal harvest time was between 4 and 5 days with a final concentration of 9.7 x 10^6cells/ml.

The infection in other cell lines was slower and more difficult to recognize than that in the MRC-5 cell line (Fig. 2.3).

MRC-5 had a probability of 100% of becoming infected, while Caco-2 45%, FHs74Int 56%, HCT-8 64%, HT-29 50%, Hs738.St/Int 38%, and Hs1.Int 50%. Neither infection nor reproduction of tachyzoites was seen in J774A.1, WEHI-265.1, and Sp2/0-Ag14 cell lines (Fig. 2.4).

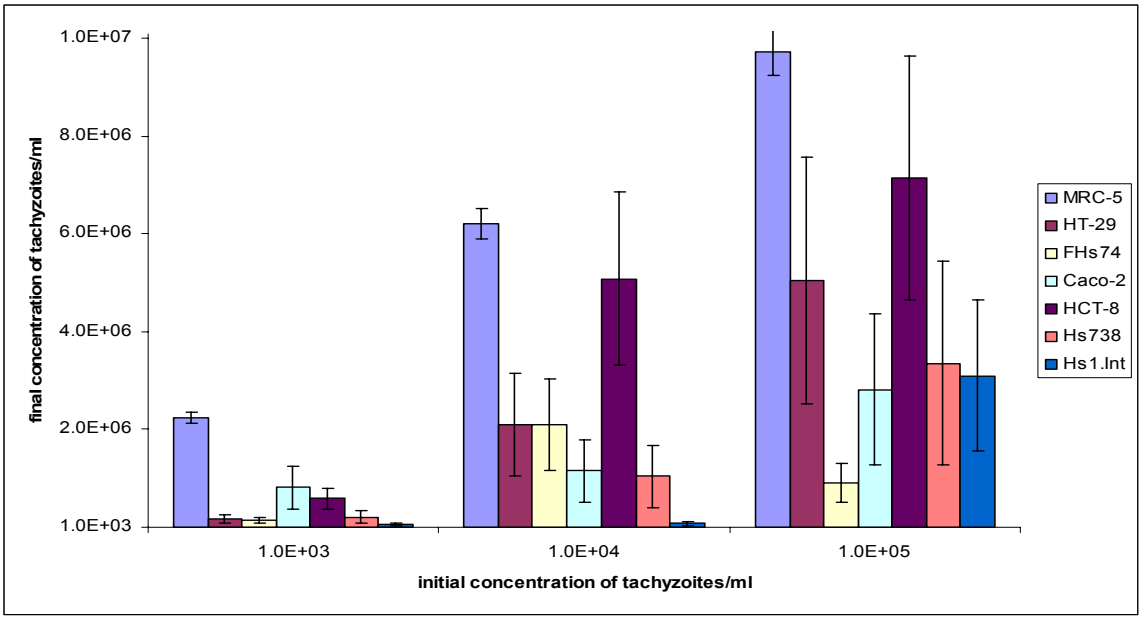

Fig.2.3. Growth of *T. gondii* tachyzoites in different human cell lines with initial tachyzoite concentrations of 10^3, 10^4, and 10^5tachyzoites/ml.

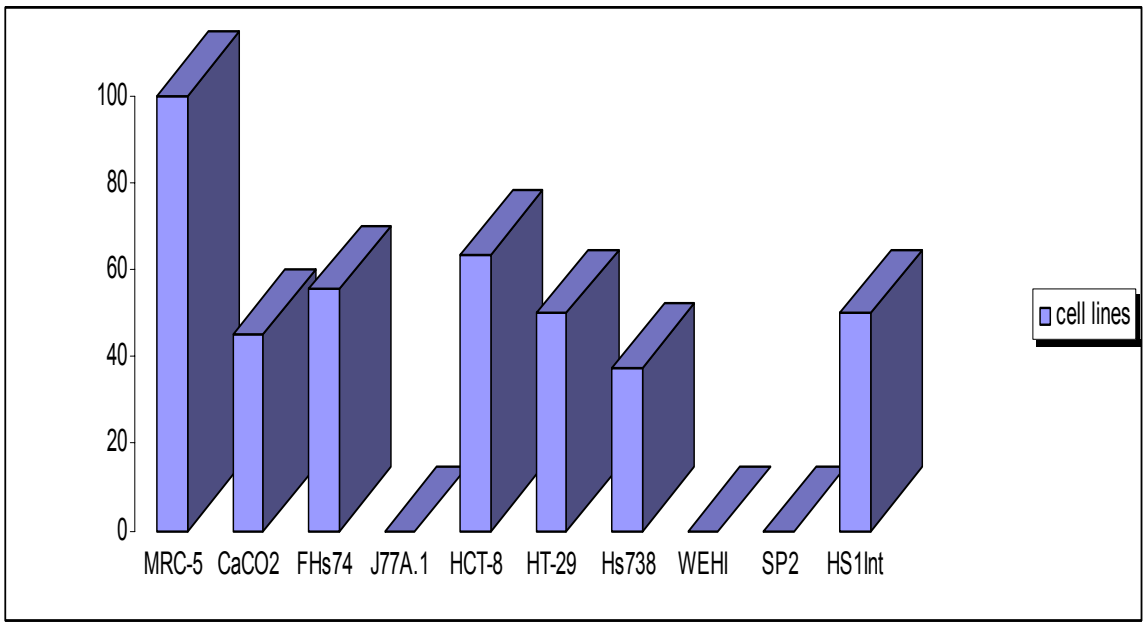

Fig.2.4.Probability of infection of *T. gondii* tachyzoites in human and mice cell lines.

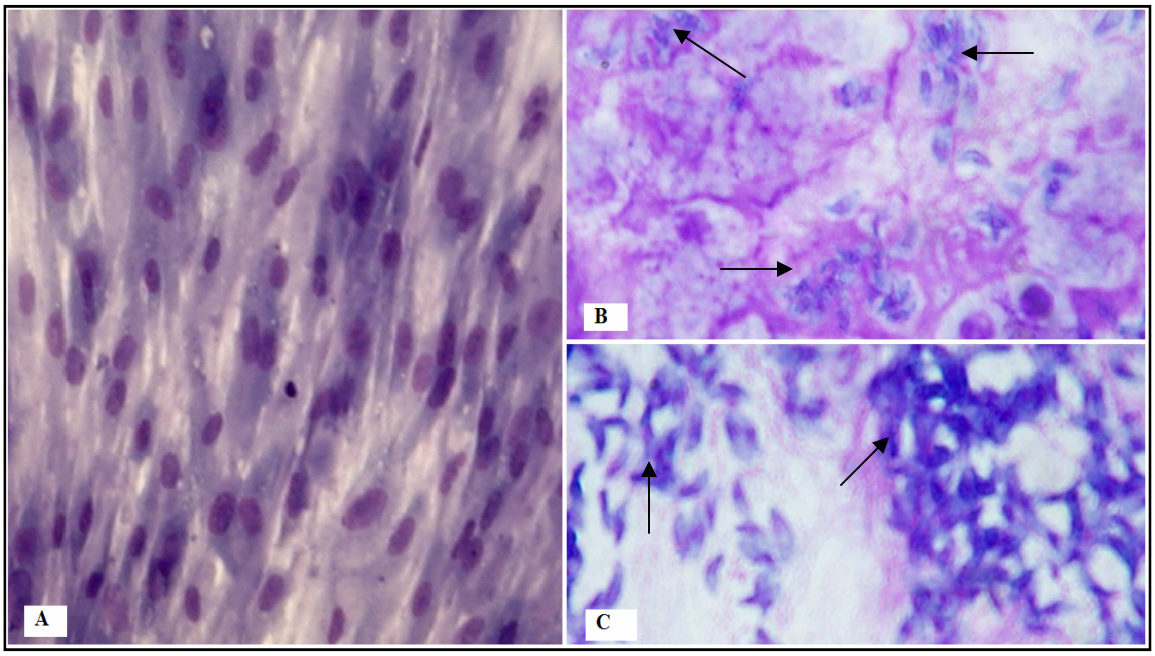

Fig. 2.5. MRC-5 lung fibroblast human cell line before and after inoculation with *T. gondii* RH tachyzoites. (A) MRC-5 in a monolayer phase. (B) Tachyzoites inside the MRC-5 cells after 2-3 days of incubation at 37°C in a 5%CO_2 atmosphere. (C) Free tachyzoites after lysis of the cells, 4-5 days of incubation.

DISCUSSION

Tissue culture is an effective method to cultivate *T. gondii* (Derouin et al., 1987; Hughes et al., 1985; Suresh et al., 1991; Ribeiro et al., 2000). *T. gondii* RH is a highly virulent strain in mice (De Champs et al., 1999; Frenkel, 1953; Dubey, 1998), hence it difficult to propagate tachyzoites from this strain in mice because of rapid death of inoculated animals. However, *in vitro* cultivation allows for a rapid and constant propagation of RH tachyzoites.

MRC-5 was determined to be the best cell line for cultivating *T. gondii* due to rapid infection, large yields of pure tachyzoites with little or no cell debris, constant

probability of infection (100%), and easy observation of tachyzoites by inverted microscopy. *T. gondii* was easily detected in MRC-5 fibroblasts cells within 2 days after inoculation, which agrees with the results of Derouin et al. (1987). The optimal initial concentration for cultivating tachyzoites was 10^5tachyzoites/ml. A concentration larger than 10^7 tachyzoites did not favor the parasite's multiplication (Chamberland and Current, 1991).

HCT-8, HT-29, and Caco-2 were able to produce similar or larger numbers of tachyzoites than MRC-5; however, the probability of infection was lower in those than in MRC-5 (Fig. 2.4.). HCT-8, HT-29 and Caco2 had an aggressive growth producing domes or overgrowth that sometimes did not facilitate observation of tachyzoites in the cells (Hughes et al., 1985). Within 2 to 4 days when *T. gondii* tachyzoites began to infect and propagate, the cells grew more quickly than the tachyzoites, with the cell monolayer dying without becoming fully infected. When infection was observed in a low density monolayer, infection was established within 3 to 10 days in HT-29, and 1 to 5 days in HCT-8. MRC-5 and Hs1.Int were similar in behavior, and in 50% of the cases propagation of tachyzoites in Hs1.Int was greater than in MRC-5; however, under the same conditions, infection was not consistently reproducible in Hs1.Int as compared to MRC-5.

