BIOLOGICAL AND SEROLOGICAL STUDIES ON *TOXOPLASMA GONDII* IN LAGOS, NIGERIA.

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CERTIFICATION

This is to certify that the Thesis titled:

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by

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CONTENTS

ABSTRACT

Sera from 775 persons in 10 age-groups, living in Lagos metropolis, were titrated for Toxoplasma antibodies, using the haemagglutination test. The overall percentage positive was 63.2%. The highest (78.4%) was found amongst the age group ranging from 36-40 years, whilst the lowest (48.2%) was recorded in the age group 11-15 years. The risk of non-immune persons acquiring infection was calculated in the different age groups using Van Der Veen's equation.

Venous blood samples, cord blood, as well as placental tissue were also collected from a total of 452 pregnant women, who attended some antenatal clinics in Lagos. Of the 452 samples studied, 60.18% were positive for Toxoplasma antibodies. 70.7% of the cord blood specimen collected were also positive.

Transmission pattern of toxoplasmosis employing cats was studied, as faecal samples of cats collected randomly from different areas of Lagos were analysed, using the Formol-Ether Concentration technique after Ritchie (1948) to detect the presence of oocysts. The cats were also bled for serological tests. Those found to be negative were fed acutely-ill mouse, and the course of development of the parasite was observed.

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The effect of Toxoplasma infection on pregnancy was observed using mice models, and it was found that when pregnant mice were experimentally infected, intraperitoneally within the different thirds of their gestation period, death occurred, beginning from the 11th to the 29th day, and not earlier.

Toxoplasma gondii trophozoites were maintained in vitro in different fluid media, with pH values ranging from 6.3 to 7.3. The length in days before the death of the infected mice was recorded and it was related to the viability and number of surviving organisms in the suspension. A graph of survival time against preservation time (both in days) was then drawn. The maximum length of survival at $4^{o}C$ was in whole human blood, as well as in Foetal Bovine Serum, for up to 56 days.

The molecular weight of the proteins present in the RH strain of Toxoplasma gondii laboratory prepared antigen (after Voller et al., 1976) was determined by gel filtration on Sephadex G200 chromatography column. The absorbance of the 3 fractions derived were read at 280 nanometers, and the molecular weights were determined by extrapolation from a standard curve of $\frac{Ve}{Vo}$ against the molecular weights of 4 standard proteins. The molecular weights ranged from 12,445 to 186,209 daltons.

CHAPTER I

GENERAL INTRODUCTION

Toxoplasmosis is a result of infection by a protozoan parasite Toxoplasma gondii, 77 years ago; (1908) it was first recognized in the rabbit by Splendore in Sao Paulo, Brazil and also in that same year by Nicolle and Manceaux (1908) in the North African rodent - Ctenodactylus gondii (after which it was named). In 1923, the organism was observed from an eye of a child in Czechoslovakia. It was called Encephalitozoon, and Wolf and Cowen (1937) recognized the organism in brains of children dying of neonatal encephalitis, and drew attention to placental transmission for the first time.

Infection in man was first proved only 46 years ago by Wolf et al (1939) who isolated T. gondii from the brain of a child (a new born baby who had suffered from fits, choroiditis and encephalomyelitis. Sabin (1949) also showed that the parasite was the cause of meningoencephalitis in 2 school children. Subsequent immunological and biological studies established the identity of the causative agents. Soon afterwards, antibody production was shown to occur first by a neutralization test in rabbit skin (Sabin and Ruchman

1942), secondly by a complement fixation test (by Warren and Sabin 1942), and thirdly by a cytoplasm modifying test, commonly called the dye test by Sabin and Feldman (1948). These discoveries led to intensive investigations of Toxoplasma infections in most parts of the world, first mainly in man, and later in other animals.

The discovery by Wolf et al (1939) that T. gondii is the etiological agent of a highly fatal disease of infants has led to the description of an increasingly large number of cases in recent years. While a majority of cases have been neonatal, characterized by hydrocephaly, cerebral calcification and chorioretinitis, (Eichenwald 1959), a few adult cases have also been reported, (Jacob and Jones, 1950).

While case studies are clarifying the clinical picture of toxoplasmosis, and while a number of studies on the immunologic diagnosis of the infection have been reported, little is known about the biology of the parasite, or its method of spread beyond the fact that it occurs in a wide variety of mammals and birds.

T. gondii is one of the commonest protozoan parasites of man and animals, and occurs worldwide, De-Roever Bonnet et al (1969), De-Roever Bonnet (1972), and Feldman and Miller (1956). Most acquired

human infections are usually mild, but clinical toxoplasmosis may present as a lymphadenopathy or chorioretinitis, while congenital toxoplasmosis might lead to malformations of the foetus. (Khordi and Matossian (1978).

