EVALUATION OF DIFFERENT SERODIAGNOSTIC METHODS OF TOXOPLASMOSIS IN PREGNANT WOMEN AND IMMUNODEFICIENT PATIENTS

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CERTIFICATE

This is to certify that the dissertation entitled, **"Evaluation of different serodiagnostic methods of toxoplasmosis in pregnant women and immunodeficient patients"** by **Dr. G. SUCILA THANGAM**, Post graduate in Microbiology (2005-2008), is a bonafide research work carried out under my direct supervision and guidance and is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in **March 2008**.

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CONTENTS

1. INTRODUCTION

Toxoplasmosis is caused by a coccidian protozoan parasite, *Toxoplasma gondii.* Humans and other warm-blooded animals are its intermediate hosts. The infection has a worldwide distribution. Approximately one-third of all humanity has been exposed to this parasite. Although usually asymptomatic in immunocompetent adults, it can cause severe disease manifestations and even death in immunocompromised patients. If acquired during pregnancy it can cause various congenital anomalies in the child.

In India, the exact seroprevalence of this infection is not known. However, using various diagnostic tests, the prevalence has been reported to be as low as 1 per cent and as high as 80 per cent in adults (Singh, 2003). There is lack of awareness and knowledge about this zoonotic infection.

Asymptomatic toxoplasmosis is the commonest type despite the "Sea of toxoplasmosis infection around us". Feline species especially cat is the "Key host" for toxoplasmosis and most of the animals, birds including human beings are acting as an intermediate host. Although toxoplasmosis infects large proportion of world's human populations, it is thought to be an uncommon cause of typical disease in early days. Nowadays, certain individuals however are at high risk for severe or life threatening disease due to this parasite.

Humans are infected either through contaminated food, water, transfusion of infected blood, organ transplantation and from mother to foetus through placenta. Congenital toxoplasmosis is the result of maternal infection acquired during gestation, an infection that is most often clinically inapparent. In immunodeficient patients, toxoplasmosis most often occurs in person with defects in T cell mediated immunity such as those with haematologic malignancies, bone marrow and solid organ transplant recipients or acquired immunodeficiency syndrome (AIDS). In immunocompetent individual, after acute infection a small percentage suffer chorioretinitis, lymphadenitis, encephalitis or even more rarely, myocarditis and polymyositis.

The prevalence of toxoplasmosis in Indian pregnant women is variably reported (Singh, 2003 and Singh and Pandit, 2004). However, the knowledge about this infection, diagnosis and interpretation of the test results is a major problem in the Indian context. Though, *Toxoplasma* infection does not cause repeated foetal losses, this is the most common indication for investigation of toxoplasmosis in India.

There are several diagnostic test kits available in Indian markets, however, their qualities is not assessed by most of the laboratories before they are procured (Singh *et al.*, 1997). There is no baseline data on seroprevalence and antibody titres of toxoplasmosis in various subpopulations in different parts of our country. There is a

lack of awareness and knowledge about this zoonotic infection. Further complicating the situation, there are several commercial organisations that are promoting their products without proper background knowledge and base line data from India.

Under this situation, it is highly imperative to assess the status of seroprevalence of toxoplasmosis in Tamil Nadu especially in pregnant women and immunodeficient patients based on suitable IgG based 'in-house' immunoassays like IFAT, ELISA and MAT, using laboratory prepared own antigen and known positive serum.

Perusal of available literature revealed no systematic studies on seroprevalence of toxoplasmosis in humans in southern parts of Tamil Nadu was undertaken. The study was aimed to bring out status of toxoplasmosis in southern districts of Tamil Nadu and possibly evaluation of different serodiagnostic methods for diagnosis of toxoplasmosis especially in pregnant women and immunodeficient patients.

2. AIM AND OBJECTIVES

The study was designed with the following objectives

- To find out the seroprevalence of toxoplasmosis in immunodeficient patients (HIV and malignant patients) and immunocompetent patients like pregnant women, ocular chorioretinitis cases and patients with lymphadenopathy in and around Tirunelveli district of Tamil Nadu.

- To evaluate the IgG based tests like Modified direct Agglutination Test (MAT), Indirect Fluorescent Antibody Test (IFAT) and Enzyme Linked Immunosorbent Assay (ELISA) for serodiagnosis of toxoplasmosis.

- To compare the utility of the IgG based tests like MAT, IFAT and ELISA for its suitability of the strategy for serodiagnosis and interpretation of toxoplasmosis.

- To compare the CD4+/CD8+ count of T lymphocytes in HIV patients of seropositive and seronegative group of toxoplasmosis.

3. REVIEW OF LITERATURE

Toxoplasmosis, an important protozoan parasitic disease is caused by *Toxoplasma gondii* the single species of genus, *Toxoplasma*. It is generally a self-limited, asymptomatic illness in immunocompetent patients, although infection can reactivate at a later time if the patient becomes immunosuppressed. Primary maternal infection during pregnancy can be transmitted to the fetus and result in serious sequelae.

3.1 HISTORY

T. gondii was first observed by Charles Nicolle and Louis Manceaux in 1908 in North African rodent, gundi (*Ctenodactylus gundi)* at the Pasteur institute, Tunis. At the same time, 1908 Alfonso Splendore described it from a laboratory rabbit at the Portuguese hospital in Sao Paulo, Brazil. In 1923, Janku, an ophthalmologist in Praque reported it in an 11 month old congenitally infected child. In 1939, Wolf, Cowen and Paige reported a confirmed case of congenital case of toxoplasmosis in a child. Vietzke *et al.* in 1968 reported several severe and potentially fatal disease of *Toxoplasma* encephalitis in patients with hematologic cancers. The complete life cycle of it was not determined until 1970 by Frenkel, Dubey, and Miller. It then became more widely recorded as a cause of morbidity in immunodeficient patients, including AIDS patients beginning in 1983. *T. gondii* continues to be an important disease in the modern world, especially

in pregnant women and immunocompromised patients. The brief review of historical landmarks of *T. gondii* discovery is presented in **Table 1.** (Dubey, 1993)

3.2 TAXONOMY

Toxoplasma belongs to the protozoan Phylum Apicomplexa Levine, 1970 which consists of intracellular parasites that have a characteristically polarized cell structure and a complex cytoskeletal and organellar arrangement at their apical end **(Plate 1).** *T. gondii* classified as a heteroxenous or two-host coccidian, forming tissue cysts. It is grouped in the Subfamily Toxoplasmatinae, Family Sarcosystidae Pouche, 1913, Suborder Eimeriorina, Order Eucoccidiorida, Subclass Coccidiasina, Class Sporozoasida of Phylum Apicomplexa.

Opinions differ regarding the placement of *T. gondii* into families and subfamilies. Various authorities have placed it in one or other of the families Eimeriidae Michin, 1903; Sarcocystidae Poche, 1913, or Toxoplasmatidae Biocca, 1956. At present the *T. gondii* is the only species assigned to the genus *Toxoplasma.* Taxonomic position commonly followed by most of the author is depicted in the **Table 2.**

3.3 MORPHOLOGICAL STAGES AND THE LIFECYCLE

There are three infectious stages of *T. gondii*; the tachyzoites (in groups or clones), the bradyzoites (in tissue cysts), and the sporozoites

(in oocysts). These stages are linked in a complex life cycle (Dubey *et al.*, 1998). **(Plate 2)**

3.3.1 Tachyzoite

The term "tachyzoite" (*tachos* = speed in Greek) was coined by Frenkel (1973) to describe the stage that rapidly multiplied in any cell of the intermediate host and in non-intestinal epithelial cells of the definitive host. The term "tachyzoite" replaces the previously used term "trophozoite" (*trophicos* = feeding in Greek). Tachyzoites have also been termed endodyozoites or endozoites. Aggregates of numerous tachyzoites are called clones, terminal colonies, or groups.

Tachyzoites **(Plate 3.1)** have also been termed endodyozoites or endozoites. Aggregates of numerous tachyzoites are called clones, terminal colonies or groups. The tachyzoite is often crescent shaped, approximately 2 - 4 by 4 - 8 µm with a pointed anterior (conoidal) end and a rounded posterior end with nucleus at the centre.

3.3.2 Bradyzoite and Tissue cyst

The term "bradyzoite" (*brady* = slow in Greek) was also coined by Frenkel (1973) to describe the organism multiplying slowly within a tissue cyst **(Plate 3.2)**. Bradyzoites are also called cystozoites.

Tissue cysts grow and remain intracellular as the bradyzoites divide by endodyogeny. Tissue cysts in the brain are often spheroidal and rarely reach a diameter of 70 µm, whereas intramuscular cysts

are elongated and may be 100 µm (Dubey, 1977). Although tissue cysts may develop in visceral organs, including the lungs, liver, and kidneys, they are more prevalent in the neural and muscular tissues, including the brain, eyes, and skeletal and cardiac muscles. Intact tissue cysts probably do not cause any harm and can persist for the life of the host without causing a host inflammatory response. The tissue cyst wall is elastic and thin (<0.5 µm thick) and it encloses hundreds of crescent-shaped bradyzoites each approximately 7 by 1.5 µm in size. The tissue cyst develops within the host cell cytoplasm (Dubey, 1988_a).

3.3.4 Oocysts

Unsporulated oocysts are sub spherical to spherical and are 10 by 12 µm in diameter. Under light microscopy, the oocyst wall consists of two colorless layers. Polar granules are absent, and the sporont almost fills the oocyst. Sporulation occurs outside the cat within 1 to 5 days of excretion depending upon aeration and temperature.

Sporulated oocysts **(Plate 3.3)** are sub spherical to ellipsoidal and are 11 by 13 µm in diameter. Each oocyst contains two ellipsoidal sporocysts without Stieda bodies. Sporocysts measure 6 by 8 µm. A sporocyst residuum is present; there is no oocyst residuum. Each sporocyst contains four sporozoites (Ferguson *et al.*, 1978).

3.4 EPIDEMIOLOGY OF TOXOPLASMOSIS

3.4.1 TRANSMISSION

T. gondii is transmitted to humans by three principal routes (Desmonts *et al.*, 1974; Dubey, 1998 and Jeffery *et al.*, 2003).

First, humans can acquire *T. gondii* by eating raw or inadequately cooked infected meat, especially pork, mutton, and wild game, or uncooked foods that have come in contact with infected meat.

Second, humans can inadvertently ingest oocysts that cats have passed in their feces, either from a litter box or from soil (e.g., soil from gardening, on unwashed fruits or vegetables, or in unfiltered water).

Third, women can transmit the infection transplacentally to their unborn fetus. In adults, the incubation period for *T. gondii* infection ranges from 10 to 23 days after the ingestion of undercooked meat and from five to 20 days after the ingestion of oocysts from cat feces.

A report from the Economic Research Service of the U.S. Department of Agriculture concluded that one half of toxoplasmosis cases in the United States are caused by eating contaminated meat (Buzby and Roberts, 1996). This conclusion is supported by the findings of a community-based epidemiologic study (Roghmann *et al.*, 1999).

Women infected with *T. gondii* before conception rarely transmit the parasite to their fetus, but those who become acutely infected or have reactivation of *T. gondii* during pregnancy (i.e., because of immunosuppression) can transmit the organism transplacentally. The risk of congenital disease is lowest (10 to 25 per cent) when maternal infection occurs during the first trimester and highest (60 to 90 per cent) when maternal infection occurs during the third trimester (Dunn *et al.*, 1999).

However, congenital disease is more severe when infection is acquired in the first trimester. The overall risk of congenital infection from acute *T. gondii* infection during pregnancy ranges from approximately 20 to 50 per cent. Immunosuppression resulting from human immunodeficiency virus (HIV) infection or therapies for malignancies, organ transplantation and lymphoproliferative disorders can result in the reactivation of latent *T. gondii* infection. Reactivation most often involves the CNS, and symptoms may include those of meningoencephalitis or a mass lesion. Women with reactivated *T. gondii* infection can transmit the organism transplacentally (Remington *et al.*, 2001).

The other miscellaneous modes of transmission of this parasite is also by transplantation of organs and transfusion of blood (Wreghitt *et al.,*1989; Singh *et al.,* 1994; Slavin *et al.,* 1994).

3.4.2 RISK FACTORS AND SOURCES OF INFECTION OF TOXOPLASMOSIS FOR HUMAN BEINGS

3.4.2.1 Vegetarian and non-vegetarian

Little is known of the sources for *T. gondii* infection for humans in India. Rawal (1959) found that the prevalence of *T. gondii* in vegetarians (37.8 per cent of 141) and non-vegetarians (37.4 per cent of 246) was similar. Akoijam *et al.* (2002) carried out a study in rural area near Delhi on primigravida women but could not find any significant difference between vegetarians and non-vegetarians. Also no difference was found on statistical analysis between women who had contact with cat and who did not have. The overall prevalence as measured by IgG antibodies was 41.2 per cent.

3.4.2.2 Undercooked meat

Epidemiologic studies from other parts of world indicate that the ingestion of undercooked meat is an important means of transmission of *T. gondii*. For example, the very high (70 to 90 per cent) seroprevalence of *T. gondii* in Paris, France is attributed to eating undercooked meat (Dubey, 1988_b).

Traditionally, meat is cooked well before human consumption in India. *T. gondii* is killed when the internal temperature of the meat reaches 66°C. However, the recent introduction of fast food chain restaurants in India might change this situation. *T. gondii* may survive in improperly grilled or barbecued meat. Such an outbreak did occur in a group of medical students in New York City who ate hamburgers

(presumably contaminated with meat from other animals) which were undercooked. *Toxoplasma gondii* is also killed by freezing; freezing in a household freezer for one day is generally sufficient to kill this parasite in meat (Dubey, 1988_b).

Poor hygiene observed in India during handling of meat from slaughter house to kitchen can be a source of *T. gondii* infection (Singh and Singh, 1994 & Singh *et al.,* 1996). Although no data are available on the consumption of undercooked meat in India, the prevalence of *Sarcocystis suihominis* oocysts in faeces of 14 of 20 children indicates that meat was consumed raw at least by some because *S. suihominis* can only be transmitted to humans by the consumption of raw pork (Banerjee, 1994).