No infection was observed in non-adherent cell lines either by inverted or bright field microscopy. However, Chamberland and Current (1991) reported that J774A.1, a non-adherent cell line, was able to support rapid growth of *T. gondii* RH in 2 –4 days. Differences in that study that may help explain differences in results include larger tachyzoite concentrations (10^6 and 10^7/ml) were used in that experiment, cells were in the

monolayer phase, and different size flasks were used. Mouse macrophage cell lines did not grow in 25 cm^2 flasks because acidification of the medium was too rapid when parasites were exposed to host cells (Chamberland and Current, 1991).

We observed 95% viability of free parasites in our study. These results are in agreement with those of Chamberland and Current (1991) who reported 90% viability of the tachyzoites.

CONCLUSION

MRC-5 was the best cell line of the cell lines evaluated to cultivate *T. gondii* tachyzoites. The overgrowth of MRC-5 cells did not affect infection or viability of *T. gondii* tachyzoites which occurred with HCT-8, HT-29, and Caco-2 cell lines. *Toxoplasma* was able to infect and reproduce in all of the cell lines of human origin where initial inoculations of 10^3, 10^4, and 10^5tachyzoites/ml were used.

Tachyzoites derived from MRC-5 cell line has demonstrated to have the same properties than tachyzoites obtained from mice. Also MRC-5 provides large yields of pure tachyzoites with little or no debris in a short time and with a constant probability of infection. These characteristics are useful in laboratories where the specific results are needed in short time.

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CHAPTER 3

DETECTION OF *TOXOPLASMA GONDII* IN MICE EXPERIMENTALLY INOCULATED WITH SMALL NUMBERS OF TACHYZOITES.[1]

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To be submitted to Journal of Parasitology

DETECTION OF *TOXOPLASMA GONDII* IN MICE EXPERIMENTALLY INOCULATED WITH SMALL NUMBERS OF TACHYZOITES.

ABSTRACT

The objective of this study was to evaluate three methods (nested–PCR, Western blot and histology) for the detection of *Toxoplasma gondii* infection in mice experimentally inoculated with small numbers of tachyzoites. Mice were infected intraperitoneally and orally with 10, 10^2, 10^3 and $2x10^5$ *T. gondii* tachyzoites. PCR amplification of *T. gondii* was determined using the B1 gene as primer. A Western blot assay was designed to detect IgG and IgM antibodies against *Toxoplasma* antigens that are conserved in the tachyzoite and bradyzoite stages. Tissues of various organs were observed by histology using hemotoxylin and eosin stain. A humoral immune response was developed in 10 of 20 (50%) intraperitoneally infected mice and 6 of 20 (30%) mice orally infected. Detection by nested-PCR was observed in 10 of 20 (50%) intraperitoneally infected mice and 3 of 20 (15%) orally infected mice. Histological examination revealed infection in 5 of 20 (25%) intraperitoneally infected mice. Infection was not observed in orally infected mice. Histology was the least sensitive and most time consuming technique for the identification of *Toxoplasma gondii.* Western blot was more sensitive than nested PCR. Western blot may become used for a rapid identification of *T. gondii* infection in clinical laboratories.

INTRODUCTION

Toxoplasmosis is one of the most common parasitic diseases in the world. In terms of deaths associated with foodborne illnesses, *Toxoplasma, Listeria and Salmonella* are the three most important pathogens in the US and possibly in Europe (Dubey, 2000). Immunocompromised individuals may experience severe symptoms, including chorioretinitis, myocarditis, pneumonitis, encephalitis and multi-system organ failure (Gagne, 2001). Immunocompetent individuals are generally asymptomatic. However, toxoplasmosis during pregnancy may lead to severe, if not fatal, infection of the fetus. When the fetus is infected in the first trimester, the result is spontaneous abortion, stillbirth, or severe disease. If infection occurs after the first trimester, disease manifestation includes epilepsy, encephalitis, retardation, blindness, and other neurological disorders (Lin et al., 2000).

Detection of *Toxoplasma*-specific antibodies is the primary diagnostic method to determine infection with *Toxoplasma* in humans and animals (Conde et al., 2001). . The immunofluorescence assay (IFA) and enzyme-linked immunoabsorbent assay (ELISA) test for IgG and IgM antibodies are the most common tests used today. Other methods of *Toxoplasma* diagnosis include histo-immunochemistry. Recently, the polymerase chain reaction (PCR) has been developed for *Toxoplasma* detection and has demonstrated a good correlation with other diagnostic techniques. In one study, PCR was more specific and sensitive in detecting the presence of *T. gondii* in large animals than was histology (Esteban-Redondo et al., 1999).

The purpose of this study was to compare three methods for detecting *T. gondii* infection in mice using two different routes (i.e., i.p. and oral) of *T. gondii* inoculation. In both routes of inoculation, different numbers of tachyzoites of the RH strain were administered. The objectives were to determine for each detection method, whether infection could be identified in different tissues containing small numbers of *T. gondii* and to observe the difference in mice response to the two routes of tachyzoite inoculation.

MATERIALS AND METHODS

Toxoplasma gondii tachyzoites. *Toxoplasma gondii* RH strain tachyzoites were obtained from Dr. R. Gilman, A.B. Prisma, Lima, Peru. They were grown in MRC-5 cells at 37°C and 5% CO_2, and harvested from the supernatant of MRC-5 cell culture after three or four days of inoculation. Tachyzoites were counted using an hemocytometer (Hausser scientific, 0.100 mm deep) and trypan blue to determine viability.

***Antigen preparation*. -** Tachyzoites (10^7 to 10^8/ml) were collected from tissue culture, passed through a 5 μm Versapor membrane Syring filter (Pall Corporation, Ann Arbor, MI) to remove culture debris and cells, and washed at 1300xg for 15 min four or five times with phosphate- buffered saline (PBS) pH 7.4 (0.1 M NaCl, 0.002 M KH_2PO_4, 0.003 M KCl, 0.01 M Na_2HPO_4, dH_2O). Tachyzoites were counted using a hemocytometer and stored at -80°C until sonication. Sonication was performed using a sonifier (model S-450A, Branson Ultrasonics Corp., Danbury, CT) with an output control of 3, duty cycle of 30% for 1 minute of sonication and 2 minutes of rest at 4°C until most of the tachyzoites were lysed. Protein concentration was determined by the micro BCA

proteins assay (Pierce, Rockford, IL) and read using a micro plate reader (Dynatech Laboratories, Chantilly, VA). Tachyzoite antigen was prepared under non-denaturing conditions. The sonicated tachyzoites were dissolved in sample buffer containing 10% sodium dodecyl sulfate (SDS), tracking dye (TD) (blue bromophenol, glycerol, 0.5M Tris, distilled water), and 6% glycerol to yield an antigen containing 0.025 ug of protein/µl. The antigen was incubated at 60°C for 20 minutes.

Mice **-** Four to five week old HSD:ICR(CD-1) female mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). Mice were maintained according to good laboratory animal practices. Fresh water and food were available at all times.

Sample collection and storage. **-** Blood samples were collected directly from the tail and in sterile Eppendorf tubes pre-infection (negative control) and post-infection two to three times a week for 2 months. The blood was centrifuged at 5223 g for 5 minutes, and sera were collected and stored at -20°C until use.

Mouse inoculation with Toxoplasma gondii. **-** In the first experiment, 5 groups of 5 mice each were inoculated intraperitoneally with 10, 10^2, 10^3, and $2x10^5$ tachyzoites. One additional mouse served as the negative control. In the second experiment, mice were grouped as described for the first experiment but tachyzoites were mixed with ground pork [the meat was mixed with PBS pH 7.2, and passed through a 5 µm Versapor membrane Syring filter (Pall Corporation, Ann Arbor, MI)] and was inoculated by oral

intubation with 25x1" (25.4mm) W/1-1/4 animal feeding biochemical needles (Popper and Sons, New Hyde Park, NY).

Collection and storage of mouse tissue.- Lung, heart, brain, kidney, spleen and liver were collected for histological examination and DNA amplification. Tissues were collected from mice that were euthanized while showing acute signs of infection, and mice that died as a result of infection. Tissues were also collected from asymptomatic mice that were euthanized at the end of the study (52-59 days post-inoculation). Tissue samples were stored using two different methods. Tissues were fixed in 10% formalin at room temperature until histology was performed, and in PBS stored at -80°C until DNA extraction was performed.

Ascites fluid from mice that died due to acute infection was collected and observed by bright field microscopy.

Positive and negative controls.- A positive human serum sample was obtained from Dr. David Peterson, University of Georgia, Athens, GA. A negative human serum sample was obtained from the University of Georgia, Griffin, GA.