Toxoplasma infection affects practically every organ of the body, including the spleen, heart, brain, intestinal epithelium and other organs. The trophozoites of T. gondii are crescentic or banana-shaped, with one end pointed and the other rounded, and are 4 - 8 by 2 - 4 microns. The nucleus is vesicular and more or less central. Locomotion is by body flexion whereby the protozoa follows a cock-screw path, rotate on their longitudinal axis or somersault (Manwell and Drobeck 1953), or by gliding. The parasites occur within vacuoles in their host cells. As the parasites multiply, they form a cyst-like structure. The trophozoites in the cysts (Bradyzoites) differ slightly from the proliferative ones in the terminal colonies, referred to as tachyzoites.

Oocysts are produced in the epithelial cells of the ileum of the cat by the union of male and female gametes. They pass out in the faeces of the cat, and are indistinguishable from those of the "small race" of Isospora bigemina (Hutchinson et al, 1970). They are

spherical at first, but after sporulation are 11-14 by 9-11 microns, containing two ellipsoidal sporocysts about 8.5 by 6 microns, each containing four sporozoites about 7 by 2 microns and a residium. Apparently, vegetative stages alone (and no sexual stages) are produced in all animals but the cat. Transmission is either by ingesting sporulated oocysts, congenitally via the placenta, or by ingesting infected meat or animals. Reproduction in the tissue is by endodyogeny, (Goldman et al, 1958). This is a process of internal budding by which two daughter cells are formed within the parent cell and are released as proliferative forms of the parasite.

The final product of sexual reproduction in the cat is the fertilized zygote that transforms to an oocyst; which is the infective stage, and is eventually extruded with faeces, in the unsporulated state. It has been shown that if a susceptible cat ingests sporulated oocysts, and developes intestinal infection the animal will pass oocysts in 21 - 24 days. (Dubey 1976). However, if a cat is fed with an acutely ill mouse which harbours proliferative forms or trophozoites of T. gondii in its tissues, oocysts will appear in the cats faeces in 9 - 11 days. Finally, when a chronically ill mouse with cysts in its tissues is fed to a cat,

oocysts are shed in the cats faeces after only 3 - 5 days (Dubey, 1976). The cat may also have an extra-intestinal infection i.e., proliferative oocysts forms in various tissues which are infective to animals eating the cat.

Although the prevalence of toxoplasmosis is very low, occasionally, epidemics occur (Maddison, 1979). For example, in 1969, 110 persons were diagnosed with acute toxoplasmosis, within a three-months study period, at a University in Sao Paulo, Brazil (Schmidt and Roberts, 1981). Most of the patients had eaten under-cooked meat, thereby implicating improperly-cooked meat as an important source of infection.

In the United States of America, a family out-break of toxoplasmosis was thought to be due to eating inadequately cooked lamb (Masur et al, 1978). Also, a study on a group of French children revealed that those who were served undercooked beef had a significantly higher incidence of toxoplasma antibodies than others who were regularly served adequately cooked beef for a period of time. When the experiment was repeated, using lamb, it was found that improperly cooked lamb gave a significantly higher incidence - about ten times that of the control population. From the above experiment,

Jacobs et al, (1975) concluded that, while beef is certainly a potential source of infection, pork and lamb are more likely to be contaminated than other kinds of meat.

Feral and domestic cats are also a source of infection to humans (Hutchinson et al, (1971).

The mode of acquired infection has now been confirmed to be by ingestion of oocysts from cats faeces contaminating food or drink, (Hutchinson et al (1969); For example filth, flies and cockroaches are capable of contaminating food and even drinks that are exposed, with oocysts of Toxoplasma (Wallace, 1971) and earthworms may also serve to expose oocysts that are buried by cats. Joel et al, (1971) showed that whole blood or leukocyte transfusions and organ transplants are also potential sources of infection, especially if the recipient is immunodeficient because of malignant disease or steroid therapy.

Evidences accumulated during recent years have helped to explain the high prevalence of Toxoplasma infection in some parts of the world. In some communities, more than 60% of the population are serologically positive, (Feldman and Miller, 1956).

Congenital transmission of toxoplasmosis accounts for relatively few of the many infections seen in man, even though such infections probably account for the majority of acute fetal infections. It is generally believed that a woman does not transmit the infection to her unborn child, unless her primary infection occurs during that particular pregnancy, (Eichenwald, 1959). The report of some workers such as Kimball et al, (1971b) suggests that habitual abortion due to chronic toxoplasmosis is uncommon.

Although there have been reports of experimental transmission (Huldt, 1967), as well as the possibilities of natural transmission through droplet inhalation or the ingestion of various body fluids, or exudates, it is likely that such routes would account for only occasional infections.