3.4.3 SEROPREVALENCE OF TOXOPLASMOSIS

3.4.3.1 Seroprevalence of toxoplasmosis in India

Until recently the prevalence of *T. gondii* in the general population of India was considered to be low compared with Western countries (Bowerman, 1991 and Mittal *et al.,* 1995). This may be partially true, but the data are based on convenience samples obtained from patients admitted to hospitals in big cities. The type of serological tests used and the titres taken as evidence of infection are also important factors. Because most of the population (75 per cent) of India lives in rural villages with little means to get to city hospitals, the data are not reflective of the population as a whole. Moreover, data

are based on low titres (1:64) in IHA Test, the specificity of which is doubtful. Three studies during 1990, however, indicate that the prevalence may be a lot higher than previously considered. (Singh and Nautiyal, 1991 and Singh *et al.,* 1994_a)

Bhatia *et al.* (1974) performed a serological study of Toxoplasmosis in South India and noted IgM antibodies by IHA were found in 12 per cent patients.

A study done in Delhi at National Institute of Communicable Diseases by Mittal *et al.* (1990), revealed an overall seroprevalence of only 1 per cent in the 200 samples studied from the general population.

In another survey of 100 asymptomatic women (83 of them 16-50 years old) from villages in Almora district, 57 per cent had IHA antibodies in titres of 162 or more (20 of them had titres of 18 to 54). Even if one discounted lower IHA titres as nonspecific, this is a high prevalence of *T. gondii* in women of child bearing age, ever reported earlier from any part of India (Singh and Nautiyal, 1991).

Banerjee *et al.*, (1994) surveyed 27 villages surrounding a hospital in Kulapur, UP. Of the 123 serum samples obtained, 18 per cent had IHA antibodies to *T. gondii* at a titre of 1:64. Prevalence increased with age. Of importance is that four of 41 (10 per cent) children below nine years of age were seropositive suggesting exposure to contaminated food or congenital infection.

In another study of children from a residential tribal school in Maharashtra, 35.1 per cent of 194 persons had *T. gondii* antibodies. Of particular interest 30 per cent prevalence rate was noticed in 10 to 12 year old children and IgG positivity in 75 per cent adult food handlers of the school mess (Singh *et al.,* 1994_b).

Shanmugam *et al.* (1995) studied the *Toxoplasma gondii* IgM antibody prevalence in patients suffering from neurological disorders in Trivandrum, Kerala by ELISA. The study revealed high prevalence rate of toxoplasmosis in neurosurgery patients (32.7 per cent) and in particular among female (35.2 per cent) than male (17.8 per cent) patients.

Mahajan (1997) reviewed the seroprevalence of toxoplasmosis in different parts of India which is presented in **Table 3**.

Meisheri *et al.* (1997) conducted a study of seroprevalence of Toxoplasmosis in general population and in HIV/AIDS patients in Bombay and reported the overall seroprevalence was 30.9 per cent (51/165) in the immunocompetent adult (34 per cent in men and 26.2 per cent in women). In HIV infected hosts the seroprevalence was 67.8 per cent.

Sharma *et al.* (1997) reported a significant increase in seropositivity in women and newborns) in Chandigarh was seen during 1989-91 as compared to 1981-88. More seropositive patients

were recorded between April-June and October-December. However, no significant correlation could be observed between rising incidence of seropositivity and the seasonal distribution or age of women. *Toxoplasma* seropositivity remained constant at 0.02 per cent from 1981 to 1987; this rate increased to 0.06 per cent in 1989, 0.08 per cent in 1990, and 0.15 per cent in 1991.

Joshi *et al.* (1998) carried out the seroprevalence of toxoplasmosis in Jodhpur. *Toxoplasma gondii* specific IgG and IgM antibodies were assayed by ELISA and seropositivity to one or both classes of antibodies was observed in 17.2 per cent cases.

Akoijam *et al.* (2002) performed a study among primigravid women attending a secondary level hospital in a district of North India using IgG ELISA and reported 41.75 per cent were seropositive for *Toxoplasma gondii* infection.

Mohan *et al.* (2002) carried out the ELISA based seroepidemiological study of toxoplasmosis in different sections of population of Union Territory of Chandigarh and reported overall prevalence of 5.4 and 4.66 per cent, IgM and IgG anti-*Toxoplasma* antibodies respectively. Amongst, significantly higher number of people in slum area (7.8 per cent) showed IgM antibodies as compared to urban and rural areas (4.2 per cent each).

Singh (2002) carried out the seroprevalence study, which was directly proportional to the age of population tested, with highest

prevalence of 77 per cent by the age of marriage (17-20 years). It was also indicated that prevalence and incidence of any of these infections are inversely correlated.

A prospective study conducted by Singh and Pandit (2004) on incidence and prevalence of toxoplasmosis in Indian pregnant women indicated that the overall IgG seroprevalence rate of toxoplasmosis was 45 per cent. Only seven women (3.3 per cent) were found to have IgM antibodies and only two of these showed low IgG avidity indicating recent infection of ≤4 months duration. One woman aborted spontaneously at her fourth month of gestation. In remaining five women the recent infection could successfully be excluded by IgG avidity testing. All these women had uneventful pregnancy.

Yasodhara *et al.* (2004) from Hyderabad, South India reported toxoplasmosis seroprevalence of 33 per cent (IgG) in lower socioeconomic group compared to higher socio-economic group (22 per cent) in 236 women with bad obstetric history.

Naveen *et al.* (2005) studied TORCH infection in 20 pregnant women with bad obstetric history in Kumaon region and revealed ELISA IgM and IgG antibodies for *Toxoplasma* were found in 20 and 55 per cent of patients respectively.

Manikandan *et al.* (2006) recently reported an outbreak of ocular toxoplasmosis in Coimbatore, Tamil Nadu, which was an acute acquired type through municipal water contamination. Out of 249

cases tested during the outbreak, 178 cases reported to had high titres of both IgM and IgG antibodies and 4 cases showed presence of IgM antibodies alone. Although all age groups were affected but a majority of them were above 20 years of age.

Rajendra *et al.* (2006) evaluated the incidence of TORCH infections in pregnancy wastage in women with bad obstetric history and noted 14.66 per cent, were seropositivity for *Toxoplasma* IgG. IgM antibodies were found in 27.27 per cent of cases with recurrent abortions.

Sundar *et al.* (2007) performed the seroprevalence of *Toxoplasma gondii* in 1,000 healthy adult population of voluntary blood donors in Karnataka, South India by ELISA and reported the overall prevalence of 20.3 per cent were positive for *T. gondii* IgG antibody, of which, 63 per cent had high and 7 per cent low avidity and 3.6 per cent IgM positive.

3.4.3.2 Seroprevalence of toxoplasmosis in other countries

Seroprevalence of toxoplasmosis studies vary according to geographic location. The global seroprevalence was reported to be 46.1 per cent (Jacquire, 1995). The incidence of primary *Toxoplasma* infection was reported as 1.1/1,000 pregnancies. In this study it was slightly higher than the earlier reports from USA where, rate of primary *Toxoplasma* infection was <1/1000 pregnancies (Lamb *et al.*, 1968; Dubey, 1988 and Remington *et al.*, 1995)

A study by Jones *et al.* (1996) in 9 United States (US) cities found that *Toxoplasma* encephalitis in HIV-infected person varied by geographic area. About 10-40 per cent of adults with AIDS were seropositive for Toxoplasmosis in US. In Europe, Latin America and Africa where the incidence of latent *Toxoplasma* infection was between 75-90 per cent, number of AIDS patients who developed toxoplasmic encephalitis may be 3-4 times higher than in US (Raffi, *et al.,* 1997).

In a study from a Scandinavian country, incidence of *Toxoplasma gondii* infections in 3,094 Swedish women during pregnancy was performed during 1992-93. Specific IgG anti-*Toxoplasma* antibodies were found in 14 per cent (450/3,094) (Evengard *et al.*, 1999).

Rey *et al.* (1999) carried out a serological survey of *Toxoplasma gondii* infection in population groups in Fortaleza, Brazil. Out of 997 persons, 22.8 per cent of children (mean age 3 to 8 years), 58.4 per cent of students (mean 11.4 years) and 71.5 per cent of pregnant and postpartum women (mean 24 years), were seropositives ($p < 0.001$). They concluded that the toxoplasmosis seroprevalence showed a rapid increase during the first ten years of life, in association with close contact with cats and larger households, probably related to inappropriate hygiene and child-care practices.

Falusi *et al.* (2002) assessed the prevalence and predictors of latent *Toxoplasma* infection in a large group of human

immunodeficiency virus (HIV)–infected and HIV-uninfected at-risk US women. The prevalence of latent *Toxoplasma* infection was 15 per cent (380 of 2525 persons) and did not differ by HIV infection status. HIV-infected women aged ≥50 years and those born outside of the United States were more likely to have latent *Toxoplasma* infection, with prevalences of 32 per cent and 41 per cent, respectively.

Nissapatorn *et al.* (2003) stated that the seroprevalence of toxoplasmosis among 406 AIDS patients was 208 (51.2 per cent) in the Hospital Kuala Lumpur (HKL), Malaysia. Their age ranged from 17 to 74 years with a median of 35 years and the majority of patients were males 172 (82.6 per cent).

Diza *et al.* (2005) estimated the seroprevalence of *Toxoplasma gondii* in the northern Greek population based on IgG-specific antibodies by IFA and found to be 37 per cent, 29.9 per cent and 24.1 per cent in 1984, 1994 and 2004, respectively, and was 35.6 per cent, 25.6 per cent and 20 per cent, respectively, in women of reproductive age (15–39 years).

Sema *et al.* (2005) stated that the seroprevalence of anti-*Toxoplasma* IgG antibodies for toxoplasmosis was 30.1 per cent among pregnant women in Aydin Province, Turkey. Seroprevalence was increased with age and with drinking water consumption other than bottled water.

Birgisdóttir *et al.* (2006) investigated the prevalence and risk factors for *T. gondii* infection in Iceland, Sweden and Estonia, from 1277 randomly selected persons. The presence of *T. gondii* IgG antibodies was determined by an ELISA method and reported the prevalence of 54.9 per cent in Tartu, 23 per cent in Uppsala and 9.8 per cent in Reykjavik in the above said countries respectively.

Francisco *et al.* (2006) reported the seroprevalence for *T. gondii* in human population from Brazil might range from 40 to 80 per cent. They analysed the seroprevalence of *T. gondii* infection in children from age one to 15 living in a low socioeconomic community, in Brazil and antibodies to *T. gondii* were found in 110 (32.4 per cent) of the 339 children tested with indirect immunofluorescent antibody test.

Studeničová *et al.* (2006) conducted prospective, cross-sectional study to evaluate the seroprevalence of *T. gondii* antibodies in a non-endemic area among 508 healthy individuals in Slovakia using ELISA to detect the levels of IgG, IgA, and IgM antibodies. IgG antibody prevalence of 24.2 per cent (123/508) was found in the study population, while examination of specific IgA and IgM antibodies was negative. They confirmed a significant increase in toxoplasmosis prevalence with increasing age.

Jeffrey *et al.* (2007) determined the prevalence of *T. gondii* infection in U.S. Of the 17,672 persons examined in 1999–2004,

15,960 (90 per cent) were retested and reported that *T. gondii* seroprevalence declined from 14.1 per cent to 9.0 per cent among U.S. born persons of ages 12-49 years. Although *T. gondii* infects many persons in the U.S., the prevalence has declined in the past decade.

3.5 DIAGNOSIS OF TOXOPLASMOSIS

Toxoplasmosis in humans was usually diagnosed by biological, serological, histological, or molecular methods, or by some combination of the above. Clinical signs of toxoplasmosis were non-specific and not sufficiently characteristic for a definite diagnosis. Toxoplasmosis in fact mimics several other infectious diseases.

3.5.1 ISOLATION OF ORGANISM

Toxoplasma gondii was usually isolated from susceptible laboratory animals. Mice were most commonly used, since they can be readily infected by intraperitoneal injections of tachyzoites or bradyzoites. Depending on the virulence of the strain, mice will develop either an acute infection with parasite-rich ascites or a chronic infection characterized by the presence of cysts in the brain. Inoculation of blood, body fluids, or tissue extracts into mice may be used for diagnosis of acute condition (Jones *et al.*, 1958 and Abbas, 1967), but the result was often delayed because the encysted parasites may not be identified before 30 days after inoculation.

Several reports demonstrated that tissue culture methods could be applied to the isolation of *Toxoplasma* organisms from blood or

infected tissues and served for diagnosis when serological tests were inconclusive. Tissue cell culture had the advantage of wide spread availability and yielded the result more rapidly within 3 to 6 days than mouse inoculation. However, mouse inoculation was more sensitive (Chang, *et al.,* 1972; Hofflin and Remington, 1985 and Shepp, *et al.*, 1985).

Francis *et al.* (1987) compared the mice inoculation method and tissue culture technique and reported that the course of infection in mice was greatly dependent on the virulence of the strain and on the parasitic stage inoculated. In the chronically infected mice, evidence of *Toxoplasma* infection was only detected 45 days after inoculation through the demonstration of cysts in the brain or the presence of specific antibodies in the serum. The tissue culture method was found to be at least as sensitive as mouse inoculation. Since *Toxoplasma* organisms may be isolated within a few days in tissue culture, it was proposed that this method should be used when early isolation of the parasite was crucial for the diagnosis of toxoplasmosis.

3.5.2 SEROLOGICAL DIAGNOSIS

The use of serological tests for the demonstration of specific antibody to *T. gondii* was the primary method of diagnosis. The problem with serologic diagnosis was that antibody to *T. gondii* was present in relatively high numbers of individuals in most human populations. These antibody titers may persist at high levels for years

even in healthy people. A large number of tests had been described, some of which were still in the experimental stage or were available only in highly specialized laboratories. Different serologic tests often measures different antibodies that possessed unique patterns of rise and fall with the time after infection. False-positive and false-negative results had been problem with certain commercial kits and laboratories in certain countries (Liesenfeld *et al.*, 2001).

There was no single serological test that can be used to support the diagnosis of acute or chronic infection of *T. gondii*. Numerous serological procedures called panel of *T. gondii* serologic profile (TSP) were available for the detection of humoral antibodies including the Sabin–Feldman dye test, the indirect haemagglutination assay (IHA), the indirect fluorescent antibody assay (IgG and IgM-IFA), the direct agglutination test (DAT or modified agglutination test (MAT), the latex agglutination test (LAT), the enzyme-linked immunosorbent assay (IgG, IgM, IgA, IgE-ELISA); the IgG Avidity test and the immunosorbent agglutination assay test (IgE and IgM-ISAGA) (Dubey, 1998).