SDS-PAGE and Western blot analysis. – Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 15% acrylamide gel under non-reducing conditions. The resolving gel was prepared with resolving buffer (Tris 1.7 M, distilled water, pH 9.2) and resolving acrylamide gel solution (0.56 M acrylamide, 0.006 M Bis-acrylamide, distilled water), TEMED (0.6 M N,N,N'N'-tetramethylthylene

diamine), 0.06 M ammonium persulfate (APS), distilled water) was prepared and allowed to polymerize for 20-30 minutes at room temperature. The stacking gel was prepared with stacking buffer (Tris 0.2 M, distilled water, pH 6.14), stacking acrylamide gel solution (0.04 M acrylamide, 0.002 M Bis-acrylamide, distilled water), 0.06 M APS (1:10), 0.6 M TEMED (1:4) was added to the top part of the resolving gel, and a white comb teflon 1.0 mm thick spacer (Biorad Laboratories, Hercules, CA) was inserted into the resolving gel and allowed to polymerize at room temperature for 20 minutes. A kaleidoscope pre-stained standard (Biorad Laboratories, Hercules, CA) was used as a molecular weigh control. Two hundred microliters of antigen was used for each gel. A mini trans-blot electrophoresis cell (Biorad Laboratories, Hercules, CA) was used for the electrophoresis. The upper chamber was filled with the upper buffer (0.04 M boric acid, 0.04 M Tris base, 10% SDS and distilled water, pH 8.6), and the lower chamber was filled with the lower buffer (resolving buffer, distilled water, pH 9.2). Gels were run at 200V at room temperature until the dye reached the bottom of the gel. The separated proteins were electrophoretically transferred to nitrocellulose membrane (0.45 μm) (Biorad Laboratories, Hercules, CA) by electroblotting at one Amp for 2 hours in a transfer buffer (lower buffer, distilled water, 6 M methanol) with a mini Trans-blot transfer cell (Biorad Laboratories, Hercules, CA). Ice was used to keep the blotting temperature at or below the ambient temperature. After transfer, membranes were washed in PBS-Tween 20 and then in PBS, cut into 0.5-1 mm strips and stored at -80°C until use. The membrane strips were individually incubated overnight in mini-incubation trays (Biorad, Hercules, CA) with 500 μl of the test serum which was diluted 1:50 in PBS Tween-20/5% low fat dry milk to block non specific antibody binding. Nitrocellulose membranes were then washed

with 50°C PBS-Tween 20 for 5 minutes, followed with PBS Tween-20 for 5 minutes three times. Peroxidase-labeled affinity purified antibody to mouse IgG (H+L) 1/1000 (1mg/ml) or IgM (μ) 1/1000 (1mg/ml) (KPL, Gaithersburg, MD) or human IgG (H+L) 1/1000 (1mg/ml) or IgM (μ) 1/1000 (1 mg/ml) (KPL, Gaithersburg, MD) was added and incubated for one hour at room temperature. The membranes were washed with PBS Tween-20, three times for 5 minutes each, and with PBS twice for 5 minutes each. The membranes were incubated with a peroxidase substrate diaminobenzidine (DAB) (Sigma-Aldrich, Steinheim, Germany) diluted in PBS with 0.02% of H_2O_2. Reaction was stopped with distilled water when bands appeared in the positive control. The membranes were allowed to dry. The molecular weight of the reactive bands was determined using the digital imaging system IS-1000 (Alpha Innotech Corporation, San Leandro, CA).

Histology. - Lung, heart, kidney, spleen and liver fixed in 10% buffered formalin, were embedded in paraffin, sectioned to 3 μm thick and hemotoxylin-eosin stained. Sections were examined with bright field microscope at 40X for the presence of *Toxoplasma gondii*.

DNA extraction. - DNA was extracted from formalin-fixed, paraffin-embedded tissues and frozen tissues. Sections of tissues embedded in paraffin were cut at 8 μm and 3 μm with microtome (Rotary microtome 100 series, Ventana, AZ, USA).

Chelex 100 extraction (Legrand et al., 2002) was used to extract DNA from tissues fixed in 10% formalin and embedded in paraffin. Briefly, the tissue was scraped into a microtube containing a preheated mix of 200μl 5% Chelex (Chelex 100, Biorad),

200 µl/ml Proteinase K and 39 mM DTT and was incubated at 60°C for 3 days. Cells were lysed by boiling at 100°C for 15 minutes. The supernatant containing the DNA was recovered after centrifugation at 15996 g for 10 min.

Organic extraction (Legrand et al., 2002) was used to extract DNA from tissues fixed in 10% formalin and embedded in paraffin. Briefly, tissue was scraped into a microtube containing 400 µl preheated (60°C) digestion buffer plus 400 µg of proteinase K/ml and 39 mM DTT. Tubes were incubated for 3 days at 60°C. The aqueous phase was recovered after two extractions with phenol/chloroform/isoamyalcohol (24:24:1). A 20% Chelex 100 solution was added to the aqueous phase (1:4) and incubated at 56 °C for 30 minutes. DNA was precipitated by adding 0.1 volume of 3M sodium acetate and 2.5 volume of 95% ethanol at 20°C and re-solubilized in 50 µl of milliQ water.

The phenol-chloroform extraction method was used for frozen tissues (Hoff-Olsen et al., 1999). Briefly, 5 ml of an extraction buffer of pH 8.0 (10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 2% sodium dodecyl sulfate) was added to the approximately $1cm^3$ tissue sample and incubated for 30 minutes at 37 °C. Fifty µl of proteinase K (20 µl/ml) was added and incubated at 37°C overnight. Ten ml of chloroform-phenol-isoamylalcohol (24:25:1.0) was added and the solution was centrifuged at 2205 g for 10 min. The upper layer was transferred to another tube and mixed with 5ml chloroform-phenol-isoamylalcohol followed by centrifugation. The upper layer was again transferred and mixed with 5 ml of chloroform isoamylalcohol (24:1) before a third centrifugation at 2205 g for 10 min. The DNA was made insoluble by the addition of two to three times the solution's volume of cold absolute ethanol to the supernatant. This solution was then centrifuged in at 4°C at 15996 g for 30 min. DNA was dried and before it was made

suspended in 50 µl of TE buffer by mixing overnight at the speed 3 in the Rocking Platform (VWR Scientific Products).

PCR Template DNA Extraction from Toxoplasma gondii tachyzoites. Tachyzoites incubated in MRC-5 cells at 37°C and 5% CO_2 were filtered through a 0.5 µm Versapor membrane Syring filter (Pall Corporation, Ann Arbor, MI) and washed with PBS 1X for 5 times at 1300 x g for 15 min. Tachyzoites were counted using a hemocytometer, and diluted to a final concentration of 300 tachyzoites/ml. Viability was determined using trypan blue (Sigma, St. Louis, MO). InstaGene matrix 505 µl; (Biorad Laboratories, Hercules, CA) was added for every 100 µl of tachyzoite suspension. DNA was released from tachyzoites by 5 cycles of a freeze and thaw method (2 min in liquid nitrogen followed by 2 min in boiling water). The preparation was centrifuged for 4 minutes at 14,000 rpm. The supernatant was used for PCR analysis.

PCR detection method. Two sets of primers of the B1 gene of *T. gondii* were used to perform nested-PCR. B1 gene is highly conserved and one of the most sensitive and specific genes of *T. gondii*. It is repeated 35 times in the genome thereby increasing the detection sensitivity of small numbers of *Toxoplasma* tachyzoites. The primer sequences were as follows: TXE1, 5'-GGAACTGCATCCGTTCATGAG-3'; TXE2, 5'-TCTTTAAAGCGTTCGTGGTC-3'; TXI1, 5'-TGCATAGGTTGCAGTCACTG-3' and TXI2, -GGCGACCAATCTGCGAATACACC-3' (Jones et al., 2000). PCR was performed with a thermal cycler (Master Cycler Gradient, Eppendorf, Hamburg, Germany).

External Amplification.- PCR assay was carried out in a total volume of 15 µl. The master mix was comprised of 4.1 µl of 10X PCR water (Sigma, MI), 1.5 µl of 10X purified BSA (New England BioLabs, Beverly, MA), 0.35 µl 2mM $Mg(OAC)_2$, 1.5 µl of 10X Taq buffer, 1 µl of 10mM dNTP mix, 1 µl of 5X Taq master, 0.1 µl of Taq DNA polymerase (5 U/µl) (Eppendorf, New York, NY), 1 µl of 3 µM Primer TXE1, and 1 µl of 3 µM Primer TXE2 (Invitrogen, Frederick, MD). Five microliters of *T. gondii* DNA (1.5 tachyzoites/5 µl) from MRC-5 cells were used per 10 µl of PCR mix. The amplification procedure included 2 min of denaturation at 94°C, followed by 40 cycles each consisting of 10 seconds of denaturation at 93°C, 10 seconds of annealing at 57°C, and 30 seconds of extension at 72°C. A final extension step at 73°C for 3 min was included. DNA extracted from *T. gondii* tachyzoites served as a positive control and blank containing water served as a negative control.

Nested Amplification. - PCR was carried out in a total volume of 15 µl. The master mix consisted of 3.8 µl of 10X PCR water (Sigma, MI), 1 µl 3 mM Mg $(OAC)_2$, 1.5 µl of 10X Taq buffer, 1 µl of 10mM dNTP mix, 1 µl of 5X Taq Master, 0.1 µl Taq DNA polymerase (5 U/µl) (Eppendorf, New York, NY), 1 µl of 3 µM Primer TXE1, and 1 µl of 3 µM Primer TXE2, (Invitrogen, Frederick, MD). DNA of the external amplification (0.5 µl) and 4.5 µl of PCR water were used per 10 µl of PCR mix. The amplification procedure included 2 min of denaturation at 94°C, followed by 40 cycles each consisting of 10 seconds of denaturation at 93°C, 10 seconds of annealing at 62.5°C, and 15 seconds of extension at 72°C. A final extension step at 73°C for 3 min was included. Positive and

negative control samples from the external amplification were included in the nested reaction. Each sample was tested three times by PCR.

Statistic analysis

All experiments were replicated four times. Differences between experiments, concentrations of tachyzoites, and methods of detection were determined with the Statistical Analysis System (SAS) using general linear model (GLM) and Pearson correlation. The level of significance reported for all tests was $\alpha=0.05$.

RESULTS

Observations in inoculated mice

Mice infected with $2x10^5$ tachyzoites by intraperitoneal inoculation were severely affected in a short period (5 days); however, two mice survived 8 and 10 days following i.p. infection, which died of acute infection and presented parasite-rich ascites. Mice infected with 10^3 tachyzoites were also severely affected in a short period of time (7 to 8 days). One mouse survived and did not develop antibodies to *T. gondii.* It was euthanized 53 days post infection. Of mice inoculated with 10^2 tachyzoites, only one mouse died with acute infection 17 days post infection and presented parasite rich ascites; the others survived until they were euthanized (53 P.I.). Mice inoculated with 10 tachyzoites survived and did not show any symptoms. They were euthanized 53 days post inoculation.

Microscopic examination of the ascites fluid from mice that died of acute toxoplasmosis, revealed presence of tachyzoites and some inflammatory cells.

Mice inoculated in the oral infection experiment with $2x10^5$ tachyzoites results in two mice dying on 10 and 12 days post injection, with acute infection. The other mice did not present any symptoms and were euthanized 59 days after inoculation.