Despite the fact that there is high prevalence of antibody to Toxoplasma in humans throughout the world, clinical toxoplasmosis is not very common. This is an indication that most infections are asymptomatic. Several factors may influence this phenomenon. For example:

(a) the virulence of the strain of Toxoplasma;

(b) the susceptibility of the individual host and

of the host species;

(c) the age of the host; and

(d) the degree of acquired immunity of the host.

Schmidt and Roberts (1981) have shown that tachyzoites proliferate in many tissues and tend to kill host cells at a faster rate than the normal turnover of such cells. Enteroepithelial cells, on the other hand, normally live only a few days, especially at the tips of the villi. Thus, in such cases, the pathology will be milder. The extraepithelial stages, particularly in sites such as the retina or brain, tend to cause more serious lesions, as epithelial cells in the retina and brain live for longer periods.

Symptomatic infections can be classified into acute; subacute; and chronic. In most acute infections, the intestine is the first site of infection. In fulminating infections, intestinal lesions can kill kittens in 2 or 3 weeks, (Schmidt and Roberts, 1981). Next, the mesenteric lymph nodes, and the parenchyma of the liver are then infected. The most common symptoms of acute toxoplasmosis is painful swollen lymph glands in the cervical, and inguinal regions. This symptom may be associated with fever, headache, muscle pain, anaemia, and sometimes lung complications, (Schmidt and Roberts, 1981).

In subacute infections, immunity develops slowly, and consequently the condition is prolonged and pathogenic lesions are more extensive. Damage in the CNS is particularly extensive unlike in other organs of the body, because of lower immuno-competence in the Central Nervous System tissues.

Chronic infections result when immunity builds up sufficiently to depress tachyzoite proliferation. This coincides with the formation of cysts, which can remain intact for years, without producing any obvious clinical effect (Remington and Cavanaugh, 1965). Repeated infections of retinal cells by tachyzoites can destroy the retina. The presence of cysts and ruptured cysts in the retina and choroid can also lead to blindness. Other forms of extensive pathology that can occur in chronic toxoplasmosis are myocarditis, with permanent heart damage, and pneumonia.

The most serious forms of this disease is congenical toxoplasmosis. Still-births and spontaneous abortions may result from fetal infection with Toxoplasma in humans and other animals. Sheep is seen to be particularly susceptible (WHO, 1969). Abortions caused by toxoplasma in this host quite often reach epidemic proportions. This disease is said to account for half of all bovine abortions in England and New Zealand.

Transmission via the placenta occurs in congenital toxoplasmosis. It is generally considered to be an accidental complication of an inapparent primary infection of a pregnant female, (Feldman and Miller, 1956).

Common lesions in congenital toxoplasmosis are hydrocephaly, microcephaly, cerebral calcifications, chorioretinitis, and psychomotor disturbances, (Feldman and Miller, 1956) and (Eichenwald, 1959). In children who survive infections, there is usually congenital damage to the brain, manifested as mental retardation and epileptic seizures.

In a case report given in Brazil, it was found that the death of a child that suffered from convulsions on the second day of life was due to Toxoplasma infection. Toxoplasma was demonstrated in the tissues of this neonate and cutaneous inoculations of the tissues into animals resulted in Toxoplasma infections.

The diagnosis of toxoplasmosis is usually based on clinical impressions, and may be confirmed in the laboratory culture of the organism from blood or cerebrospinal fluid, by inoculation of infected tissue into mice, by histological examination of lymph node or by serological tests (Alford et al, (1973). Determination of serum concentrations of antibodies is another way of confirming

exposure to Toxoplasma infection. Occasionally, Toxoplasma can be isolated and identified in smears of lymph nodes, bone marrow, spleen, brain or other material. The organisms are readily identified by impression smears or smears of fluid materials; however, in histologic preparations of fixed tissues, they do not exhibit the typical morphology. Body fluids or ground tissues may be inoculated intraperitoneally into young laboratory mice from a Toxoplasma-free colony, and after 7 - 10 days; the peritoneal fluid and smears of lung, spleen, and liver are examined for the proliferative forms of the parasite. Serum from the inoculated animals may also be tested for the presence of antibodies. Serological tests may be done in all cases of suspected toxoplasmosis, and a variety of good tests are now available. For example:

(i) a complement fixation test (CFT) (Warren and Russ, 1948).

(ii) the dye test (Sabin and Feldman, 1948).

(iii) the Indirect Haemagglutination Test (IHAT) (Jacobs and Lunde, 1957).

(iv) the Indirect Fluorescent Antibody Test (IFAT) (Remington, 1969 and Kelen et al, 1962).

(v) the Direct Agglutination Test (DAT) (Desmonts and Remington, 1980).

(vi) and most recently the ELISA test for detection of IgM antibodies to T. gondii (Lin et al, 1980).