3.5.2.1 INDIRECT FLUORESCENT ANTIBODY (IFA) TECHNIQUE

The indirect fluorescent antibody (IFA) test measured the IgG antibodies as the dye test. Titers were parallel to dye test titers. The IgM fluorescent antibody test was used to detect IgM antibodies within the first week of infection, but titers fall within a few months.

The indirect fluorescent antibody technique was used to titrate patients' antibody against *T. gondii*. The reactions of the gamma globulin of human immune serum with *T. gondii* and fluorescent rabbit anti-human gamma globulin were thus shown to be specific. Thereafter the technique could be used with confidence to detect the reaction between doubling dilutions of patient's serum and smears of unfixed *T. gondii*. In this way, titres were obtained with the indirect fluorescent antibody technique which agreed well with those of the dye test at both low and high levels of antibody. Compared with the dye test, the indirect fluorescent antibody technique had many advantages. The end-point was sharp and obviates the counting of stained and unstained organisms; supplies of antibody-free accessory factor sera were not needed; prozones undetected by the dye test were strongly positive at screening dilutions, the reagents kept indefinitely and lend themselves to preparation, standardization, and issue by a central reference laboratory (Fletcher, 1965).

Fausto *et al.* (1971) noted that in IFA tests false-positive results may occur with sera that contain antinuclear antibodies and false-negative result may occur in sera with low IgG antibody titers. For this reason in patients with connective tissue disorders like systemic lupus erythematous, a DT or ELISA can be performed to document a positive result on IFA testing.

Sulzer *et al.* (1971) stated that to avoid misinterpretation of the polar staining of organism that was due to naturally occurring IgM antibodies, flurescein-tagged conjugate should be only anti-IgG.

Shirley *et al.* (1982) compared ELISA and IFA tests, for detection of antibodies to *T. gondii* and reported that both the ELISA and the IFA correlated well with serologically positive (IFA titer 1:32) and negative sera. The ELISA was more specific, but less sensitive, than the IFA. However, the IFA was more reproducible and more rapid than the ELISA.

Mittal *et al.* (1995) considered the titer of 1:64 or more were positive whereas most of recent authors such as Sema *et al.* (2005) and Cavalcante *et al.* (2006) taken dilutions of 1:16 and higher were as positive for IFAT.

3.5.2.2 DIRECT AGGLUTINATION TEST (AG / DAT /MAT)

The *Toxoplasma* agglutination (AG) test was first described by Fulton and Turk (1959). In this method whole killed organisms were used and it was routinely performed in France with an antigen that had been prepared as described by Ardoin, *et al.* (1967) and that was commercially available (BioMerieux, Charbonnieres-les-Bains, France). The method was very simple and useful but as presently used had two drawbacks. First, it lacks sensitivity; the titer in the AG test was usually much lower than that in the dye test (DT) or the conventional immunofluorescent-antibody (IFA) test. As a

consequence, some sera that were positive in the latter two tests were reported as negative in the AG test. Second, it lacks specificity; some sera that were negative in the DT and IFA test were reported as positive in the AG test (Couzineau, *et al.*, 1973). This was shown to be due to the binding of normal immunoglobulin M (IgM) ("natural IgM antibody") to the surface of the parasite (Desmonts, *et al* , 1974).

Desmonts and Remington (1980) described a method for preparing antigen which increases the sensitivity of the AG test and a method for suppressing nonspecific agglutination by the use of a buffer containing 2-mercaptoethanol (2ME). When the AG test was modified by these two methods, its specificity and sensitivity parallel those of the DT. The technique of modified agglutination test (MAT/MAGT) and the reading were so simple and accurate that, if the antigen was made available, the MAT method would be convenient for laboratories that perform serology only occasionally as well as for those that perform large-scale surveys.

Desmonts and Remington (1980) also concluded that the AG test titer was most often lower than the DT titer during acute (recent) infection and the AG test titer was most often higher than the DT titer in older or chronic infection. If the MAT test antigen using 2ME can be made available, the test would be ideal for use as a screening test and would provide a simple and inexpensive means for the surveillance of

seronegative women during pregnancy and for detection of seroconversions.

Johnson *et al.* (1989) stated that AG test titers were usually markedly lower than those obtained with the DT or IFA test and that "negative" results were often obtained in DT-positive sera. For AG test doubling dilutions started from 1:20 and a positive direct agglutination test result was defined as that with titre of ≥ 40.

Singh *et al.* (1996) modified the direct agglutination test described by Desmonts and Remington, 1980 and reported that antibody titers of 1:20 or more were taken as positive.

Sema *et al.* (2005) considered titers of ≥ 20 were positive for the direct agglutination test using formalin fixed tachyzoites as antigen.

3.5.2.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

In the ELISA test the soluble antigen is absorbed on plastic surface and the antigen-antibody reaction is enhanced by the addition of secondary enzyme linked antibody- antigen system and the reaction can be assessed objectively by the quantitation of the colour that develops. ELISA is first described by Engvall and Perlmann in 1971 as a modification of the radioimmunosorbent technique (RIST), in which an enzyme is substituted for the radiolabel of the antiserum. Enzyme-linked immunosorbent assay (ELISA) technology has largely replaced other methodologies in the routine clinical laboratory. It has been

used successfully to demonstrate IgG, IgM, IgA and IgE antibodies in the pregnant women, foetus and new born.

Most workers have employed an enzyme-conjugated antibody directed against human IgG or against total immunoglobulins. Titers in the IgG ELISA correlated well with the titers in the DT, IHA, IFA, CF tests in some studies but not in others.

IgM antibodies are measured by the "double-sandwich" or "immuno-capture" IgM-ELISA method. This method avoids false positive results due to the presence of rheumatoid factor and antinuclear antibodies. In patients with recently acquired infection, IgM *T. gondii* antibodies are detected initially and, in most cases, these titers become negative within a few months. However, in some patients, positive IgM *T. gondii* specific titers can be observed during the chronic stage of the infection. IgM antibodies have been reported to persist as long as 12 years after the acute infection. Persistence of these IgM antibodies does not appear to have any clinical relevance and these patients should be considered chronically infected. Their reliability for detection of IgM antibodies varies considerably however; false positive results have been a serious problem.

IgA antibodies may be detected in sera of acutely infected adults and congenitally infected infants using ELISA or ISAGA methods. As is true for IgM antibodies to the parasite, IgA antibodies may persist for many months to more than one year. For this reason they are of little

additional assistance for diagnosis of the acute infection in the adult. In contrast, the increased sensitivity of IgA assays over IgM assays for diagnosis of congenital toxoplasmosis represents an advance in diagnosis of the infection in the fetus and newborn. In a number of newborns with congenital toxoplasmosis and negative IgM antibodies, the serological diagnosis has been established by the presence of IgA and IgG antibodies.

IgE antibodies are detectable by ELISA in sera of acutely infected adults, congenitally infected infants, and children with congenital toxoplasmic chorioretinitis. The duration of IgE seropositivity is less than with IgM or IgA antibodies and hence appears useful as an adjunctive method for identifying recently acquired infections.

Nowadays, various commercial ELISA kits are widely available for detection of IgG or IgM antibodies.

Voller *et al.* (1976) first to initiate preliminary studies on enzyme immunoassay method in a microplate for diagnosis of *Toxoplasma* infection and reported that there was a positive correlation between the enzyme immunoassay results and dye test and haemagglutination test titers.

Walls *et al.* (1977) described a detail ELISA procedure for toxoplasmosis in which an antigen derived from solubilized whole organisms utilized for the test. They have successfully used both the

tube and microtitration procedures for assay and reported that ELISA results were equivalent to those found in the indirect immunofluorescence test with 98 per cent specificity.

Balsari *et al*. (1980) reported that the ELISA specificity looks comparable with that of the reference tests, as no sample classified as negative by all the reference tests was ELISA-positive and *vice versa*; ELISA appears to correlate better with haemagglutination and immunofluorescence, on the basis of the respective class frequencies; in particular, the number of positives, which was much lower for the dye-test and crossover-linked immunoassay, suggests that a higher sensitivity was reached in the former cases.

Naot and Remington (1980) developed ELISA for detection of IgM antibodies to *T. gondii*, and stated that the IgM-ELISA was more sensitive than the IgM-IFA test in the diagnosis of recently acquired infection with *T. gondii.*

Pappas *et al*. (1986) evaluated Dot-ELISA and compared with ELISA and IFA. They stated that the Dot-ELISA correlated well with the IFA test (correlation coefficient = 0.895) and the ELISA correlated slightly higher with the IFA test (correlation coefficient = 0.910) for detection of IgG antibodies to *Toxoplasma*.

Susanto and Muljono (2001) attempted to detect *T. gondii* circulating antigen in 60 samples by using ELISA and it was shown that circulating antigen was found in 27 (90 per cent) from 30

samples which contained both IgG and IgM, whereas only 2 (66 per cent) from 30 samples which contained only IgG showed positive results. Therefore, circulating antigen detection can be used to identify the acute phase of infection.

Filice *et al.* (1983) compared ELISA and IFA for the quantitation of IgG and IgM anti-*toxoplasma* antibodies on whole sera and purified IgM fractions. They stated that the conventional IgM ELISA was constantly negative when performed on both whole sera or pure IgM IFA positive fractions of the same newborns.

Kodym *et al.* (2007) reported that detection of IgE ELISA appears to be a highly specific test for confirming the acute nature of *Toxoplasma* infections that have been detected by other sensitive methods like IgA and IgM ELISAs, IgG avidity assay and CFT.

3.6 $CD4^+$ / $CD8^+$ COUNT OF T LYMPHOCYTES IN HIV PATIENTS WITH SEROPOSITIVE TOXOPLASMOSIS

$CD4^+$ T-lymphocyte count is the standard method to evaluate the immunologic response to antiretroviral treatment and the ability to predict the development of opportunistic infections and viral load and can provide a good indicator of long-time prognosis of disease progression (Post *et al.*, 1996). This group of blood cells is the target of HIV and a $CD4^+$ count below 200 cells per µl makes it necessary to initiate anti-retroviral treatment. Otherwise, opportunistic infections which correlate to specific $CD4^+$ counts can decrease the patient's survival expectation.

Hughes (2003) also stated that patients with $CD4^+$ count of <100 per µl have more chance to develop toxoplasmosis, cryptococcosis and candidal esophagitis.

Kumarasamy *et al.* (2003) reported from South India that the 3.5 per cent of person with HIV (n=20) having mean $CD4^+$ count of 135 cells/ µl had CNS toxoplasmosis. They also stated that there was a progressive and highly significant inverse relationship between the $CD4^+$ count with the increasing number of this opportunistic infection (p=0.001).

Nissapatorn *et al.* (2003) reported an association between the level of $CD4^+$ cell count and toxoplasmic encephalitis (p=0.019; OR=2.6; 95 % CI=1.14-6.02).

Kasper (2005) quoted that Toxoplasmic Encephalitis (TE) occurs in 10–50 per cent of HIV-infected patients who were seropositive for antibodies to *T. gondii* and who had $CD4^+$ count <100 cells/mm3. The greatest risk was among patients with a $CD4^+$ T-lymphocyte count <50 cells/mm^3. The highest risk for HIV-infected patients with latent infection was when $CD4^+$ count was <100 cells/mm^3. Patients with positive IgG antibody to *T. gondii* and $CD4^+$ count <200 cells/mm3 should receive empiric therapy.

4. MATERIALS AND METHODS

The present study was conducted at Tirunelveli Medical College Hospital, Palayamkottai, Tirunelveli District, Tamil Nadu from May' 2006 to October' 2007 to assess the seroprevalence of toxoplasmosis in and around Tirunelveli by using in-house IgG assays *viz.,* IFAT, MAT and ELISA using laboratory own prepared antigen. Various risk factors associated with in the study group was statistically analysed and results were interpreted.

4.1 MATERIALS

4.1.1 Study place and test material

A total of 350 peripheral blood samples for this study were collected from 175 immunodeficient patients (HIV and malignant patients) and 175 immunocompetent patients like pregnant women (135), ocular chorioretinitis cases (20) and patients with lymphadenopathy (20), in and around Tirunelveli district of Tamil Nadu those admitted at Tirunelveli Medical College Hospital.

4.1.2 Parasites

Mouse adapted RH strain of *Toxoplasma gondii* maintained at the Department of Veterinary Parasitology, Madras Veterinary College, Chennai, which was originally obtained from All India Institute of Medical Sciences, New Delhi was used in this study.

4.1.3 Laboratory/experimental animals

Swiss albino mice were procured from the Madras Veterinary College, Chennai. They were maintained on concentrate feed, fully soaked White Bengal gram and water *ad lib*.

4.1.4 Cell lines

Fibroblast cell lines, Madin Darby Canine Kidney (MDCK) and Murine Macrophage cell lines were procured from Department of Animal Biotechnology, Madras Veterinary College, Chennai.

4.1.5 Chemicals, Immunobiologicals and plasticwares

Chemicals were purchased from Himedia and Qualigens. Immunobiologicals were obtained from Bangalore Genei. Plastic tissue culture wares of Tarson and Laxbro make were used.

4.1.6 Equipments

The following equipments were used :

Bright field microscope (Labomed), Inverted tissue culture microscope (Olympus ck-40), Laminar clean air-work station (Klenzaids), Fluorescent microscope (Labomed), Table top centrifuge (Remi, R8C), Carbondioxide incubator (Forma Scientific), Balance (Mettler, Toledo), Incubator (Tempo), ELISA reader and washer (BioRad, France), Sonicator (B. Braun, Germany) and Flowcytometer (Becton Dickinson, USA).

4.1.7 Reference sera

The known positive and known negative *Toxoplasma* reference sera were gifted by Dr. C. Sreekumar, Toxo Laboratory, USDA, Beltsville, Maryland, U.S.A.

METHODS

4.2 General safety precautions and ethical clearance

As all the stages of *T. gondii* are infective, the following safety precautions were followed during the course of the present study. All objects used in tachyzoite handling were washed thoroughly with water, followed by swabbing with alcohol. Face mask and goggles were used during cell culture work. Litter from mouse cages and floors of sheds were collected daily and disposed off after adding concentrated ammonia. All cages were thoroughly washed with 30 per cent ammonia solution between various batches of animals. Dead laboratory animals were immersed in 10 per cent formalin before disposal.

Due consideration for animal ethical concern was made before implication of this study for rearing experimental animal, mice. The Institutional Ethical committee clearance was obtained for this study.