Mice orally inoculated with 10^3, 10^2, and 10 tachyzoites did not present any symptoms. They were euthanized 59 days post injection.

Histology

Tissues from mice that were sacrificed while moribund, mice that recently died and all that were euthanized at the end of the studies were stained with hemotoxylin and eosin and examined microscopically.

Infection was observed in 4 of 5 mice inoculated i.p. with $2x10^5$tachyzoites, in 3 of 5 mice inoculated with 10^3 tachyzoites and in only one mouse inoculated with 10^2 tachyzoites (Fig. 3.1). Lung and liver were the only tissues infected by *T. gondii* as determined by histological examination. The only tissue affected in all cases was the lung and in one case the liver (one mouse inoculated with 10^3 tachyzoites). Changes were characterized by necrosis and the presence of numerous tachyzoites within hepatocytes and Kupffer cells. The tachyzoites appeared as small (1-25 μ) dots. No bradyzoites or tissue cysts were identified in either experiment. No organisms were observed in tissues of mice inoculated orally.

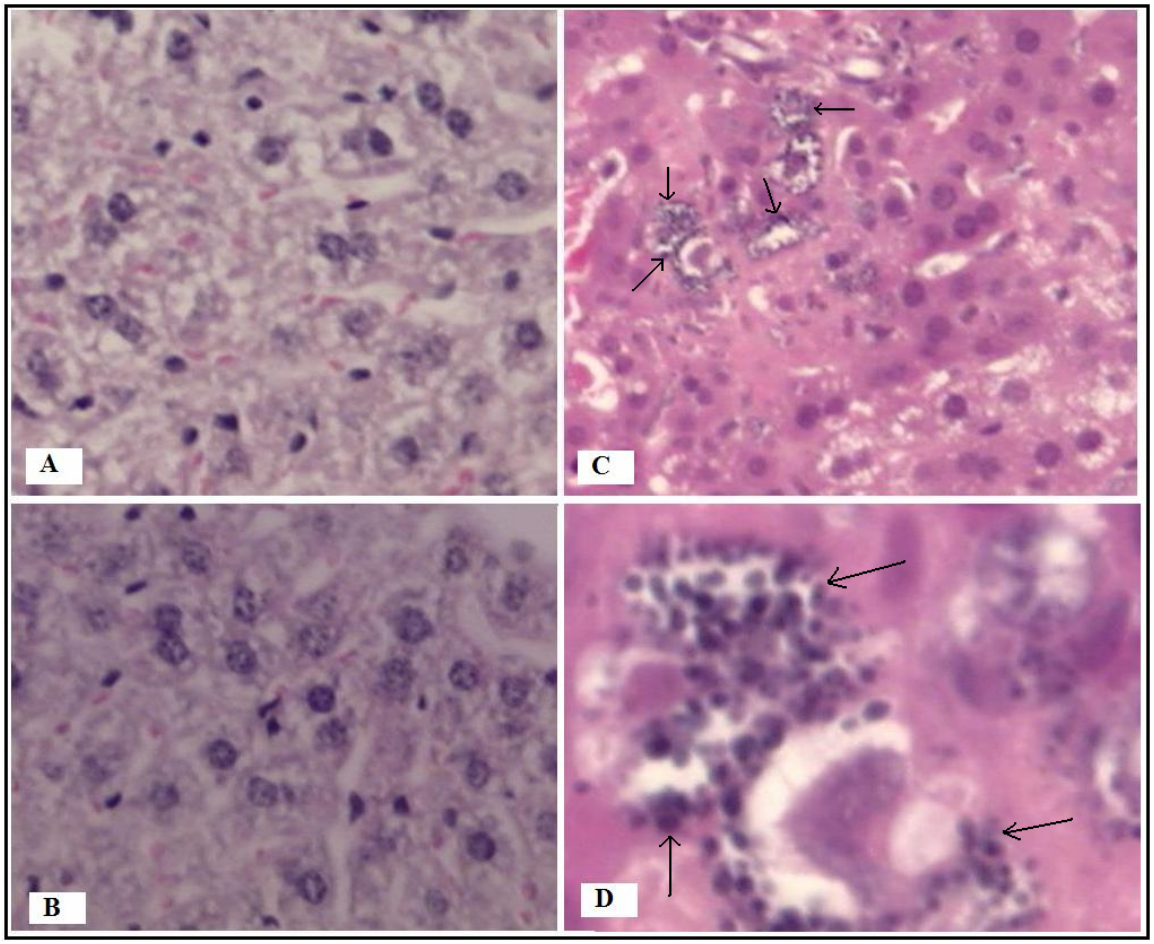

Fig. 3.1. Hemotoxylin and eosin stain of histological sections (A), (B) liver, uninfected mouse, 20 X (C, D) groups of tachyzoites (arrows) present in the liver of a mouse i.p. infected with 10^3 tachyzoites, 9 days P.I., (C) 20X, (D) 40X.

Western blot

Serum with antibodies to *T. gondii* was used as a positive control (human serum) was reactive with antigens of molecular weights 82kDa, 68kDa, 63kDa, 53kDa, 44kDa, 40kDa, 32-29kDa, 26kDa, 24-22kDa, 17kDa, and 6-2kDa. A strong reaction was observed with 31, 24, and 6 KDa antigens. Only the band 31 kDa was recognized by serum obtained from mice inoculated intraperitoneally or orally. IgG responses appeared

at the fourth or fifth day of infection, and IgM appeared the first or second day post infection following i.p. inoculation. IgG antibodies to *T. gondii* in mice orally infected were detected at the eighth day and IgM at the fourth day post infection. No samples were collected previous to day four.

Fifty percent (n=10) of the mice inoculated intraperitoneally and 30% (n=6) of the mice orally infected produced antibodies to *T. gondii.*

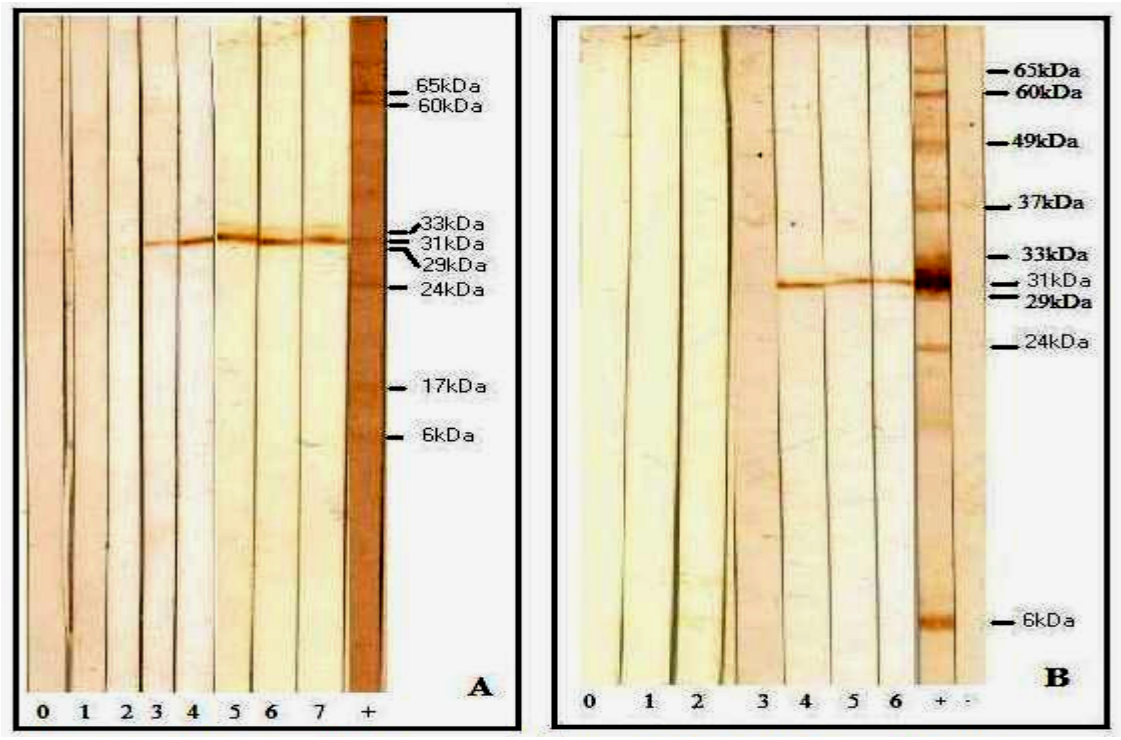

Fig. 3.2. Western blot of IgM and IgG antibodies to *T. gondii* obtained from a mouse infected i.p. with $2x10^5$ RH tachyzoites. Lane 0, negative control (pre-inoculation); lanes 1-7, post inoculation; Lane (+), human serum positive control; Lane (-), negative control. Fig. A, IgM antibodies from day 0 to day 7. Fig. B, IgG antibodies from day 0 to day 6.

PCR

All tissue samples of infected and control mice that were examined by histology, were also assayed by nested-PCR.

The sensitivity of nested-PCR was 0.1 pg of pure DNA, which is equivalent to one tachyzoite (Cornelissen et al., 1984). DNA was detected in 50% (n=10) of the mice inoculated i.p. and in 15% (n=3) of the mice orally infected (Table 3.1). External PCR amplified a single 220-bp band, and nested-PCR amplified a 120-bp single band of *T. gondii*.

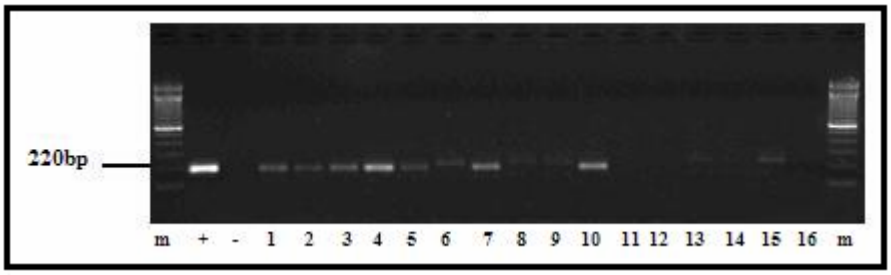

Fig 3.3. Product of external PCR of Chelex-extracted DNA from paraffin-formalin-embedded tissues from mice inoculated i.p. Lanes 1-16, mice tissue samples; (m), molecular marker, 100 bp; (+), positive control; (-), negative control.

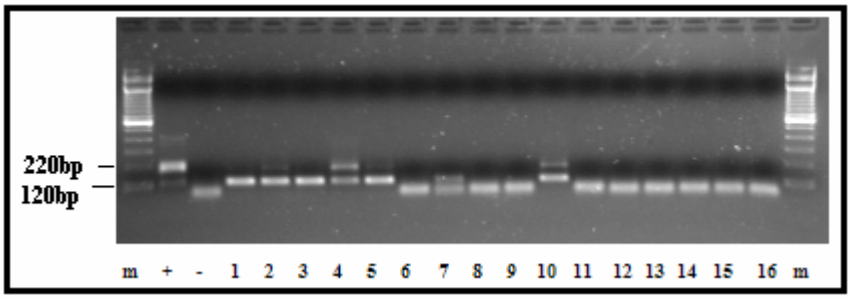

Fig. 3.4. Product of nested-PCR of phenol-chloroform extracted DNA from paraffin-formalin embedded tissues from i.p. inoculated mice. Lanes 1-16, mice tissue samples; (m), molecular marker, 100 bp; (+), positive control; (-), negative control.

Table 3.1. Detection of *T. gondii* infection in mice by histology, western blot and nested-PCR.

	Number of positive tests obtained by:							
	Ascites		Histology		Western blot		Nested-PCR	
Dilutions	i.p.	oral	i.p	oral	i.p.	oral	i.p.	oral
10	ND	ND	0/5	0/5	0/5	0/5	0/5	0/5
10^2	1/5	ND	1/5	0/5	1/5	1/5	1/5	0/5
10^3	4/5	ND	3/5	0/5	4/5	1/5	4/5	1/5
$2x10^5$	5/5	ND	4/5	0/3[b]	5/5	2/5	5/5	2/5

[a]ND

[b]Tissues samples of 2 mice were not examined.

DISCUSSION

In the present study, determination of the presence of necrosis or parasites (tachyzoites and/or bradyzoites) by microscopic examination of tissues stained by eosin and hemotoxylin, by Western blot, of specific IgM and IgG antibody responses and by nested PCR of DNA revealed level of *T. gondii* tachyzoites inoculated intraperitoneally or orally inoculated in mice correlated with degree of virulence.

Presence of necrosis or tachyzoites occurred only in tissues of mice inoculated intraperitoneally and was not detected in mice inoculated orally when H and E stained

tissues were examined microscopically. Lung and liver were the only tissues affected. Infection was observed only in mice inoculated i.p. with $2x10^5$, 10^3, and 10^2 tachyzoites. We observed a significant difference ($p<0.05$) between different doses. Dubey et al. (1996) reported that *T. gondii* organisms are found more in muscle than in neural tissues. Dubey et al. (1984), recovered *T. gondii* from brain, heart, tongue, thigh muscle, diaphragms, kidney, spinal cords, liver, small intestine, eyes and salivary glands, with the brain, heart and tongue being the most heavily infected tissues. Dubey et al. also indicated that a difference of 100-fold in dose did not affect the persistence of *T. gondii* in porcine tissues. Lunden and Uggla (1992) determined that at acute stages of infection, before detectable levels of antibodies or cysts develop, the identification of proliferate *T. gondii* in tissue sections is difficult without specific staining. Hemotoxylin and eosin staining has been used in other studies (Lindsay and Dubey, 1990; Dubey, 2002; Dubey and Frenkel, 1976) with successful results, however, doses of inoculation were higher than those used in our study.

In our study, bradyzoites were not observed in any of the tissue samples removed from mice. Dubey et al. (1999) did not identify tissue cysts in impression smears of the brain of any rats injected with the RH strain. Cysts are not usually observed in histological sections; therefore it is not recommended to look for cysts in mammalians inoculated with *T. gondii* RH strain (Lin and Kuo, 2001), but frequently used. The RH strain is a highly virulent strain that destroys most cells, therefore bradyzoites are not able to encyst (Dubey et al., 1999, De Champs et al., 1997, Ware and Kasper, 1987).

Mice were susceptible to infection with *T. gondii* tachyzoites by oral and intraperitoneal infection, although there was a significant difference ($p<0.05$) between the

two experiments. Only two mice infected orally died 10 days post-injection, and the remainder of the mice remained alive until the study was terminated. Half of the mice inoculated i.p. died between the 4th or 9th day post-injection. These results agree with those reported by Lindsay et al. (1990). They reported a similarity in infection between subcutaneous and oral infection; however, i.p. inoculation differed from both in that mice died earlier in the infection. Tachyzoites are not considered an important source of oral transmission of *T. gondii* because they are rapidly killed outside the host and because they are considered sensitive to proteolytic enzymes. (Powell et al., 2001) However, higher doses (≥1000) of extracellular tachyzoites of *T. gondii* administered were infectious to mice and cats administered by the oral route (Dubey, 1998). Also, Bonametti et al. (1997) reported a case of toxoplasmosis in a breast milk-fed child whose mother had recently acquired toxoplasmosis.

Differences in infectivity between this study and other studies may be attributed to the mice being infected with tachyzoites of the RH strain which were cultivated in MRC-5 cells and passed three to five times, which can result in loss of virulence. Dubey (1998) reported that tachyzoites from passaged several times in cell lines were capable of division, but did not retain infectivity or induce antibody formation. However, Chamberland and Current (1987) and Hughes et al. (1986) reported that in vitro cultivation of *T. gondii* does not affect virulence. In this study tachyzoites remained infectious and were capable of producing ascites fluid and death in i.p.- inoculated mice.

Four bands were commonly recognized in Western blots using antibodies from sera of patients positive for toxoplasmosis. These bands have approximate molecular weights of 28–32 kDa, 26-27 kDa, 22-24 kDa, and 2-6 kDa. One band was recognized in

all positive mice (28-32 kDa) which agrees with the findings of Wastling et al. (1994). Sharma et al. (1983) identified three major antigens with apparent molecular weights of 32 kDa, 22 kDa, and 6 kDa. The human immune response, however, is more evident than the mosue immune response in developing multiple bands.

Polypeptides of low molecular weights of IgM antibodies were identified on day 1-4 post-infection in mice intraperitoneally inoculated and on days 4-8 post-infection in mice orally infected. These results differ from those described by Hafid et al. (2001). They identified three polypeptides with molecular weights of 110, 87 and 75 kDa in sera from all animals on days 1-7 post-infection; however, polypeptides of lower molecular weight (48, 30, 24 and 22 kDa) appeared from day 5. In our study, there was no response to antigens of high molecular weights.

B1 gene was used as a primer for PCR because it appears to be the most sensitive in the detection of *T. gondii* DNA, more so than P30 and 18S rDNA which were able to detect 1 pg genomic *T. gondii* DNA. The sensitivity of the nested-PCR to detect *T. gondii* in this study was 1 tachyzoite, which is 0.1 pg of pure DNA (Cornelissen et al., 1984). These results were corroborated by Hafid et al. (2001), Hitt and Filice (1992), and El Sayed Khlaifa et al. (1994) who determined detection limits of 0.5 to 5 tachyzoites. Moreover, primers based on the B1 gene maintained a level of sensitivity in both normal and inflamed aqueous tissues. Also, B1 primers did not amplify sequences from fungal, bacterial or human lymphocyte DNA (Jones et al., 2000).

In the present study, *Toxoplasma* DNA from fixed tissues could be amplified by PCR even when tissues were stored for up to 1 month, contrary to results of other investigators (Coombs et al., 1999; Koshiba et al., 1993; Romero et al., 1991; NCI) who

described DNA degradation when tissues were stored 2 -3 days in 10% formalin. For one mouse, the DNA signal was the same whether the tissue was stored frozen or in formalin.

The phenol-chloroform and the Chelex methods are effective in extracting DNA from fixed tissues. DNA amplification was detected in the same tissues by both methods of extraction; however the Chelex extraction method was more sensitive than the phenol method. Two bands of 220 bp and 120 bp were detected with the Chelex method, whereas only one band of 120 bp was detected using the phenol chloroform method.

Western blot and nested-PCR were able to detect infection in mice inoculated both i.p. and orally. Histological examination only detected infection in mice inoculated i.p. Among the three techniques, Western blot was the most sensitive. IgG and IgM antibodies were detected by Western blot of sera from mice infected orally with 10^2 tachyzoites. Nested-PCR detected infection in mice orally infected with 10^3 and $2x10^5$ tachyzoites. Hafid et al. (2001) detected *T. gondii* infection at 18 hours by PCR versus 24 h post-infection by capture ELISA and immunoblotting. The investigators concluded that PCR is more sensitive than ELISA, immunoblotting or cell cultures assays to detect *T. gondii* in serum specimens. Early infection dynamics by nested-PCR or by histology were not examined in this study.

Western blot has been demonstrated to be a good technique in the detection of *T. gondii* infection. This technique can be used for a rapid identification of infection with *T. gondii* especially in pregnant and immunosuppressed patients.

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CHAPTER 4

SEROPREVALENCE OF *TOXOPLASMA GONDII* IN SWINE AND CATTLE FROM PERU AND THE UNITED STATES OF AMERICA.[1]

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SEROPREVALENCE OF *TOXOPLASMA GONDII* IN SWINE AND CATTLE FROM PERU AND THE UNITED STATES OF AMERICA.

ABSTRACT

Toxoplasma gondii is an important pathogen transmitted by food, with raw or undercooked meat as the main foodborne source of toxoplasmosis. In Peru, 2 to 4 million people have antibodies to *T. gondii.* It is believed that more than 60 million people in the US are infected with *Toxoplasma gondii*. In this study, the prevalence of *Toxoplasma gondii* in pigs and cows from Peru and the US was determined by Western blot of IgG antibodies from serum samples. Blood samples were collected from 137 pigs and 253 cows at a slaughterhouse in Lima, Peru, and 152 pigs and 23 cattle at a slaughterhouse in Georgia, USA. Of the serum samples from Peru, 27.7% (n=38) of swine and 51.4% (n=130) of cattle were positive for *T. gondii.* Of the serum samples from the USA, 16.4% (n=25) of swine and 26% (n=6) of cattle were positive for *T. gondii*. Pork and cattle represent a significant source of human infection with *T. gondii* in Peru and the USA.