4.3 Maintenance of *T. gondii* (RH strain)

The recipe of the buffers used is appended in **Annexure I.**

4.3.1 *In vivo* propagation of *T. gondii*

Mice infected with RH strain of *T. gondii* were maintained in the small laboratory animal facility and examined for signs of infection as per the method adopted by Derouin *et al.* (1987). The animals showing extreme dullness were euthanized by chloroform anaesthesia and the peritoneal cavity was opened aseptically **(Plate 4).** Using an insulin syringe, the ascitic fluid (peritoneal exudate) was aspirated and diluted in about 1 ml of sterile phosphate buffered saline (PBS, pH 7.2). A drop of this fluid was examined microscopically for the presence of tachyzoites and there after the dried smear was stained with Leishman's and Giemsa's stain **(Plate 5)**. About 0.3-0.5 ml of the ascitic fluid preparation, containing 5-10 parasites per high power field, was inoculated intraperitoneally to healthy mice for further maintenance and production of tachyzoites. The tachyzoites thus harvested were counted (as described below in 4.3.3) and prepared as antigen which was used for immuno assays.

4.3.2 *In vitro* cultivation of *T. gondii*

Seed cultures of fibroblast (MDCK) cell lines were maintained as per the procedure of Hughes *et al.* (1986) in prescription and/or milk dilution bottles in RPMI-1640 medium supplemented with 5 per cent neonatal calf serum, 25 mM HEPES, 2 mM L-glutamine and 50 µg/ml gentamicin. Cultures were incubated in a BOD incubator at 37°C. Fresh monolayers were split from confluent seed cultures and used for *in vitro* infection with *T. gondii* tachyzoites, as described below.

Infected mice were sacrificed *in extremis* by chloroform anaesthesia. They were thoroughly scrubbed with absolute alcohol to remove extraneous dirt and other body fluids. The mice were then dipped once in alcohol before transfer to a laminar clean air work station and placed on dorsal recumbency. Using sterile forceps, the skin over the abdomen was lifted and incised with a pair of scissors to expose the abdominal muscles. The peritoneal cavity was then opened by cutting through the muscle layers, exposing the visceral organs. A drop of the peritoneal fluid was then aspirated into a sterile insulin syringe containing about 0.5 ml of plain medium. If peritoneal fluid was scanty, then a small quantity of plain medium was instilled onto the visceral organs and immediately aspirated. Care was taken not to puncture the intestines during this process.

A drop of the fluid thus collected was transferred to a glass slide (sterilized over flame) and examined for the presence of tachyzoites and absence of gross bacterial contamination. After ensuring the sterility of the tachyzoite preparation, it was directly transferred to 25 cm^2 culture flasks containing confluent monolayers. Coverslip cultures of murine macrophage cell line, grown in 24-well plates with 0.5 ml of medium, were also infected as per the method of Chamberland and Current (1991). The cultures were incubated in Co^2 incubator at 5 per cent Co_2 tension and 37^oC.

The medium was replaced after 24 hr and the cultures were examined daily for signs of infection under an inverted microscope **(Plate 6)**. The medium from the infected culture flasks was partially replaced as and when acidic pH conditions were noticed. The rate of multiplication of the tachyzoites was periodically monitored. Coverslips from the 24 -well plates were removed at intervals, fixed in Bouin's or methanol, stained with 10 per cent Giemsa solution and examined using a bright field microscope **(Plate 6)**. Once the monolayers of host cells were totally taken over and disrupted releasing the tachyzoites, a small aliquot of the supernate (~0.2 ml)

was transferred to a freshly split monolayer to maintain the infection for subculture.

4.3.2.1 Recovery of tachyzoites from i*n vitro* culture

Culture supernates were harvested from flasks previously infected with *T. gondii* tachyzoites, after the complete disruption of monolayers. The medium from all the flasks was removed after vigorous pipetting to release all the attached cells. These supernates, consisting mainly of free tachyzoites and heavily infected macrophage cells were washed twice in PBS (pH 7.2). The material was centrifuged at 500 g for 5 minutes and the pellet was resuspended in 1 ml of PBS. The number of free tachyzoites was then counted.

4.3.3 Counting of tachyzoites

The tachyzoite suspension harvested from mice inoculation or by tissue culture was thoroughly pipetted to break down clumps and two dilutions, 10-fold and 100-fold, were prepared for counting. The first dilution was made by adding 10 µl of tachyzoite suspension to 90 µl of Trypan blue (0.4 per cent) solution (10-fold dilution). The second dilution was then made by adding 10 µl of this tachyzoite preparation (10-fold dilution) to 90 µl of Trypan blue (100-fold dilution). Ten microlitres of each of these dilutions were charged to the two chambers of a haemocytometer. After allowing the tachyzoites to settle, the unstained (viable) tachyzoites were counted in four corner squares (WBC chamber) using 40x objective. From this value, the total

number of tachyzoites/ml of suspension was calculated using the following formula:

Tachyzoites per millilitre = Average number of tachyzoites (from 4 corner squares) x Dilution factor x 10^4.

Values were considered only if those of the two dilutions were concordant.

4.3.4 Preparation of tachyzoites antigen

After counting the number of free tachyzoites, the material was resuspended in an appropriate volume of PBS and centrifuged again. After the centrifugation tachyzoites were suspended in 6 per cent formaldehyde solution. The tachyzoites were kept overnight in this solution. After 16 hours of suspension in formaldehyde, they were centrifuged and resuspended in PBS. The sediment was washed thrice in PBS, to remove cell debris and the formaldehyde. Finally the tachyzoite antigen was resuspended in an appropriate volume of PBS, made as different aliquots and stored at -20°C until use. The antigen thus prepared was used for the immunoassay.

4.4 Collection of test material from patients

4.4.1 Study place and period

The study was conducted at Tirunelveli Medical College Hospital, Palayamkottai, Tirunelveli District, Tamil Nadu from May' 2006 to October' 2007.

4.4.2 Study population

A total of 350 peripheral blood samples for this study were collected from 175 immunodeficient patients (HIV and malignant patients) and 175 immunocompetent patients like pregnant women (135), ocular chorioretinitis cases (20) and patients with lymphadenopathy (20) in and around Tirunelveli district of Tamil Nadu those admitted at Tirunelveli Medical College Hospital.

4.4.3 Socio-demographic, Clinical and Behavioral data

The history of illness and patients information and socio-demographic data like epidemiological risk, antenatal risk, HIV risk and clinical risk assessment factors were collected in the specially designed data sheet **(**appended in **Annexure II)** for this purpose. Socio-demographic data including age, education, occupation, parity, residency and related risk factors including source of drinking water, obstetrical history, frequency and type of meat, vegetables, fruits and milk consumption, cooking preferences, owning cat, history of cleaning cat litter box or feeding raw meat scraps, history of blood transfusion, organ transplant etc., were collected for further analysis.

4.4.3 Separation of sera

About 2-5 ml of blood collected from each patient was separated after centrifugation. The sera collected were inactivated at 56°C for 30 minutes in water bath and stored in small aliquots at –20°C.

4.5 EVALUATION OF IgG BASED ASSAYS FOR SERODIAGNOSIS OF TOXOPLASMOSIS

4.5.1 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

4.5.1.1 Standardization of IFAT

The recipe of the buffers used is appended in Annexure l.

4.5.1.1.1 Preparation of antigen slides

Antigen slides were prepared as per the procedure outlined in USHDEW manual (1976) with minor modifications.

The formalin killed *T. gondii* tachyzoite antigen harvested from the cell line culture was resuspended in an appropriate volume of PBS (so as to obtain 100-200 tachyzoites per high power field). The suspension was then drawn into a capillary tube and spotted onto grease-free Teflon coated slides on which circular wells were previously etched using a glass marking pencil. Once the spots had dried up, the slides were placed in cold acetone overnight. The fixed slides were air-dried, wrapped in aluminium foil and stored at –20^{o}C until further use.

4.5.1.1.2 Reference sera

The known positive and negative sera obtained from the reference lab were aliquoted and stored at –20^{o}C until use.

4.5.1.1.3 Conjugates

Anti-human IgG-FITC conjugate raised in goat was obtained from Bangalore Genei.

4.5.1.1.4 Standardisation of IFAT

Optimum working dilution of conjugate was standardised. The conjugate dilutions of 1:25, 1:50, 1:100 and 1:200 were tested with known positive and negative sera. The optimum dilution, which gave the highest titre with positive sera and lowest negative sera titre, was selected. The sensitivity, specificity and positive and negative predictive value of the assay were calculated using 10 known positive and negative reference sera.

4.5.1.2 IFA Test procedure

IFAT was performed as described by Renterghem and Nimmen (1976) and USHDEW manual (1976) with minor modifications. Briefly, the following procedure was adapted.

- The formalin killed acetone fixed antigen slides were thawed at room temperature before use.
- Ten µl of diluted sera was added to the spots. Test sera were used in 3 dilutions of 1 in 8, 1 in 16 and 1 in 32 in PBS.
- Positive and negative control sera were diluted to 1 in 16 and 1 in 8, respectively.
- The slides were then incubated for 1 hr at 37^{o}C in a humid chamber.
- They were then washed in distilled water and PBS.

- After drying on a blotting paper, 10 µl of Goat Anti-human IgG-FITC conjugate of 1 in 50 dilution was added to the antigen spots.
- The slides were washed as before and dried.
- A drop of buffered glycerol was added to the spots and a coverslip was placed.
- The slides were examined immediately at 40x magnification using a fluorescent microscope.
- Positivity and negativity were determined as per the guidelines described (USHDEW manual, 1976).
- Test sera samples that were positive at 1:16 dilution were further tested at two fold serial dilutions until an end point was reached.

4.5.2 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

4.5.2.1 Standardization of IgG ELISA

The recipe of the buffers used is appended in Annexure 1.

4.5.2.1.1 Preparation of tachyzoite soluble antigen (TSA)

Purified formalin killed tachyzoites were lysed with distilled water and then disrupted by six successive cycles of freezing and thawing. The material was centrifuged at 3,000 g at 4°C for 30 minutes. The sediment was resuspended in 4 ml PBS and was sonicated (B. Braun Sonifier, Germany) at constant power on high setting with repeated duty cycles per 0.3 seconds for 5 minutes for 3 cycles in ice bath of 4^0C. The disruption was confirmed by light

microscopic examination. The sonicated material was then centrifuged at 15,000 rpm at 4^0C for 30 minutes. The supernatant fluid was taken as tachyzoite soluble antigen (TSA) and stored at –20^0C until use.

4.5.2.1.2 Protein estimation

The protein content of tachyzoite soluble antigen (TSA) as determined by the Lowry *et al.* (1951) method was approximately 0.63 mg/ml.

4.5.2.1.3 Conjugate

Commercially available horseradish peroxidase conjugated goat anti-human IgG (Genbio, Taiwan) was used for the ELISA.

4.5.2.1.4 Substrate

Commercially available substrate solution Tetramethyl benzididine (TMB, Genbio, Taiwan) of 0.6 mg / ml of TMB in citric acid buffer plus 0.02 per cent H_2O_2 was used.

4.5.2.1.5 Standardization of ELISA by Chequer board titration

Optimum working dilution of antigen, sera and conjugate was determined by chequer board titration using serial dilution of serum, antigen and conjugate.

The sera were diluted as 1:50, 1:100, 1:200 and 1:400 and these were tested in a chequer board titration with known positive antigen serially diluted. The optimum dilution, which gave maximum

difference between negative and positive antigen was selected as the working dilution of serum for use.

Similarly, the antigen dilution was also standardized at various concentrations of 5, 10, 20 and 40 μg/ml. The conjugate dilutions of 1:500, 1:1,000, 1:5,000 and 1:10,000 were tested by chequer board titration with serially diluted known positive and negative sera and antigen. The optimum dilution, which gave the highest ratio between positive and negative control sample was selected.

The sensitivity, specificity and positive and negative predictive value of the assay were calculated using 10 known positive and negative reference sera.

4.5.2.2 Test procedure for in-house IgG Micro-ELISA

In-house IgG Micro-ELISA was performed as described by Voller *et al.* (1976) with minor modifications. Briefly, the following procedure was followed.

- The 96-well flat bottom polystyrene microtitre (ELISA) plate (Tarsons, India) was recharged by filling up the wells with lukewarm water at least for 15 minutes. Thereafter the water was discarded by inverting and shaking the plate.

- The predetermined antigen solution (10 μg of TSA/ml) was prepared in 0.05 M Carbonate-bicarbonate buffer. Hundred μl of

antigen was coated in the ELISA plate and incubated at 4^0C for overnight.

- The plate was then washed with PBS – T for three times.
- Thereafter 100 µl of 1 per cent skimmed milk solution in PBS-T was added into the plate and incubated for 1 hour at 37^0C.
- The plate was then washed with PBS – T for three times.
- Then 100 µl of test sera prepared at 1:100 dilution was added to the plate. The positive and negative sera controls of same dilutions were added in the respective wells.
- The plate was incubated for one hour at 37^0C. The plate was washed with PBS –T for 3 times.
- One hundred µl of 1:1,000 goat antihuman IgG HRP conjugate diluted in PBS-T was added on each well and incubated at 37^0C for one hour.
- The plate was washed with PBS – T for 3 times.
- One hundred µl of TMB substrate solution was added in each well and incubated in a dark room at room temperature for 15 minutes and the reaction was stopped by adding 100 µl of 1M Sulphuric acid.
- The substrate and conjugate control was maintained in each plate.

☛ The optical density (OD) values were read in Multiscan ELISA reader (Biorad, France) at 450 nm reference filter.

4.5.2.3 Interpretation of ELISA

The cutoff value was calculated by adding mean OD value of two negative controls and mean OD value of 3 positive controls and the sum is divided by six. The test sera with absorbance values less than the cutoff value were considered nonreactive and test sera with absorbance values equal or greater than the cutoff value were considered reactive.

4.5.3 MODIFIED DIRECT AGGLUTINATION TEST (MAT)

4.5.3.1 Standardization of MAT

The recipe of the buffers used is appended in Annexure l.

4.5.3.1.1 Preparation of MAT antigen

Direct agglutination method described by Desmonts and Remington (1980) was followed with minor modification (Singh *et al.,* 1996).