INTRODUCTION

Toxoplasmosis is among the most widespread zoonotic diseases in the world. *T. gondii* is capable of infecting most warm-blooded vertebrates, including humans (Dubey, 1993; Jungersen et al, 2002; Skjerve et al., 1998). Toxoplasmosis is ranked as a leading cause of death in immunocompromised individuals, especially of acquired immune deficiency syndrome (AIDS) patients (Gellin and Soave, 1992). Lymphadenopathy, fever, encephalitis, rash, myositis and myocarditis can develop in immunocompetent individuals after infection with *T. gondii* (Masur et al., 1978; De Silva et al., 1984; Choi et al., 1997; Luft et al., 1983).

Infection can be transmitted by ingestion of *T. gondii* oocysts in food and water contaminated with cat feces or by ingestion of tissue cysts in raw or undercooked meat (Dubey and Beattie, 1998; Suarez-Aranda et al, 2000; Skjerve et al, 1998; Lunden et al., 2002). *Toxoplasma gondii* has been associated with the meat of pigs, goats, lambs, ewes, and cattle. Among meat-producing animals, sheep and pigs are the most frequent carriers of the parasite (Lunden et al., 2002; Dubey and Towle, 1986), whereas cattle appear to be relatively more resistant to the infection it causes (Lunden et al., 2002).

Congenital disease due to *T. gondii* infection is rarely reported in cattle, whereas the parasite is a major cause of abortion and neonatal mortality in sheep. It is believed that sheep remain chronically infected for life. Cattle are thought to harbor fewer parasite tissue cysts which may not persist for the lifetime of the host (Dubey, 1990). Therefore, cattle are believed to pose less of a risk for human infection than sheep (Esteban-Redondo et al., 1999). Gagne (2001) reported that *Toxoplasma* is not present in cattle in the United States. However, a seroprevalence of toxoplasmosis in cattle between <1 to

100% was determined by the Sabin Feldman dye test (DT) or indirect hemagglutination test (IHA), or modified agglutination test (MAT), from different states within the United States (Dubey, 1990).

Several studies have been done on of the prevalence of *T. gondii* in swine in Europe, e.g., in Sweden a seroprevalence of 3.3% was found in fattening pigs and 17.3% in adult swine. Antibodies were detected in samples from all but two of the abattoirs tested, indicating that the parasite is present in all parts of the country. In Denmark, a seroprevalence of 3.1% was detected in fattening pigs and 11.9% in sows. In Finland, a seroprevalence of 2.5% was detected in both swine and fattening pigs by a enzyme-linked immunosorbent assay (ELISA) (Lunden et al, 2002).

In the USA, the prevalence of *T. gondii* infection in swine was determined to range from as low as <1 to as high as 69% by an indirect fluorescence antibody test (IFA), the Sabin Feldman dye test (DT), an indirect hemagglutination test (IHAT), and a modified agglutination test (MAT) (Dubey, 1990). Zimmerman et al. (1990) and Gamble et al. (1999) reported found a seroprevalence between 3.5 to 48% as determined by ELISA or MAT; Dubey et al. (1990) and Weigel et al. (1995) found infection in 3-23% of market age pigs and 11-42% of breeding pigs based on a serological survey of IgG in swine by MAT. Dubey et al. (1995) determined by MAT in the United States a seroprevalence of 22% in sows. In Canada, a seroprevalence of 9.4% in sows was determined by MAT (Smith, 1991), and 3.5 to 13.2% in market age pigs by the latex agglutination test (LAT) (Gajadhar et al., 1998). However, in the US and Europe, the prevalence of *T. gondii* in swine appears to have declined during the last 30 years (Lunden et al., 2002).

Goats represent an other major source of *T. gondii* in some geographical areas such as the Grand Canary Island where the seroprevalence of goat toxoplasmosis approaches a mean value of 63.3% by ELISA (Rodriguez-Ponce et al, 1993).

Few studies on have been conducted in South America; however, Suarez-Aranda et al. (1998) determined by ELISA and Western blot a seroprevalence of *T. gondii* in swine of 32.3% in Peru and 9.6% in Brazil. In Peru, a prevalence of 25.2% in swine reared in good sanitary conditions, and 14.8% in swine reared in poor conditions tested by indirect hemagglutination methods (Bustamante and Suarez, 2000).

Weight, age and sex are important factors related to the prevalence of *Toxoplasma* infection (Suarez-Aranda et al, 1998).

The aim of this study was to provide a better understanding of the risk at which the populations of Peru and the USA are exposed by the consumption of raw or undercooked meat. The prevalence of antibodies to *T. gondii* in swine and cattle naturally infected in Peru and USA was determined by Western blot.

MATERIALS AND METHODS

Collection of samples from Peru. – During September 2002 and January 2003, 137 porcine blood samples and 253 bovine blood samples were collected at an abattoir in Lima, Peru. Serum samples were heated at 60°C for 60 min before shipping to the USA. The samples were stored at -80°C until use.

Collection of samples from USA.- During December 2002 and March 2003, 152 porcine blood samples and 23 bovine blood samples were collected at a slaughterhouse located

in Georgia, USA. The samples were centrifuged at 2500 rpm for 10 min and sera were stored at -80°C until use.

***Antigen preparation*.-** Tachyzoites of *T. gondii* strain RH (10^7 to 10^8) obtained from Dr. R. Gilman, A.B. Prisma, Lima, Peru, were grown in MRC-5 fibroblast lung cells at 37°C and 5% CO_2. Supernatants of cultures were collected, passed through a 5 µm Versapor membrane Syring filter (Pall Corporation, Ann Arbor, MI), and tachyzoites were washed four or five times with PBS, pH 7.4, following sedimentation by centrifugation at 1300xg for 15 min. Tachyzoites were counted in a hemocytometer and stored at -80°C until sonication. Sonication was done with a sonifier (model 450, Brandon) with an output control of 3, duty cycle of 30%. at 4°C with a series of 1 min sonication treatments followed each time by 2 min of rest until most of the tachyzoites were lysed. Protein concentration was determined by Micro BCA protein assay (Pierce, Rockford, IL) and read using a microplate reader (Dynatech Laboratories, Chantilly, VA). The sonicated antigen was prepared under non-denaturing conditions, dissolving in sample buffer [10% sodium dodecyl sulfate (SDS), 6% glycerol, and tracking dye (TD) (blue bromophenol, glycerol, 0.5M Tris, distilled water, pH 8.0)] to yield an antigen containing 0.025 ug of protein/µl. The antigen was incubated at 60°C for 20 min.

Positive and negative controls.- *T. gondii* positive human serum sample obtained from Dr. David Peterson, University of Georgia, Athens, GA, served as the positive control. The negative human serum sample was obtained from The University of Georgia, Griffin, GA.

SDS-PAGE and Western blot analysis. – Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 15% acrylamide gel under non-reducing conditions. The resolving gel was prepared with resolving buffer (1.7 M Tris, distilled water, pH 9.18), resolving acrylamide gel solution (0.56 M acrylamide, 0.006 M Bis-acrylamide, distilled water), 0.6 M TEMED (N,N,N'N'-tetramethylthylene diamine), 0.06 M ammonium persulfate (APS), and distilled water) and allowed to polymerize for 20-30 min at room temperature. The stacking gel was prepared with stacking buffer (0.2 M Tris, distilled water, pH 6.14), stacking acrylamide gel solution (0.04 M acrylamide, 0.002 M Bis-acrylamide, distilled water), 0.06 M APS (1:10), 0.6 M TEMED (1:4)) and added to the top position of the resolving gel. A white comb teflon 1.0-mm thick spacer (Biorad, Hercules, CA) was inserted in the resolving gel and allowed to polymerase at room temperature for 20 min. A kaleidoscope pre-stained standard (Biorad, Hercules, CA) was used as molecular weight markers. Antigen (200µl) was used for each gel. A mini-trans-blot electrophoresis cell (Biorad, Hercules, CA) was used for the electrophoresis. The upper chamber was filled with the upper buffer (0.04 M boric acid, 0.04 M Tris base, 10% SDS and distilled water, pH 8.6), and the lower chamber was filled with the lower buffer (1.7 M Tris base, distilled water, pH 9.2). Gels were run at 200 V at room temperature until the dye reached the bottom of the gel. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (0.20 µm) (Biorad Laboratories, Hercules, CA), by a mini trans-blot cell (Biorad Laboratories, Hercules, CA) using transfer buffer (lower buffer, distilled water, 6 M methanol). Membranes were washed in PBS-Tween 20, cut into 0.5-1 mm strips, and stored at –80°C until use. Membrane strips were individually incubated overnight with 500 µl of

test serum diluted 1:50 in PBS-Tween 20/5% low fat dry milk. The nitrocellulose membrane was washed with PBS-Tween 20 at 50°C for 5 min, followed by three washes with PBS-Tween 20 at room temperature for 5 min. Peroxidase-labeled affinity purified antibody to pig IgG diluted 1/8000 (2.6 mg/ml) or goat antibovine IgG diluted 1/1000 (1 mg/ml) (KPL, Gaithersburg, MD) or human IgG diluted 1/1000 (1 mg/ml) (KPL, Gaithersburg, MD) was added and incubated for 1 h at room temperature. The membranes were washed with PBS-Tween 20 three times for 5 min each followed by PBS twice for 5 min. Membranes were incubated with diaminobenzidine (DAB) (Sigma Aldrich, Milwaukee, WI) in PBS with 0.02% of H_2O_2 at room temperature until bands appeared in the positive control (about 30 seconds to 2 min); the reaction was stopped with distilled water. The membranes were allowed to dry. The molecular weight of the reactive bands was determined with a digital imaging system (Model IS-1000, Alpha Innotech Corporation, San Leandro, CA).