Ten mice were infected with RH strain of *T. gondii* maintained in the small laboratory animal facility and examined for signs of infection. On 5 days post infection the mice were euthanized by chloroform anaesthesia and the peritoneal cavity was opened aseptically. Using an insulin syringe, the ascitic fluid (peritoneal

exudate) was aspirated and diluted with sterile phosphate buffered saline (PBS, pH 7.2). A drop of this fluid was examined microscopically for the presence of tachyzoites. Repeated washing of the peritoneal cavity with sterile PBS was carried out to collect maximum tachyzoites. The tachyzoites thus harvested were counted and the Formalin killed freshly prepared antigen was reconstituted in sterile PBS so as to reach the final concentration of 1 x 10^7 tachyzoites per ml, which was used for the MAT.

4.5.3.1.2 Preparation of MAT antigen mixture

Antigen diluting Borate buffer was prepared by dissolving 7.01 g of Sodium chloride, 3.09 g Boric acid, 2 g Sodium azide in 900 ml deionised water. Twenty four ml of 1N NaOH was added to this and the pH was adjusted to 8.95. The volume was made up to 1 litre and used as stock solution which was stored at room temperature.

For the preparation of working antigen diluting buffer, 0.4 g of Bovine Serum Album in was dissolved in 100 ml of Borate buffer and stored at 4°C. For each plate, 2.5 ml of antigen diluting buffer was mixed with 35 µl of 2-mercaptoethanol, 50 µl of Evans blue dye solution as tachyzoite colouring agent and 0.15 ml of Formalin killed antigen, which was used as antigen mixture for the MAT.

4.5.3.1.3 Standardisation of MAT

The sensitivity, specificity and positive and negative predictive value of the assay were calculated using 10 known positive

and negative reference sera. End titre of positive sera control was standardised.

4.5.3.1.1 Test procedure of MAT

- The serum samples were diluted with sterile serum diluting buffer (PBS of pH 7.2) in microtitre plates. The initial dilution was started at 1:20.
- Twenty five µl of antigen mixture was added to each well on U bottom microtitre plate (Tarson) immediately after mixing.
- Twenty five µl of serum of all dilutions prepared as above were added.
- The antigen and sera were mixed by repeated pipetting action and the plate was covered with sealing tape.
- The positive and negative controls were maintained in each plate
- The plate was read after overnight incubation at 37°C.
- The negative reaction was indicated by formation of blue button at the bottom of the well whereas; the positive reaction had matted appearance.
- Antibody titres of 1:20 or more were taken as significant for reporting positive results. Those test samples positive at 1:20 were further serially two fold diluted until the end titre was reached.

4.5.4 $CD4^+/CD8^+$ COUNT OF T LYMPHOCYTES IN HIV PATIENTS OF SEROPOSITIVE AND SERONEGATIVE GROUP OF TOXOPLASMOSIS

$CD4^+$ and $CD8^+$ count of T lymphocytes in peripheral blood was assessed by flow cytometry in HIV positive patients. The $CD4^+$ and $CD8^+$ count in the seropositive and seronegative toxoplasmosis group was compared and interpreted.

4.5.4.1 Immuno cytometry

4.5.4.1.1 Collection of blood

The blood samples of the patients were collected with all bio-safety precautions; initially 3 ml, in plastic vials for HIV testing and later 2 ml, in K-3 liquid EDTA vacutainer (Becton Dickinson, Mountain View CA) for $CD4^+/CD8^+$ testing.

4.5.4.1.2 HIV testing

Sera were separated on the same day and tested for HIV antibodies on the same/following day of collection, by WHO approved ELISA/Rapid kits supplied by MicroElisa, New Delhi, following its testing guidelines. All samples were tested by the same kit manufactured by the same company.

4.5.4.1.3 $CD4^+/CD8^+$ testing

The blood samples were processed immediately within 2 hours of collection, for determining the absolute counts of CD4+ and CD8+ cells and their ratios by two colour immunophenotyping on the single

platform fluorescence activated cell sorting (FACS) count system (Becton Dickinson Pvt. Ltd., Mountain View, CA. **Plate 7**), using fluorochrome labeled monoclonal antibodies to $CD4^+/CD8^+$ T-cells, as per the method of Kumarasamy *et al.* (2003). The same batches of antibodies were used for testing the samples of subjects of all the groups. FACS count protocol software versions 1.0 (2005) (Becton Dickinson) were used for data acquisition and analysis.

4.5.4.1.4 Statistical analysis

Differences between mean $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ count of seropositive and seronegative toxoplasmosis patient in HIV group was compared and analysed.

4.5.5 EVALUATION OF DIFFERENT SERODIAGNOSTIC METHODS FOR SEROPREVALENCE OF TOXOPLASMOSIS

The sensitivity, specificity, positive predictive value and negative predictive value of the three IgG based assays *viz.,* IFAT, MAT and ELISA for seroprevalence of toxoplasmosis was compared and the testing efficiency of the assays was statistically interpreted.

4.5.5.1 Statistical analysis

4.5.5.1.1 Test of significance of IFA, MAT and ELISA by McNemar's test for paired samples

The testing efficiency of the three assays IgG based assays *viz.,* IFAT, MAT and ELISA for seroprevalence of toxoplasmosis was

statistically analysed for its significance by McNemar's test (Armitage and Berry, 1987).

The 'z' value was calculated by the following formula

$$z = \frac{r - \frac{1}{2}n}{\frac{1}{2}\sqrt{n}}$$

follows normal distribution with degree of freedom (n-1)

where,

z = Calculated value

r = Number of seropositivity of the first test and seronegativity of the compared test (test 2)

n = Number of untied pairs

and the test of significance at $p<0.05$ / $p<0.01$ was interpreted.

4.5.6 SEROPREVALENCE OF TOXOPLASMOSIS IN TIRUNELVELI DISTRICT

All the collected sera (n=350) were subjected into three IgG based assays *viz.*, IFAT, MAT and ELISA for seroprevalence of toxoplasmosis. The results of the three assays were compared and statistically interpreted.

Seroprevalence of toxoplasmosis in the different groups of patients in and around Tirunelveli District of Tamil Nadu using three IgG based assays *viz.*, IFAT, ELISA and MAT was statistically analysed. Seroprevalence of toxoplasmosis in pregnant women based on gravida, trimester of pregnancy and bad obstetric history was

compared. The various socio demographic risk factors associated with seroprevalence of toxoplasmosis in Tirunelveli District was analysed.

4.5.6.1 Statistical analysis

The odds ratio (OR) and 95 % confidence interval (CI) range was calculated for each risk factors of different category of case control study using David Hutchon's odds ratio calculating software.

The Chi-squared test was calculated for the analytic assessment by SPSS 11.5 version software and the p value was calculated using VassarStat software. The differences were considered to be statistically significant when the p value obtained was less than 0.05 ($p<0.05$).

5. RESULTS

5.1 COLLECTION OF TEST MATERIAL FROM PATIENTS

The study was conducted at Tirunelveli Medical College Hospital, Palayamkottai, Tirunelveli District, Tamil Nadu from May 2006 to October 2007. A total of 350 peripheral blood samples for this study were collected from 175 immunodeficient patients (HIV and malignant patients) and 175 immunocompetent patients like pregnant women (135), ocular chorioretinitis cases (20) and patients with lymphadenopathy (20) admitted at Tirunelveli Medical College Hospital those residing in and around Tirunelveli District of Tamil Nadu **(Fig. 1a and 1b).**

5.2 PREPARATION OF *T. GONDII* TACHYZOITE ANTIGEN

The *T. gondii* (RH strain) tachyzoite antigen was prepared by *in vivo* mice propagation and *in vitro* cell culture system in MDCK fibroblast cell line. The tachyzoites count was 1 x 10^7 and 1 x 10^9 per ml of antigen by *in vivo* and *in vitro* methods respectively. Formalin – killed *T. gondii* tachyzoite antigen was prepared for IFAT and MAT. The tachyzoite soluble antigen (TSA) with protein concentration of 0.63 mg/ml was prepared and used for ELISA.

5.3 EVALUATION OF IgG BASED ASSAYS FOR SERODIAGNOSIS OF TOXOPLASMOSIS

5.3.1 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

5.3.1.1 Standardization of IFAT

An IFAT was standardized using reference control positive and negative sera. The optimum dilution of conjugate was standardized. The conjugate dilutions of 1:25, 1:50, 1:100 and 1:200 were tested with known positive and negative reference sera. The optimum conjugate dilution, of 1 in 50 which gave the highest titre with positive sera and lowest negative sera titre, was selected. The positive sera from minimum dilution of 1:16 to maximum up to 1:512, showed good immunoreactivity with either bright yellow-green fluorescence over the entire tachyzoite or distinct yellow-green fluorescence around the entire periphery of the organism (which appeared reddish). With negative control serum (1:8), the organisms did not show any fluorescence or sometimes polar fluorescence was also seen **(Plate 8).** Therefore the cut-off titre for positive was arrived at 1:16 and above.

5.3.1.2 Evaluation of IgG IFAT

The sensitivity, specificity, positive and negative predictive value of IgG IFAT in detecting toxoplasmosis was 80, 90, 88.89 and 81.81 per cent, respectively **(Table - 4).**

5.3.2 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

5.3.2.1 Standardization of IgG ELISA

The tachyzoite soluble antigen (TSA) was prepared and the protein concentration was determined which approximately amounts to 0.63 mg/ml. The antigen concentration was standardised at

10 μg/ml. The optimum dilution of serum and conjugate were found to be 1:100 and 1:1,000 respectively, by chequer board titration.

5.3.2.2 Interpretation of IgG ELISA

The cutoff value was calculated by adding mean OD value of two negative controls and mean OD value of 3 positive controls and the sum is divided by six. The cutoff value for the IgG ELISA was found to be 0.144. The test sera with absorbance values less than this cutoff value were considered nonreactive and test sera with absorbance values equal or greater than this cutoff value were considered reactive **(Plate 9).**

5.3.2.3 Evaluation of IgG ELISA

The sensitivity, specificity, positive and negative predictive value of IgG ELISA in detecting toxoplasmosis was 90, 100, 100 and 90.9 per cent, respectively **(Table - 5).**

5.3.3 MODIFIED AGGLUTINATION TEST (MAT)

5.3.3.1 Standardization and interpretation of MAT

The formalin killed antigen mixture containing $1x10^7$ tachyzoites per ml with 2-mercaptoethanol and Evans blue was prepared and used for MAT. The negative reaction was indicated by formation of blue button at the bottom of the well whereas, the positive reaction had matted appearance **(Plate 10).** Antibody titers of

1:20 or more were taken as significant for reporting positive results. Those samples positive at this dilutions were further diluted up to 1:10,240 to get the end titer.

5.3.3.2 Evaluation of MAT

The sensitivity, specificity, positive and negative predictive value of MAT in detecting toxoplasmosis was 80, 90, 88.89 and 81.81 per cent, respectively **(Table - 6).**

5.3.4 COMPARATIVE EVALUATION OF THREE IgG BASED ASSAYS FOR ITS SENSITIVITY AND SPECIFICITY FOR DETECTING TOXOPLSMOSIS

The comparative evaluation of three IgG based assays *viz.*, IFAT, ELISA and MAT for detecting toxoplasmosis is presented in the **Table 7 and Fig. 2.** Among the three assays, IgG ELISA was found to be highly sensitive (90 per cent) and specific (100 per cent) in detecting toxoplasmosis, whereas the IgG IFAT and MAT were equal in sensitivity (80 per cent) and specificity (90 per cent). The positive and negative predictive value of ELISA was 100 and 90.9 per cent, respectively.

5.4. SEROPREVALENCE OF TOXOPLASMOSIS

5.4.1 Seroprevalence of toxoplasmosis by IgG IFAT

Out of 350 patients tested by IgG IFAT, 41 patients (11.71 per cent) had antibodies for toxoplasmosis with mean IFAT titre of 43.42 ± 58.7 and the titre ranged from 1:16 to 1:256. Among the

immunocompetant group of 175 patients, 19 patients (10.86 per cent) had antibodies to toxoplasmosis whereas in immunodeficient group of 175 patients, 22 patients (12.57 per cent) had antibodies for toxoplasmosis (**Table 8 and Fig. 3).**

Out of 135 pregnant women tested, 13 (9.63 per cent) were seropositive with mean IFAT titre of 51.69 ± 74.23 (titre ranged from 1:16 to 1:256). Among the 20 cases of lymphadenopathy, only 3 (15 per cent) were seropositive with mean titre of 26.6 ± 9.23 (titre ranged from 1:16 to 1:32). Of the 20 ocular cases tested, only 3 (15 per cent) were seropositive with mean titre of 58.67 ± 60.57 (titre ranged from 1:16 to 1:28).

Among the 160 HIV positive individuals tested by IFAT, 19 (11.88 per cent) were seropositive with mean titre of 45.47 ± 61.33 (titre ranged from 1:16 to 1:256). Out of 15 cases of malignancy, only 3 (20 per cent) were seropositive with mean titre of 26.6 ± 9.23 (titre ranged from 1:16 to 1:32).

5.4.2 Seroprevalence of toxoplasmosis by IgG ELISA

Out of 350 patients tested by IgG ELISA, 46 patients (13.14 per cent) had antibodies for toxoplasmosis with mean OD value of 0.2 ± 0.073 and the OD value ranged from 0.144 to 0.444. Among the immunocompetant group of 175 patients, 19 patients (10.86 per cent) had antibodies to toxoplasmosis whereas, in immunodeficient group of

175 patients, 27 patients (15.43 per cent) had antibodies for toxoplasmosis (**Table 9 and Fig. 5).**

Out of 135 pregnant women tested, 13 (9.63 per cent) were seropositive with mean OD value of 0.191 ± 0.069 (OD value ranged from 0.148 to 0.357). Among the 20 cases of lymphadenopathy, only 3 (15 per cent) were seropositive with mean OD value of 0.162 ± 0.029 (OD value ranged from 0.144 to 0.196). Of the 20 ocular cases tested, only 3 (15 per cent) were seropositive with mean OD value of 0.179 ± 0.043 (OD value ranged from 0.147 to 0.228).

Among the 160 HIV positive individuals tested by IgG ELISA, 24 (15 per cent) were seropositive with mean OD value of 0.212 ± 0.079 (OD value ranged from 0.146 to 0.444). Out of 15 cases of malignancy, only 3 (20 per cent) were seropositive with mean OD value of 0.173 ± 0.025 (OD value ranged from 0.146 to 0.196).