Statistical analysis

The statistical analysis system (SAS) using general linear model (GLM) and Pearson correlation was used to analyze the data. The level of significance for the test in all cases was α=0.05.

RESULTS

Pig serum samples.- Three bands were most frequently observed by Western blot of swine sera i.e., 30-32 kDa, 26 kDa, and 4-6 kDa; however, other bands such as 22-24 kDa, 15-17kDa, and 10kDa were also observed (Fig. 4.1 and 4.2). The 30 to 32 kDa band

which is the most prominent antigen may correspond to the 30 kDa membrane glycoprotein (p30 or SAG1). This protein is specific to tachyzoites and is conserved in other strains of *T. gondii* (Wastling et al. (1994) as determined in ewes vaccinated with tachyzoites of strain S48. The SAG1 or P30 was identified in all of the positive samples, with MW of approximately 28 to 32 kDa (Fig. 4.1 and Fig 4.2). These bands were also recognized by human sera. Immunoglobulin G (IgG) antibodies to *T. gondii* were detected in 38 (27.7%) of 137 pigs serum samples from Peru and in 25 (16.4%) of 152 pig sera samples from the USA (Fig. 4.4).

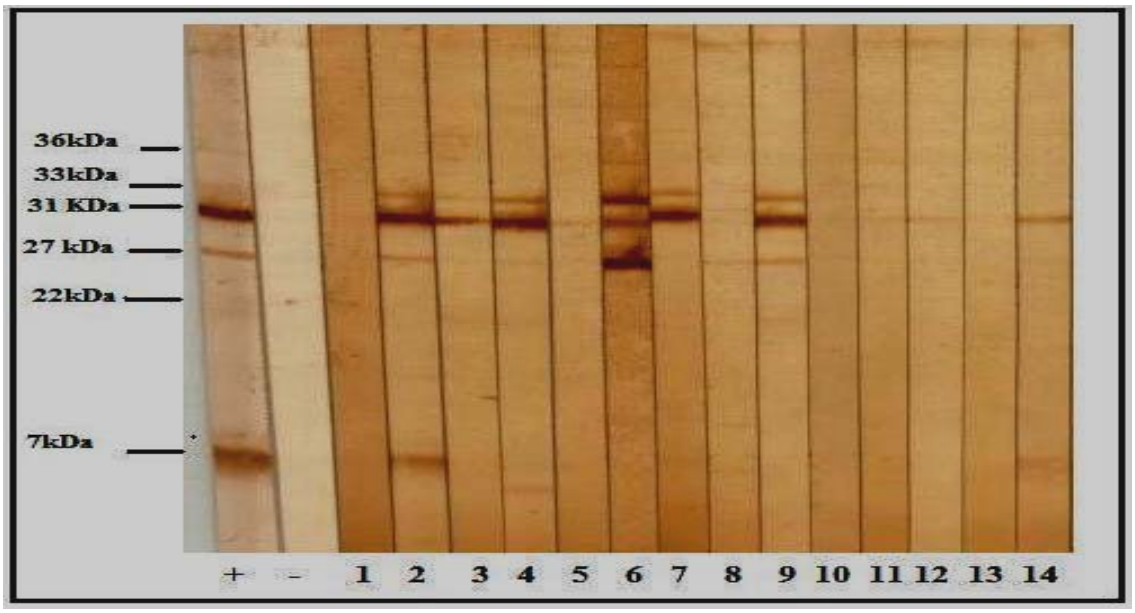

Fig 4.1. Western blot IgG antibodies to *T. gondii* in swine from Peru; Lane (+), human positive sample; Lane (-), negative control. Lane 1-14, different pig serum samples.

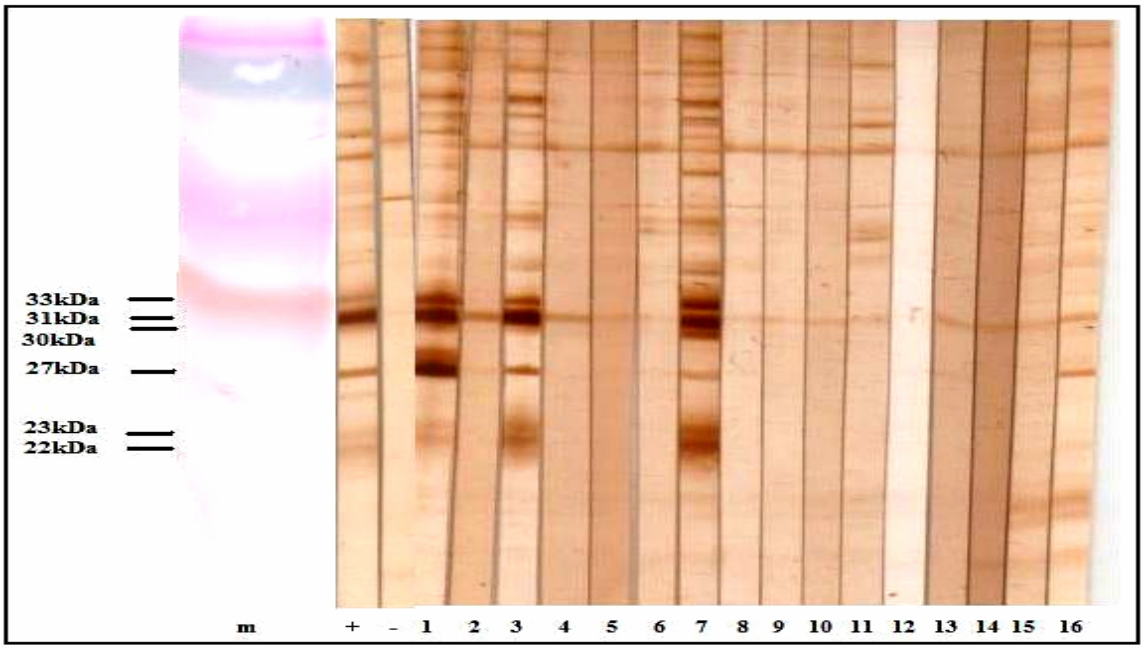

Fig 4.2. Western blot IgG antibodies to *T. gondii* in swine from USA; Lane (+), human positive control. Lane (-), negative control. Lane 1-16, different pig serum samples.

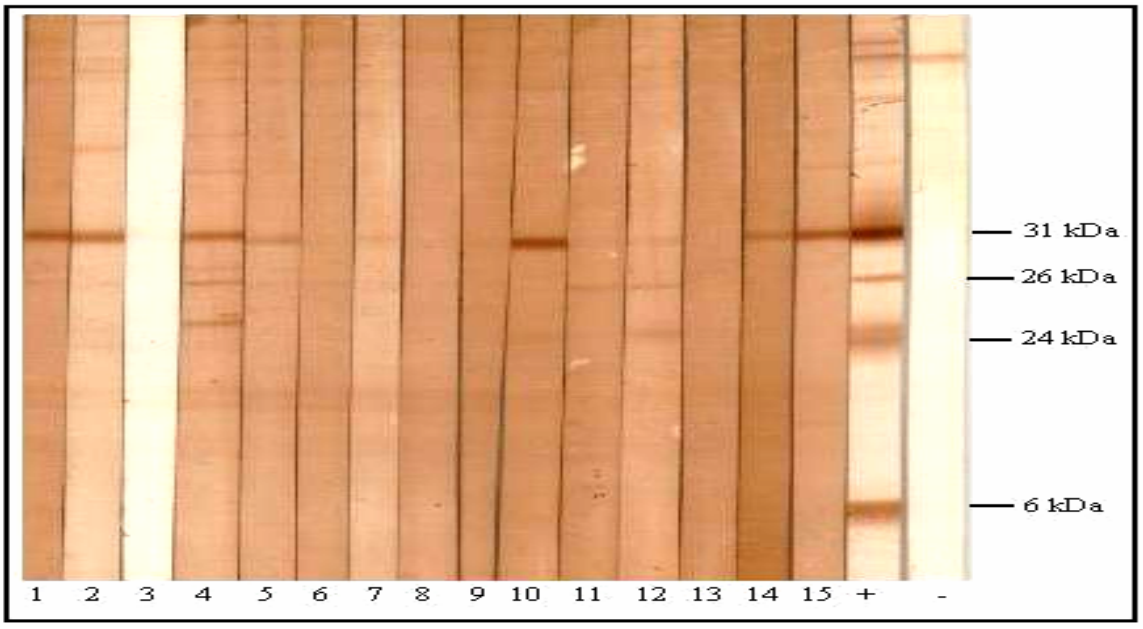

Fig. 4.3. Western blot IgG antibodies to *T. gondii* in cattle from Peru; Lane (+), human positive control. Lane (-), Negative control. Lane 1-21, different cattle serum samples.

Cattle serum samples.- Similar bands to those observed with swine sera were detected by Western blot of cattle sera from Peru and the USA (Fig. 4.3). Immunoglobulin G (IgG) antibodies to *T. gondii* were detected in 130 (51.4%) of 253 cattle from Peru, and in 6 (26%) of 23 cattle serum samples from the USA.