5.4.3 Seroprevalence of toxoplasmosis by MAT

Out of 350 patients tested by MAT, 39 patients (11.41 per cent) had antibodies for toxoplasmosis with mean MAT titre of 53.85 ± 55.28 and the titre ranged from 1:20 to 1:160. Among the immunocompetant group of 175 patients, 18 patients (10.29 per cent) had antibodies to toxoplasmosis whereas in immunodeficient group of 175 patients, 21 patients (12.0 per cent) had antibodies for toxoplasmosis (**Table 10 and Fig. 4).**

Out of 135 pregnant women tested, 12 (8.89 per cent) were seropositive with mean MAT titre of 55 ± 63.32 (titre ranged from 1:20 to 1:160). Among the 20 cases of lymphadenopathy, only 3 (15 per cent) were seropositive with mean titre of 33.33 ± 11.54 (titre ranged from 1:20 to 1:40). Of the 20 ocular cases tested, only 3 (15 per cent) were seropositive with mean titre of 73.33 ± 75.71 (titre ranged from 1:20 to 1:160).

Among the 160 HIV positive individuals tested by MAT, 18 (11.25 per cent) were seropositive with mean titre of 50 ± 51.44 (titre ranged from 1:20 to 1:160). Out of 15 cases of malignancy, only 3 (20 per cent) were seropositive with mean titre of 33.33 ± 11.54 (titre ranged from 1:20 to 1:40).

5.4.4 Comparison of three IgG based assays for seroprevalence of toxoplasmosis in and around Tirunelveli

The three IgG based assays *viz.*, IFAT, ELISA and MAT for detection of toxoplasmosis was compared and is presented in **Table 11 and Fig. 6.** Among the three assays, IgG ELISA was detected 46 (13.14 per cent) seropositive cases out of 350 patients tested, whereas in IFAT it was 11.71 per cent and in MAT it was only 11.14 per cent. Therefore, IgG ELISA detected more number of seropositive cases than the other tests. The overall seroprevalence of toxoplasmosis in and around Tirunelveli District of Tamil Nadu was arrived as 13.14 per cent based on IgG ELISA.

5.4.5 Comparison of testing efficiency of three IgG based assays by McNemar's test of significance

The testing efficiency of the three IgG based assays *viz.*, IFAT, ELISA and MAT was statistically compared for its test of significance by McNemar's test, which is depicted in the **Table 12**. Statistically there was no significant difference between IgG IFA and MAT in detecting toxoplasmosis. The significant difference was noticed between IFA and ELISA at $p<0.05$ whereas, the significant difference at $p<0.01$ was found between MAT and ELISA in detecting toxoplasmosis. Statistically the testing efficiency of IgG ELISA for detecting toxoplasmosis was higher than the other two tests.

5.5. ANALYSIS OF VARIOUS RISK FACTORS ASSOCIATED WITH THE SEROPREVALENCE OF TOXOPLASMOSIS IN AND AROUND TIRUNELVELI

5.5.1. Seroprevalence of toxoplasmosis in pregnant women

The seroprevalence of toxoplasmosis in pregnant women in and around Tirunelveli based on gravid, trimester of pregnancy and bad obstetric cases is presented in **Table 13a, 13b and 13C and Fig 7a, 7b and 7c.**

Among the 135 pregnant women tested, 13 (9.63 per cent) were seropositive for toxoplasmosis. Out of 52 primi tested, 5 (9.62 per cent) and 21 Gravida-3 (G3) tested 2 (9.52 per cent) were seropositive for toxoplasmosis.

Seroprevalence of toxoplasmosis during I, II and III Trimester of pregnancy was noticed in 9.1, 13.6 and 7.5 per cent cases respectively. Out of 19 cases with bad obstetric history (like abortion / premature delivery), only 3 (15.79 per cent) were seropositive for toxoplasmosis.

5.5.2. Analysis of socio demographic risk factors associated with the seroprevalence of toxoplasmosis

Various socio demographic risk factors like age, gender, residence, water source, diet and animal contact, associated with the seroprevalence of toxoplasmosis in and Tirunelveli is presented in **Table 14.** The statistical analysis was worked out by Odds ratio (OR) with 95 per cent Confidence interval (CI) range and the p value was arrived by Chi-squared test for significance.

There was a significant difference between sex of the study population and drinking water source. Seroprevalence was increased in the male (19.12 %, p=0.0075, OR=2.316, 95 % CI=1.2362-4.3405) than the female and with river water consumption other than bore water (12.5 %, p=0.0483, OR=0.857, 95 % CI=0.1853-3.4641). No significant relations were observed between anti-*Toxoplasma* IgG antibodies and age, residence, diet and animal contact in the study population.

5.5.2.1. Seroprevalence of toxoplasmosis according to age and sex

The seroprevalence of toxoplasmosis according to age and sex of the study group was depicted in the **Table 15.** Among the different

age groups (<20 – 80 years) studied, there was significant difference between 21-30 years group than the other (p=0.02, OR=3.46, 95 % CI=1.08 -11). In this age group, there was 26.32 and 9.33 per cent of seroprevalence in male and females respectively.

5.5.2.2. Seroprevalence of toxoplasmosis in different regions in Southern Districts of Tamil Nadu

Seroprevalence of toxoplasmosis in different regions in Southern Districts of Tamil Nadu is presented in **Table 16**. Out of 208 patients belonging to rural area and 56 cases of urban area of Tirunelveli District tested, 33 (15.71 per cent) and 9 (10.71 per cent) were had anti-*Toxoplasma* antibodies, respectively.

There was a significant difference between the urban and rural areas of Tuticorin District with respect to the seroprevalence of toxoplasmosis, at p=0.0161, OR=1.09, 95 % CI=0.116-10.26. Out of 65 patients belonging to rural area and 12 cases of urban area of Tuticorin District tested, 5 (7.69 per cent) and 1 (8.33 per cent) were had anti-*Toxoplasma* antibodies, respectively. Among the 5 patients belonging to rural area of Kanyakumari District tested, only 1 (20 per cent) had anti-*Toxoplasma* antibodies.

5.5.2.3 Analysis of risk factors in HIV patients with seropositive and seronegative group of toxoplasmosis

The various risk factors like sexual behaviour, vertical transmission, blood transfusion, I/V drug abuser, Needle injury/

surgery and different stages of HIV infection and their influence in toxoplasmosis seropositive group is depicted in **Table 17.**

Male HIV patients with multiple heterosexual behaviour were observed to be having higher seropositivity for toxoplasmosis (25.58 per cent) than single sexual behaviour. Among the different stages of HIV infections, seroprevalence of toxoplasmosis was found in 22.91 per cent of males and 9.09 per cent of females in Stage III group.

5.6 COMPARISON OF $CD4^+/CD8^+$ COUNT OF T-LYMPHOCYTES IN HIV PATIENTS OF SEROPOSITIVE AND SERONEGATIVE GROUP OF TOXOPLASMOSIS

The $CD4^+$, $CD8^+$ count of T-lymphocytes and $CD4^+/CD8^+$ ratio in HIV patients of seropositive and seronegative group of toxoplasmosis is presented in **Table 18.** The data were statistically interpreted to assess the risk of development of toxoplasmosis as opportunistic infection in HIV patients.

The mean $CD4^+$ count of HIV patients in toxoplasmosis seropositive male and female group was found to be 111.05 ± 79.96 and 79.5 ± 27.36 respectively, whereas in seronegative group it was 218.23 ± 169.38 and 326.57 ± 259.51. Statistically there was significant difference between mean $CD4^+$ count in seropositive and seronegative group of toxoplasmosis was noticed (at p=0.0001, OR=2.09, 95 % CI=1.5-2.9). Similarly significant difference was also noticed in the mean $CD8^+$ count and $CD4^+/CD8^+$ ratio in the study group.

6. DISCUSSION

The study was conducted to assess the seroprevalence of toxoplasmosis in and around Tirunelveli District of Tamil Nadu. Hitherto, no systematic study on seroprevalence of toxoplasmosis was carried out in this southern most district of Tamil Nadu, which necessitates the present study.

6.1 PREPARATION OF *T. GONDII* TACHYZOITE ANTIGEN

The *T. gondii* (RH strain) tachyzoite antigen was prepared by *in vivo* mice propagation and *in vitro* cell culture system and used for IFAT and MAT. The above said method was widely accepted for preparation of antigen and most of the workers *viz.*, Shirley *et al.* (1982), Mittal *et al.* (1995) and Singh *et al.* (1996) also followed the same procedure. The tachyzoite soluble antigen (TSA) with protein concentration of 0.63 mg/ml was prepared and used for ELISA which was in accordance with Loon and Veen (1980), they also used TSA of 0.61 mg/ml of protein for ELISA.

6.2 EVALUATION OF IgG BASED ASSAYS FOR SERODIAGNOSIS OF TOXOPLASMOSIS

6.2.1 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

The IgG IFAT was standardized and the cut-off titre for positive was arrived at 1:16 and above. The sensitivity, specificity of IgG IFAT in the present study in detecting toxoplasmosis was 80 and 90 per cent, respectively. Mittal *et al.* (1995) considered the titer of 1:64 or more were positive whereas, most of recent authors such as

Sema *et al.* (2005) and Cavalcante *et al.* (2006) taken dilutions of 1:16 and above were as positive for IFAT.

6.2.2 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The in-house IgG ELISA for toxoplasmosis was standardised and the cutoff value for the ELISA was found to be 0.144 in the present study. The sensitivity and specificity of ELISA in detecting toxoplasmosis was found to be 90 and 100 per cent, respectively. This was in accordance with the findings of Shirley *et al.* (1982), who reported that ELISA was more specific (100 per cent).

6.2.3 MODIFIED AGGLUTINATION TEST (MAT)

The formalin killed antigen mixture with 2-mercaptoethanol and Evans blue was prepared and used for MAT. In the present study, MAT titers of 1:20 and above were considered as positive. The sensitivity and specificity of MAT in detecting toxoplasmosis was 80 and 90 per cent, respectively. Desmonts and Remington (1980), Singh *et al.* (1996) and Sema *et al.* (2005) modified the DAT and reported that antibody titers of 1:20 or more were taken as positive.

6.3. COMPARATIVE EVALUATION OF THREE IgG BASED ASSAYS

In the current study, among the three assays, the ELISA was found to be highly specific (100 per cent) and sensitive (90 per cent) for detecting toxoplasmosis. This was in accordance with the findings of Shirley *et al.* (1982) who reported that the ELISA was more specific,

than the IFA. However, the IFA was more reproducible and more rapid than the ELISA.

6.4. SEROPREVALENCE OF TOXOPLASMOSIS

In the present study, out of 350 patients tested, 41 (11.71 per cent), 46 (13.14 per cent) and 39 (11.41 per cent) patients were found to be seropositive for toxoplasmosis by IgG IFAT, ELISA and MAT, respectively. The overall seroprevalence of toxoplasmosis in and around Tirunelveli District of Tamil Nadu was arrived as 13.14 per cent based on IgG ELISA.

In India, the exact seroprevalence of toxoplasmosis was not known. However, using various diagnostic tests, the prevalence has been reported to be as low as 1 per cent and as high as 80 per cent in adults (Singh, 2003). Until recently the prevalence of *T. gondii* in the general population of India was considered to be low when compared with Western countries (Bowerman, 1991 and Mittal *et al.,* 1995). This may be partially true, but the data are based on convenience samples obtained from patients admitted to hospitals in big cities. The type of serological tests used and the titres taken as evidence of infection are also important factors. However, only studies during 1990 indicated that the prevalence may be a lot higher than previously considered (Singh and Nautiyal, 1991 and Singh *et al.,* 1994).

Bhatia *et al.* (1974) reported only 12 per cent seropositivity for toxoplasmosis in South India, whereas, Mittal *et al.* (1990), revealed

an overall seroprevalence of only 1 per cent from Delhi. Singh and Nautiyal (1991) noted a high prevalence (75 per cent) of *T. gondii* in Almora, North India in women of child bearing age, ever reported earlier from any part of India. Singh (2002) reported the seroprevalence study, with highest prevalence of 77 per cent from North India.

In this study, among the immunocompetant group of 175 patients, 19 patients (10.86 per cent) had antibodies to toxoplasmosis whereas, in immunodeficient group of 175 patients, 27 patients (15.43 per cent) had antibodies for toxoplasmosis. Meisheri *et al.* (1997) reported the overall seroprevalence from Bombay was 30.9 per cent (51/165) in the immunocompetent adult and in immunodeficient patients it was 67.8 per cent.

Out of 135 pregnant women tested, 13 (9.63 per cent) were seropositive in the current study. The prevalence of toxoplasmosis in Indian pregnant women was variably reported (Singh, 2003). Chakraborty *et al.* (1995) stated that 7.1 per cent prevalence of toxoplasmosis in pregnant women from Calcutta whereas, Singh and Pandit (2004) reported 45 per cent of prevalence in pregnant women by using IgG Avidity ELISA test.

However, Singh, (2003) quoted that the knowledge about this infection, diagnosis and interpretation of the test results in pregnant women was a major problem in the Indian context. Though,

Toxoplasma infection does not cause repeated foetal losses, this is the most common indication for investigation of toxoplasmosis in India. In this present study also only 3 out of 19 (15. 79 Per cent) cases with bad obstetric history were seropositive for toxoplasmosis. So BOH was not alone taken as criteria for routine screening of toxoplasmosis.

In the present study, among the 20 cases of lymphadenopathy, only 3 (15 per cent) were seropositive. Of the 20 ocular cases tested, only 3 (15 per cent) were seropositive. Recently, Manikandan *et al.*, (2006) reported an outbreak of ocular toxoplasmosis in Coimbatore, Tamil Nadu, about 71.5 per cent of particular area were had high titres of both IgM and IgG antibodies which was an acute acquired type through municipal water contamination.

Present study revealed, 15 per cent seropositive among the HIV positive individuals tested by IgG ELISA and out of 15 cases of malignancy, only 3 (20 per cent) were seropositive. Meisheri *et al.* (1997) from Bombay reported that the seroprevalence rate of toxoplasmosis was 67.8 per cent in HIV patients.

In this study, the significant difference was noticed between IgG IFA and ELISA at $p<0.05$ whereas, the significant difference at $p<0.01$ was found between MAT and IgG ELISA in detecting toxoplasmosis. Statistically the testing efficiency of IgG ELISA for detecting toxoplasmosis was higher than the other two tests. Similar results were observed by Pappas *et al.* (1986) and Shirley *et al.* (1982).

6.5. ANALYSIS OF VARIOUS SOCIO DEMOGRAPHIC RISK FACTORS

In this study, there was a significant difference between sex of the study population and drinking water source. Seroprevalence was increased in the male than the female and with river water consumption other than bore water. There was significant difference between 21-30 years group than the other. About 15 per cent of patients belonging to rural area of Tirunelveli District had anti-*Toxoplasma* antibodies.

As in the study of Bobic *et al*. (1998) and Sema *et al*. (2005) that have found that prevalence increases as the age increases, as the age effect was observed in the current study. The reason might be increasing risk of exposure with age.

In the current study, no statistical meaningful difference was observed between urban and rural areas for toxoplasmosis seroprevalence in Tirunelveli District. Baril *et al.* (1999) have found similar results with the current study; however, Ades *et al*. (1993) have found higher seroprevalences of toxoplasmosis in urban areas.

Sema *et al.* (2005) also reported higher prevalence of toxoplasmosis in municipal network water users which was well correlated with the current study. Frequent consumption and type of meat in non-vegetarian group were identified as the principle risk factor in several recent studies of *T. gondii* infections in humans (Bobic *et al.*, 1998 and Sema *et al.*, 2005). No relation was observed

between seroprevalence of vegetarian and non-vegetarian in the current study and most of the study population (99 .5 per cent) was belonging to non-vegetarian.

The association of cats and human toxoplasmosis was difficult to assess by epidemiological surveys because soil, not the cats, is the main culprit. In the current study, the number of cat owners was just a few and no relation was detected which was similar to the findings of Sema *et al*. (2005).

6.6 COMPARISON OF $CD4^+$/$CD8^+$ COUNT OF T-LYMPHOCYTES IN HIV PATIENTS

In the current study, the mean $CD4^+$ count/µl of HIV patients of seropositive (Male 111.05 and Female 79.5) and seronegative (Male 218.23 and Female 326.5) group of toxoplasmosis was interpreted to assess the risk of development of toxoplasmosis as opportunistic infection. The similar observation of CD4+ count of 135/µl was observed in HIV patients by Kumarasamy *et al*. (2003).

As per the observation made by Kasper (2005), HIV Patients with $CD4^+$ T cell counts <100/µl may develop highest risk for toxoplasmosis. HIV Patients with IgG antibody to *Toxoplasma* should receive prophylaxis for toxoplasmosis with Trimethoprim/ Sulfamethoxazole. Those who are seronegative should be counseled about ways to minimize the risk of primary infection including avoiding the consumption of undercooked meat and careful hand washing after contact with soil or changing the cat litter box.

7. SUMMARY

The present study was conducted at Tirunelveli Medical College Hospital, Palayamkottai, Tirunelveli District, Tamil Nadu from May' 2006 to October' 2007 to assess the seroprevalence of toxoplasmosis in and around Tirunelveli using in-house IgG based assays *viz.*, IFAT, ELISA and MAT.

- A total of 350 peripheral blood samples for this study were collected from 175 immunodeficient and 175 immunocompetent patients.
- The *Toxoplasma gondii* (RH strain) tachyzoite antigen was prepared by *in vivo* mice propagation and *in vitro* cell culture system in MDCK fibroblast cell line.
- Formalin–killed *T. gondii* tachyzoite antigen for IFAT and MAT and the tachyzoite soluble antigen (TSA) for ELISA were prepared.
- The IgG IFAT, ELISA and MAT were standardized and the cut-off titre for IFAT positive was arrived at 1:16 and above. The cutoff value for the IgG ELISA was found to be 0.144. MAT antibody titers of 1:20 or more were taken as significant for reporting positive results.
- Out of 350 patients tested, 41 (11.71 per cent), 46 (13.14 per cent) and 39 (11.41 per cent) patients were found to be seropositive for toxoplasmosis by IgG IFAT, ELISA and MAT respectively.

- Among the three assays, IgG ELISA was found to be highly sensitive (90 per cent) and specific (100 per cent) in detecting toxoplasmosis, whereas the IgG IFAT and MAT were equal in sensitivity (80 per cent) and specificity (90 per cent).
- Statistically the testing efficiency of IgG ELISA in detecting toxoplasmosis was higher ($p<0.05$) than the other two tests.
- The overall seroprevalence of toxoplasmosis in and around Tirunelveli District of Tamil Nadu was arrived as 13.14 (46/350) per cent based on IgG ELISA. Seroprevalence of toxoplasmosis in immunocompromised and immunocompetent patients were of 15.43 (27/175) and 10.86 (19/175) per cent, respectively.
- The seroprevalence of toxoplasmosis in pregnant women was found to be 9.63 (13/135) per cent. Only 3 out of 19 (15. 79 per cent) cases with bad obstetric history (BOH) were positive for toxoplasmosis.
- There was a significant difference between sex of the study population and drinking water source. Seroprevalence was increased in the male (19.12 %, $p=0.0075$, OR=2.316, 95 % CI=1.2362-4.3405) than the female and with river water consumption other than bore water (12.5 %, $p=0.0483$, OR=0.857, 95 % CI=0.1853-3.4641).
- Among the different age groups (<20 – 80 years) studied, there was significant difference between 21-30 years group than the others ($p=0.02$, OR=3.46, 95 % CI=1.08 -11).

- Out of 208 patients belonging to rural area and 56 cases from urban area of Tirunelveli District tested, 33 (15.71 per cent) and 9 (10.71 per cent) were had anti-*Toxoplasma* antibodies, respectively.
- The seroprevalence of toxoplasmosis in HIV patients was found to be 15 per cent (24/160). Male HIV patients with multiple heterosexual behaviour were observed to be having higher seropositivity for toxoplasmosis (25.58 per cent) than single sexual behaviour.
- The mean $CD4^+$ count of HIV patients of seropositive (Male - 111.05 ± 79.96 and Female- 79.5 ± 27.36) and seronegative (Male- 218.23 ± 169.38 and Female- 326.5 ± 259.51) group of toxoplasmosis was statistically interpreted to assess the risk of development of toxoplasmosis as opportunistic infection.

8. CONCLUSION

- The study has highlighted an overall 13.14 per cent of seroprevalence of toxoplasmosis, which constitutes 15.43 per cent in immunocompromised and 10.86 per cent in immunocompetent patients in and around Tirunelveli District of Tamil Nadu. The study underlines the importance of screening of this parasite especially in the immunocompromised patients.
- The seroprevalence of toxoplasmosis in pregnant women was about 10 per cent, which necessitates initial screening for IgG antibodies and then a paired sera sample after 3 weeks should be tested for rise in titer. Thereafter, the IgM antibodies should be tested to exclude the recent infection. All seropositive women who seroconvert during pregnancy should be followed and their amniotic fluid / foetus should be screened by PCR and Ultrasound, respectively.
- Only 3 out of 19 (15. 79 Per cent) cases with bad obstetric history (BOH) were positive for toxoplasmosis, so BOH is not alone taken as criteria for routine screening of toxoplasmosis.
- The in-house IgG ELISA was found to be 90 per cent sensitivity and 100 per cent specificity. IFAT and MAT were equal in sensitivity and specificity. Since in-house assays were cost effective and can be used for routine serological screening.
- Fifteen per cent of HIV patients were found to have anti-*Toxoplasma* antibodies, so routine screening for serology for toxoplasmosis should be done especially when the CD4+ count was less than 200/µl, who may have a risk for development of toxoplasmosis as opportunistic infection.

Table 1

Historical landmarks of *Toxoplasma gondii*
(Dubey, 1993)

Contributors and year	Contribution
Charles Nicolle and Louis Manceaux (1908)	Discovered in gundi
Alfonso Splendore (1908)	Discovered in rabbit
Mello (1910)	Described in domestic animal, dog
Janku (1923)	Identified in human eye
Wolf, Cowen and Paige (1939)	Congenital transmission documented
Pinkerton and Weinman (1940)	Fatal disease described in adult human
Sabin (1942)	Disease characterised in man
Sabin and Feldman (1948)	Dye test described
Siim (1952)	Glandular toxoplasmosis in man
Weinman and Chandler (1954)	Suggested carnivores transmission
Hartely and Marshall (1957)	Abortion in sheep recognised
Beverley (1959)	Repeated congenital transmission
Jacobs *et al.* (1960)	Tissue cysts characterised
William McPhee Hutchison (1965)	Faecal transmission recognised; nematode egg suspected for transmission
Hutchison *et al.* (1969, 1970, 1971); Jack Frenkel *et al.* (1970); Dubey *et al.* (1970); Sheffield and Melton (1970), Overdulve (1970)	Described the coccidian phase
Jack Frenkel *et al.* (1970); Miller *et al.* (1972)	Definitive and intermediate host defined
Dubey and Frenkel (1972)	Five *T. gondii* types described from feline intestinal epithelium
Wallace (1969); Munday (1972)	Confirmed the epidemiological role of cats from studies on remote islands

Table 2

Taxonomic classification of

Toxoplasma gondii Nicolle and Manceaux, 1908

Kingdom	**Protista**
Subkingdom	**Protozoa**
Phylum	**Apicomplexa**
Class	**Sporozoasida**
Subclass	**Coccidiasina**
Order	**Eucoccidiorida**
Suborder	**Eimeriorina**
Family	**Sarcocystidae**
Subfamily	**Toxoplasmatinae**
Genus	***Toxoplasma***
Species	***T. gondii***

Table - 3

Report on seroprevalence of toxoplasmosis in human beings in India
(Mahajan, 1997)

Sl. No.	Area	Number tested	Test	Per cent positive	Author(s) and year
1.	Bombay	141 (Veg) 246 (Non-veg)	DT	21.0 18.0	Rawal (1954)
2.	Delhi	135 (Veg) 400 (Non-veg)	CFT	1.5 4.5	Kalra (1957)
3.	Delhi	60	DT	16.7	Prakash and Choudhary (1969)
4.	Hissar	185	IHA≥1:16	18.9	Bhardwaj (1974)
5.	South India	130	IHA≥1:128	12.0	Bhatia *et al.* (1974)
6.	Chandigarh	641	IHA≥1:128	8.1	Mahajan *et al.* (1976)
7.	Delhi	116	IHA≥1:16	5.2	ICMR (1976)
8.	Rotak	141	IHA≥1:16	4.2	Chugh *et al.* (1978)
9.	Kashmir	100	ELISA ≥1:128	1.0	Thokar *et al.* (1988)
10	Kumaon	200	IHA≥1:18	77.0	Singh and Nauttiyal (1991)

Table – 4

EVALUATION OF IgG IFAT FOR DETECTING TOXOPLASMOSIS

True Positive (a) **8**	False Positive (b) **1**
False Negative (c) **2**	True Negative (d) **9**

$$\text{Sensitivity of IgG IFAT} = \frac{a}{a+c} \times 100 = 80\ \%$$

$$\text{Specificity of IgG IFAT} = \frac{d}{b+d} \times 100 = 90.0\ \%$$

$$\text{Positive predictive value of IgG IFAT} = \frac{a}{a+b} \times 100 = 88.89\ \%$$

$$\text{Negative predictive value of IgG IFAT} = \frac{d}{c+d} \times 100 = 81.81\ \%$$

Table – 5

EVALUATION OF IgG ELISA FOR DETECTING TOXOPLASMOSIS

True Positive (a) **9**	False Positive (b) **0**
False Negative (c) **1**	True Negative (d) **10**

$$\text{Sensitivity of IgG ELISA} = \frac{a}{a+c} \times 100 = 90.0\ \%$$

$$\text{Specificity of IgG ELISA} = \frac{d}{b+d} \times 100 = 100\ \%$$

$$\text{Positive predictive value of IgG ELISA} = \frac{a}{a+b} \times 100 = 100\ \%$$

$$\text{Negative predictive value of IgG ELISA} = \frac{d}{c+d} \times 100 = 90.9\ \%$$

Table – 6

EVALUATION OF MAT FOR DETECTING TOXOPLASMOSIS

True Positive (a) **8**	False Positive (b) **1**
False Negative (c) **2**	True Negative (d) **9**

Sensitivity of MAT $= \frac{a}{a + c} \times 100 = 80.0\ \%$

Specificity of MAT $= \frac{d}{b + d} \times 100 = 90.0\ \%$

Positive predictive value of MAT $= \frac{a}{a + b} \times 100 = 88.89\ \%$

Negative predictive value of MAT $= \frac{d}{c + d} \times 100 = 81.81\ \%$

Table – 7

EVALUATION OF THREE IgG BASED ASSAYS (IFAT, MAT & ELISA) FOR ITS SENSITIVITY AND SPECIFICITY FOR DETECTING TOXOPLASMOSIS

Sl. No.	Category	IFAT	MAT	ELISA
1.	Sensitivity (%)	80.0	80.0	**90.0**
2.	Specificity (%)	90.0	90.0	**100**
3.	Positive predictive value (%)	88.89	88.89	**100**
4.	Negative predictive value (%)	81.81	81.81	90.9

Table – 8

SEROPREVALENCE OF TOXOPLASMOSIS BY IgG IFAT

Sl. No.	Category of patients	Number of patients examined	Number of patients positive	Per cent positive (%)	Mean titre with ± SE and Range of positive patients
	Immnocompetant				
1.	Pregnant women	135	13	9.63	51.69 ± 74.23 (1:16 – 1:256)
2.	Lymphadenopathy	20	3	15.0	26.6 ± 9.23 (1:16-1:32)
3.	Ocular	20	3	15.0	58.67 ± 60.57 (1:16-1:128)
	Immunodeficient				
4.	HIV positive individuals	160	19	11.88	45.47 ± 61.33 (1:16-1:256)
5.	Malignancy	15	3	20.0	26.6 ± 9.23 (1:16-1:32)
	TOTAL	**350**	**41**	**11.71**	**43.32 ± 58.7 (1:16-1:256)**

Table – 9

SEROPREVALENCE OF TOXOPLASMOSIS BY IgG ELISA

Sl. No.	Category of patients	Number of patients examined	Number of patients positive	Per cent positive (%)	Mean OD value with ± SE and Range of positive patients
	Immnocompetant				
1.	Pregnant women	135	13	9.63	0.191 ± 0.069 (0.148 – 0.357)
2.	Lymphadenopathy	20	3	15.0	0.162 ± 0.029 (0.144 – 0.196)
3.	Ocular	20	3	15.0	0.179 ± 0.043 (0.147 – 0.228)
	Immunodeficient				
4.	HIV positive individuals	160	24	15.0	0.212 ± 0.079 (0.146 – 0.444)
5.	Malignancy	15	3	20.0	0.173 ± 0.025 (0.146 – 0.196)
	TOTAL	**350**	**46**	**13.14**	**0.2 ± 0.073 (0.144 – 0.444)**