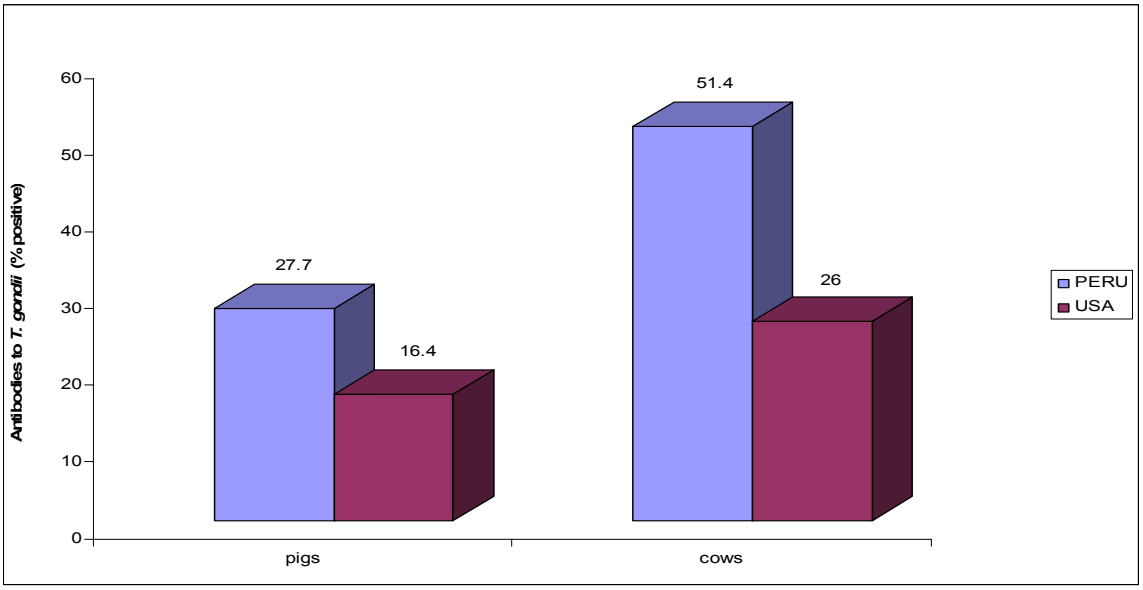

Fig. 4.4. Prevalence of antibodies to *T. gondii* in swine and cattle from the USA and Peru.

DISCUSSION

Serological surveys are commonly used in the determination of *Toxoplasma* infection. Western blot was used in this study as it biochemically characterizes antigens from a complex mixture, showing specific antigen recognition during infection as described in Chapter 3. Other methods such as ELISA, radioimmunoassay (RIA), and hemagglutination are particularly useful for quantitating the amount of certain known antigens (Steward and Male, 1989). This study has determined the exposure of swine to

T. gondii in the USA (16.4%) and Peru (27.7%). The higher prevalence in Peru than in the USA may be attributed to the age of pigs at the time of slaughter. In the USA, most pigs are slaughtered at 6 months of age; however, in Peru most pigs are older and larger at the time of slaughter than those in the USA. D'Angelino and Ishisuka (1986) determined the relationship of weight and age of swine to occurrence of toxoplasmosis and reported a higher proportion of infected pigs among larger or older pigs. A higher prevalence of *Toxoplasma* was observed in pigs from Peru than in Brazil where pigs were slaughtered at a younger age than those in Peru (Suarez-Aranda et al., 2000). The age of an animal, breed, environmental conditions, and management practices appear to be major determinants influencing the prevalence of antibodies against *T. gondii* (Arko-Mensah et al., 2000).

Results of this study agree with those of previous studies in Peru where a seroprevalence of 32.3% was found in swine tested by ELISA and Western blot (Suarez-Aranda et al., 1998). Interestingly, a prevalence of 25.2% was found in pigs reared under good sanitary conditions compared to 14.8% for pigs reared under poor conditions when tested by indirect hemagglutination methods (Bustamante and Suarez, 2000).

In our study, the prevalence of *T. gondii* in swine from the USA was 16.4%, which is in agreement with previous USA studies where the prevalence was shown to be between 4 and 69% (Dubey, 1990). Zimmerman et al. (1990), Gajadhar et al. (1998) and Gamble et al. (1999) have found a seroprevalence between 3.5 to 48% for *T. gondii* in swine; Dubey et al. (1990) and Weigel et al. (1995) found infection in 3-23% of market age pigs and 11-42% of breeding pigs.

In our study, a seroprevalence of 51.4% and 26% was found in cattle from Peru and the USA, respectively. Gondim et al. (1999a) found a prevalence of 1.0% in cattle in Brazil by latex agglutination test (LAT) and he concluded that *T. gondii* in cattle is not high. Lunden et al. (2002) and Esteban-Redondo et al. (1999) reported that cattle are more resistant to *T. gondii* than other species. Dubey (1986) suggested that cattle are not a favored host for *T. gondii* as demonstrated by Hashemi-Fesharki (1996), who tested sera of 2000 cows and did not detect *T. gondii* by direct microscopic examination of fetal tissues. Dubey (1990) concluded that *T. gondii* is quickly eliminated or reduced in bovine tissues after 1 week of inoculation. He reported a serologic prevalence between <1 to 100% in cattle in the US, with 83% in the state of Georgia. Huong et al (1998) found *T. gondii* antibodies in 10.5% of sera of cattle in the USA.

In an experiment done with pork and beef meat inoculated experimentally with *T. gondii* tachyzoites (data not shown). *T. gondii* remained viable for up to 7 days at 4°C, and for up to 4-8 hours at -15 and 42°C in pork meat; however, at 55 and 75°C, tachyzoites were not viable. Infectivity remains viable at 42 and 55°C in beef meat tissue. At 75°C, tachyzoites were not viable. Tachyzoites were not viable when the internal temperature was –18°C or less for 24 hours or more. Kotula et al. (1991) reported that *T. gondii* tissue cysts remain viable for up to 22.4 days at –1 and –3.9°C, and for 11.2 days at –6.7°C, but were usually rendered nonviable by freezing at –12.37°C. Only one of 15 inoculated samples had viable *T. gondii*.when treated at –12.2°C for 8.5 h. Lunden et al. (1992) demonstrated loss of infectivity at –20°C for 54 hours *T. gondii* loses its infectivity when heated at temperatures above 65°C (Lunden et al. 1992).

It is unknown whether there is a cross reaction with antibodies to parasites that are close relatives of *T. gondii* such as *Neospora caninum, Hammondia sp., Besnoitia, and Sarcocystis sp.* that can also infect animals. In (3/9) pig sera, a cross-reactive antibody response was observed with sera of *Sarcocystis*-infected pigs (Lind et al., 1997). Also a moderate cross-reactive response was observed in an indirect ELISA with sera from *Sarcocystis*-infected calves, based on a soluble antigen from sonicated *Toxoplasma* tachyzoites (Uggla et al., 1987). Swine and cattle sera tested with antigens and positive sera for the parasite *Neospora caninum* revealed that there was no cross-reaction between the parasites (data not shown). One of the pigs that were positive for *N. caninum* was negative for *T. gondii*, and in an other case, *T. gondii* and *N. caninum* was positive for the same pig. The remainder of the pigs that were positive for *T. gondii* was negative for *N. caninum*. Most of the surface antigens are homologous to one another and are predicted to share similar sets of surface conformation. A similar set of surface antigens are also present in the tachyzoite stage of *Neospora caninum*. Using SDS-PAGE, the two major surface antigens of *T. gondii* migrate at approximately 29 kDa and 35 kDa and have been referred to as p29 and p35, respectively (Howe and Sibley, 1999). A similar set of antigens in *N. caninum* have named been Nc-p36 and Nc-p43. Surface antigens of *T. gondii*, p29 and p35, are highly immunogenic and are also recognized by antisera from *Neospora*-infected animals (Howe et al, 1998). Cross-reactions between *N. caninum* and *T. gondii* have not been recognized by IFAT (Trees et al., 1994; Buxton et al., 1997), Gondim et al. (1999b) detected *T. gondii* antibodies by LAT in only three of the 63 *Neospora* positive samples, those animals had double infection, which indicates a lack of cross-reactivity between *N. caninum* and *T. gondii* antibodies (Dubey et al., 1996).

Similar results were reported by Reichel and Drake (1996), who found no cross-reactions between *Neospora* and *Toxoplasma* antibodies in sera from bovine in New Zealand. On the other hand, antigen preparations may include somatic antigens released when tachyzoites are lysed (Buxton et al., 1988; Pare et al., 1995; Dubey et al., 1996). *T. gondii* is well-known as a major cause of abortion in sheep and goats, but does not appear to be involved in abortion or clinical illness in cattle (Dubey, 1986). On the contrary, *N. caninum* is an important cause of abortion and neonatal mortality in cattle (Huong et al, 1998). *T. gondii* antibodies were found in 10.5% of sera of cattle, whereas 5.5% were positive for *Neospora caninum* (Huong et al, 1998). These results agree with results obtained in this study.

CONCLUSIONS

Swine and cattle from Peru and the USA are exposed to *Toxoplasma gondii* as confirmed by the Western blot technique. This supports the suggestion that raw or undercooked pork and beef represent a risk of human infection with *Toxoplasma gondii*, especially for pregnant women and immunosuppressed people.

The number of pigs and cattle used in this study was small, and may only provide a valid estimate for the prevalence of *T. gondii* in the slaughterhouses studied. One slaughterhouse may not be representative of the overall bovine and porcine populations in the USA and Peru; however, this study provides a better understanding of the risk at which the human populations of Peru and the United States of America are exposed to *T. gondii* the consumption of raw or undercooked meat.

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CHAPTER 5

CONCLUSIONS

MRC-5 can be used as a cell line to cultivate *Toxoplasma gondii* tachyzoites. The overgrowth of MRC-5 cells did not affect infection or viability of *T. gondii* tachyzoites as was observed when HCT-8, HT-29, and CaCO2 cell lines were used. *Toxoplasma* was able to infect and reproduce in all of the human cell lines evaluated, each susceptible to infection when exposed to 10^3, 10^4, and 10^5 tachyzoites/ml**.**

There was a significant difference ($p<0.05$) in mice infection with *T. gondii* between the methods of inoculation, as well as among the initial concentrations of tachyzoites administered to the mice. No significant difference ($p> 0.05$) was observed among Western blot and nested-PCR results for the detection of *T. gondii*. Both methods were able to detect infection in both, mice inoculated i.p. and mice infected orally. However, histology only detected infection in mice inoculated i.p. Of the three techniques, Western blot was the most sensitive, and histology was the least sensitive and the most-time consuming. Western blot was able to detect infection when mice were inoculated with 10^2 tachyzoites; however, nested-PCR detected infection only when mice were inoculated with 10^3 and $2x10^5$ tachyzoites.

According to the results of this research, pigs and cattle from Peru and the USA are exposed to *Toxoplasma gondii* as demonstrated by the Western blot technique. This

supports that raw or undercooked pork and beef represent a high risk of human infection with *Toxoplasma gondii*, especially for pregnant women and immunosuppressed people.

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