Table – 10

SEROPREVALENCE OF TOXOPLASMOSIS BY MAT

Sl. No.	Category of patients	Number of patients examined	Number of patients positive	Per cent positive (%)	Mean titre with ± SE and Range of positive patients
	Immnocompetant				
1.	Pregnant women	135	12	8.89	55 ± 63.32 (1:20 – 1:160)
2.	Lymphadenopathy	20	3	15.0	33.33 ± 11.54 (1:20-1:40)
3.	Ocular	20	3	15.0	73.33 ± 75.71 (1:20-1:160)
	Immunodeficient				
4.	HIV positive individuals	160	18	11.25	50 ± 51.44 (1:20-1:160)
5.	Malignancy	15	3	20.0	33.33 ± 11.54 (1:20-1:40)
	TOTAL	**350**	**39**	**11.14**	**53.85 ± 55.28 (1:20-1:160)**

Table – 11

COMPARISON OF THREE IgG BASED ASSAYS (IFAT, MAT & ELISA) FOR DETECTING TOXOPLASMOSIS

Sl. No.	Category of patients	Number of patients examined	Number of positive patients and per cent (%) positive		
			IFAT	MAT	ELISA
1.	**Immnocompetant** Pregnant women	135	13 (9.63 %)	12 (8.89 %)	13 (9.63 %)
2.	Lymphadenopathy	20	3 (15 %)	3 (15 %)	3 (15 %)
3.	Ocular	20	3 (15 %)	3 (15 %)	3 (15 %)
4.	**Immunodeficient** HIV positive individuals	160	19 (11.88 %)	18 (11.25%)	24 (15 %)
5.	Malignancy	15	3 (20 %)	3 (20 %)	3 (20 %)
	TOTAL	**350**	**41 (11.71 %)**	**39 (11.14%)**	**46 (13.14 %)**

Table – 12

COMPARISON OF TESTING EFFICIENCY OF THREE IgG BASED ASSAYS (IFAT, MAT & ELISA) BY McNEMAR'S TEST

Sl. No.	Test (n = 350, degree of freedom =349)	Calculated z value	Tabulated value		Remark (Test of significance)
			at 0.01 level	at 0.05 level	
1.	**IFA with MAT**	0.357 NS	2.58	1.96	**Not-significant**
2.	**IFA with ELISA**	**2.232 ***	2.58	1.96	***Significant at p<0.05**
3.	**ELISA with MAT**	**2.646 ****	2.58	1.96	****Significant at p<0.01**

Table – 13

SEROPREVALENCE OF TOXOPLASMOSIS IN PREGNANT WOMEN

a. Based on gravid

Sl. No.	Gravida	No. of patients tested	No. of patients positive for IgG	Percentage (%)
1.	Primi	52	5	9.62
2.	G_2	56	4	7.14
3.	G_3	21	2	9.52
4.	G_4	3	2	66.67
5.	G_5	3	0	0
	Total	**135**	**13**	**9.63**

b. Based on trimester of pregnancy

Sl. No.	Trimester of pregnancy	No. of patients tested	No. of patients positive for IgG	Percentage (%)
1.	I Trimester	11	1	9.1
2.	II Trimester	44	6	13.6
3.	III Trimester	80	6	7.5
	Total	**135**	**13**	**9.63**

c. Based on bad obstetric history (Abortion/premature delivery)

Sl. No	Trimester of pregnancy	No. of patients with bad obstetric history (Abortion/PMD)	No. of patients positive for IgG	Percentage (%)
1.	I Trimester	**3**	**1**	**33.34**
2.	II Trimester	**8**	**-**	**-**
3.	III Trimester	**8**	**2**	**25.0**
	Total	**19**	**3**	**15.79**

Table – 14

ANALYSIS OF SOCIO DEMOGRAPHIC RISK FACTORS ASSOCIATED WITH THE SEROPREVALENCE OF TOXOPLASMOSIS

Sl. No.	Risk factors	No. of patients tested (n = 350)	No. of patients positive for IgG	Percentage (%)	Odds ratio	95 % CI	p value (χ2 test) #
1.	**Age** a. >20 years b. <20 years	 19 331	 3 43	 15.79 12.99	**1.255**	0.3512-4.4901	0.7255
2.	**Gender** a. Male b. Female	 136 214	 26 20	 19.12 9.35	**2.316**	1.2362-4.3405	**0.0075 ***
3.	**Residence** a. Rural b. Urban	 278 72	 39 7	 14.02 9.72	**1.515**	0.6476-3.5450	0.3351
4.	**Water source** a. River water b. Bore water	 336 14	 42 2	 12.5 14.29	**0.857**	0.1853-3.4641	**0.0483 ***
5.	**Diet** a. Vegetarian b. Non-vegetarian	 2 348	 1 45	 50.0 12.93	**6.73**	0.4138-109.569	0.1218
6	**Animal contact** a. Yes b. No	 40 310	 8 38	 20.0 12.26	**1.789**	0.768-4.1695	0.1726

Results of χ2 test and Fisher's Exact test by p value of < 0.05 as significant difference*****

Table – 15

SEROPREVALENCE OF TOXOPLASMOSIS ACCORDING TO AGE AND SEX

(n= 350)

Sl. No.	Age	No. of patients		No. of patients positive for IgG		Percentage (%) of positivity		Odds ratio	95 % CI	p value (χ2 test) #
		Male	Female	Male	Female	Male	Female			
1.	**<20 years**	6	7	2	1	33.34	14.29	3	0.19-45.2	0.41
2.	**21 – 30 years**	19	150	5	14	26.32	9.33	3.46	1.08-11.0	**0.02 ***
3.	**31 – 40 years**	54	38	10	4	18.19	10.52	1.93	0.55 -6.69	0.29
4.	**41 – 50 years**	29	6	4	1	13.79	16.67	0.8	0.07-8.75	0.85
5.	**51 – 60 years**	14	8	3	-	21.43	-	5.67	0.47-6.73	0.15
6.	**61 – 70 years**	12	3	2	-	16.67	-	3.84	0.01-1.40	0.44
7.	**71 – 80 years**	2	2	-	-	-	-	-	-	-
	Total	**136**	**214**	**26**	**20**	**19.12**	**9.34**	**2.31**	**1.2 - 4.3**	**0.007**

Results of χ2 test and Fisher's Exact test by p value of < 0.05 as significant difference

Table – 16

SEROPREVALENCE OF TOXOPLASMOSIS IN DIFFERENT REGIONS IN SOUTHERN DISTRICTS OF TAMIL NADU

Sl. No.	Region	No. of patients tested	No. of patients positive for IgG	Percentage (%)	Odds ratio	95 % CI	p value (χ2 test) #
1.	**Tirunelveli** **a. Urban** **b. Rural**	 56 208	 6 33	 10.71 15.87	0.636	0.252 - 1.6048	0.3349
2.	**Tuticorin** **a. Urban** **b. Rural**	 12 65	 1 5	 8.33 7.69	1.09	0.116-10.26	**0.0161 ***
3.	**Kanyakumari** **a. Urban** **b. Rural**	 4 5	 0 1	 0 20	0.165	0.003-8.5371	0.3248
	TOTAL	350	46	13.14			

Results of χ2 test and Fisher's Exact test by p value of < 0.05 as significant difference *

Table – 17

RISK FACTORS IN HIV PATIENTS WITH SEROPOSITIVE AND SERONEGATIVE GROUP OF TOXOPLASMOSIS

Sl. No.	Risk factor	No. of patients tested		No. of patients positive for IgG		Percentage (%)	
		Male	Female	Male	Female	Male	Female
1.	**Sexual behaviour** **a. Single** **b. Multiple**	 54 43	 55 2	 8 11	 3 1	 14.81 25.58	 5.45 50.0
2.	**Vertical transmission**	2	4	1	-	50.0	-
3.	**Blood transfusion**	4	-	-	-	-	-
4.	**I/V drug abuser**	1	-	-	-	-	-
5.	**Needle injury/surgery**	4	6	-	-	-	-
6.	**Stages of HIV infection** **1. Stage I** **2. Stage II** **3. Stage III** **4. Stage IV**	 35 10 48 6	 42 1 15 3	 7 2 11 -	 3 - 1 -	 20.0 10.0 22.91 0	 7.14 - 9.09 -

Table – 18

COMPARISON OF $CD4^+$ and $CD8^+$ COUNT [Mean count (cells/µl) ± SE] **IN HIV PATIENTS WITH SEROPOSITIVE AND SERONEGATIVE GROUP OF TOXOPLASMOSIS**

Sl. No.	Category Mean count (cells/µl)	Seropositive toxoplasmosis group		Seronegative toxoplasmosis group		Odds ratio	95 % CI	p value ($\chi 2$ test) #
		Male	Female	Male	Female			
1.	CD4+	111.05 ± 79.96	79.5 ± 27.36	218.23 ± 169.38	326.57 ± 259.51	2.09	1.5-2.9	0.0001 *
2.	CD8+	621.8 ± 379.88	514 ± 183.48	986.17 ± 493.86	1062.63 ± 494.21	1.30	1.1-1.5	0.0004 *
3.	CD4+/CD8+	0.18 ± 0.09	0.16 ± 0.01	0.27 ± 0.16	0.32 ± 0.24	1.33	0.0-0.63	0.0349 *

Results of $\chi 2$ test and Fisher's Exact test by p value of < 0.05 as significant difference *

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ANNEXURE I

RECIPE FOR BUFFERS AND SOLUTIONS USED

Phosphate buffered saline (PBS) without Ca++ and Mg++ (1x, pH 7.2)

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Disodium hydrogen orthophosphate dihydrate	1.41 g
Potassium dihydrogen orthophosphate	0.20 g
Distilled water to make (adjust pH to 7.2)	1000 ml

PBS – T

PBS 10X solution	100 ml
Tween – 20	0.5 ml
Triple glass distilled water to	1 litre

pH adjusted to 7.2

Buffered glycerol (pH 9.0, for IFAT)

Phosphate buffer [Na_2HPO_4 - 284g /10ml H_2O (pH 9.0)]	1 volume
Glycerol	9 volumes

Carbonate-bicarbonate buffer for ELISA

Sodium carbonate	1.59 g
Sodium bicarbonate	2.93 g
Distilled water to make up to	1 litre
Adjust the pH to	9.6

Skimmed Milk 1 per cent for ELISA

Skimmed Milk powder	1g
PBS –T to	100 ml

Hydrofluric acid 0.1 M for ELISA

Hydrofluric acid (Concentrated)	3.5 ml
Triple glass distilled water to	1 litre

Borate buffer for MAT

Sodium chloride	7.01 g
Boric acid	3.09 g
Sodium azide	2 g
1N NaOH	24 ml
Deionised water to make up to	1 litre
Adjust the pH to	8.95

Working antigen diluting buffer for MAT

Bovine Serum Albumin	0.4 g
Borate buffer	100 ml

Antigen mixture for the MAT

Antigen diluting buffer	2.5 ml
2-mercaptoethanol	35 µl
Evans blue dye solution	50 µl
Formalin killed antigen	0.15 ml

ANNEXURE II

DATA SHEET FOR COLLECTION OF SOCIO-DEMOGRAPHIC, CLINICAL AND BEHAVIORAL DATA

FOR P.G. DISSERTATION WORK ON

"Evaluation of different serodiagnostic methods of Toxoplasmosis in pregnant women and immunodeficient patients"

I. GENERAL INFORMATION

Lab No. :

Name : **Age :** **Sex: M / F**

Address : **Occupation :**

Referred by : **UNIT** **WARD** **OP/IP No. :**

Income : **Urban/ Rural / Migrant**

Educational status : **Occupation of the partner:**

Marrital status : Married / Unmarried Duration :

II. EPIDEMIOLOGICAL RISK FACTOR ASSESSMENT

Food habits : Vegetarian / Non-vegetarian

Type of food : Salad / greens / Meat type Cooked / half cooked

Drinking water source : Well / River / others

Close contact with cat : Owner of cat / access to cat :

Close contact / access with domestic animal / chicken :

Whether consumed raw goat milk :

Habits : Alcohol / Smoking/ Tobacco / Drugs

Organ transplantation :
Family H/o :

Specific Medical history :

III. ANTENATAL RISK ASSESSMENT :

If married women
Pregnant / Non pregnant/

Menstrual history :

LMP : **Gravida:**

EDD : **Para :**

CEDD: **Live :**

Abortion / still birth **MTP :**

LSCS: **LCB :**

Previous obstetric history :

Status of baby born :

Illness of present or previous baby :

H/o Congenital infection (maternal infection acquired during pregnancy)

H/o Mother to foetus transmission / placental transmission

IV. HIV RISK ASSESSMENT :

Height : **Weight :**

Sex behaviour : Hetero/Homo/ Bisexual/ No sex

Partner : Single / Multiple

Blood transfusion : **Vertical transmission :**

I/V drug user: **Needle Injury :**

Injection : **Hospital acquired injury :**

Others (specify) :

V. CLINICAL ASSESSMENT :

History of Previous clinical illness with duration :

Present Clinical symptoms :

Fever

Lymphadenopathy / lymphadenitis

Ocular lesions / chorioretinitis

Neurological symptoms / Encephalitis

Haematological malignancy

Myocarditis

Polymyositis

Others (specify if any)

VI. CLINICAL DIAGNOSIS :

Tentative clinical diagnosis :

Concurrent infections (if any) :

VII. TREATMENT / ADVISE GIVEN IN THE UNIT:

VIII. FOLLOW UP OF A PATIENT (if needed) :

IX. LAB INVESTIGATIONS

SAMPLES COLLECTED :

BLOOD for Serum / Peripheral blood for flowcytometry

RESULTS OF HIV ANTIBODY TEST

Name of the test : Qualitative MICROELISA

Result : Seropositive / Seronegative

RESULTS OF FLOWCYTOMETRY

$CD4^+$, count : cells/µl
$CD8^+$, count : cells/µl
$CD4^+/CD8^+$ ratio :

RESULTS OF CONCURRENT INFECTIONS (if any)

RESULTS OF specific blood parameters (if any)

RESULTS OF ultra scan /CT scan/ MRI (if any)

RESULTS OF SERODIAGNOSIS OF TOXOPLAMOSIS

Date of the test :

	MAT	IgG IFAT	IgG ELISA
RESULT			
TITRE			
Follow up Titre (if any)			

Date:
Signature :