Serum titres for the dye tests, indirect fluorescent antibody test, and the IHA tests can be detected earlier i.e. are more sensitive, reach much higher levels, and drop much later than do those obtained with the CFT, (Walton et al, 1966 ; Fletcher 1965). Tests of serum taken at intervals of several weeks can be useful in determining the course of infection. Also, demonstration of the organism at necropsy or biopsy is definitive diagnosis. In the case of a congenital infection, it is important to diagnose the infection at birth, since early specific treatment may be helpful. In such a case, routine examination of the umbilical cord blood is particularly desirable.

Prevention and control methods to avoid Toxoplasma infection includes freezing of meat at $-4^{o}C$ for even a few hours, (Schmidt and Roberts (1981) as this apparently kills all cysts. To avoid a multitude of parasites, persons who insist on eating undercooked meat should see that it has been hard-frozen. Also, efforts should be made to keep down the number of stray cats in our environment as these serve as reservoirs of several diseases. Filth flies and cockroaches should also be avoided

(Wallace, 1971) as they can also transfer Toxoplasma oocysts from cats' faeces to the dinner table.

Simple precautions for those at risk include not eating undercooked meat and, if possible, avoiding contact with cats' faeces. Hand washing before food, especially after gardening should be mandatory for those susceptible, if there is a cat in the house.

For treatment, pyrimethamine with triple sulphonamide has been shown to give good results in ocular infections, but it may cause macrocytic anemia (Eyles and Coleman, 1955). Although clinical improvement or care has been obtained by the combined use of pyrimethamine and sulfonamides which act synergistically, there is evidence that the parasite may not be completely eliminated, (Frenkel and Weber, 1960). It is probable that the resistant cyst forms persist and these may later initiate an active infection in that host.

Most Toxoplasma infections are self-limiting, and treatment may not be necessary. The exceptions are patients with eye disease and those who are immunocompromised. A combination of sulphamerazine, pyrimethamine and folinic acid is the most successful, (Remington and Klein, 1976).

Other drugs that have been tried include co-trimoxazole and the macrolide antibiotic - spiramycin, which is used in France for treating infections in pregnancy

when folic acid inhibitors are contraindicated. None of these drugs is of proven efficacy (Feldman, 1968).

Spiramycin is an erythromycin-like antibiotic, and is said to act on parasites on the maternal side, and not known to cross the placenta; (Alford et al, 1969). Its dosage and timing by and large are arbitrary..

Toxoplasma gondii antibodies were first shown to be present in the Nigerian human population, through a serological survey carried out by Ludlam (1965) in the Delta Region of Nigeria. He reported 64% of the 62 cases he studied as positive. Ogunba and Thomas (1979) showed the prevalence in healthy blood donors to be about 20.6% in Ibadan area, whereas, in patients with eye infections and with choriocarcinoma, its prevalence increased to 44.39% and 45.9% respectively. Olurin et al, (1971) in another study of the disease in Ibadan showed a high prevalence of Toxoplasma antibodies in patients with chorioretinitis. The zoonotic effect of T. gondii was also demonstrated by Okoh et al, (1981) who did a serological survey on various animals and indicated that clinically significant titres of Toxoplasma antibodies were present in our Nigerian animal population. This survey was however restricted to the Northern part of the country.

The role of Toxoplasma infection in the epidemiology of habitual abortion was also investigated in Ibo Women in the Eastern part of Nigeria by Megafu and Ugbuegbulam (1981), and they concluded that there was a higher incidence of the titer in Women with a history of recurrent abortions than in the control group. Also, patients in the low income groups were shown to have a much higher incidence of the titer than patients in the higher income group.

Despite the fact that few people have reported on the presence of toxoplasmosis in the Nigerian population, the studies have been localised, and restricted to certain regions, therefore to get a clearer picture of the infection pattern in Nigeria, it becomes necessary to fill in the gaps and study areas that have not been investigated.

In spite of the work done by the earlier mentioned authors, the unanswered questions with regards to toxoplasmosis in Nigeria include the study of the infection in Lagos, with the following objectives:- (i) To determine the age prevalence levels of Toxoplasma antibodies in the general population, as well as in pregnant women; and also to correlate antibody presence with susceptibility to infection.

(ii) To determine the prevalence of toxoplasmosis in stray and domesticated cats; thereby determining the sources and modes of infection in the community, and transmission mechanism.

(iii) To find the effect of Toxoplasma infection in pregnant mice; with reference to recurrent abortions.

(iv) In-vitro culture pattern of T. gondii in fluid media.

(v) To separate and identify (using molecular weights) the different chromatographic bands resulting from the laboratory prepared Toxoplasma gondii antigen, after the method of Voller et al, (1976).

CHAPTER II

LITERATURE REVIEW

Transmission Patterns and Factors Affecting it:

Since 1970, when T. gondii was demonstrated to be an Isosporan organism similar to the small form of Isospora bigemina, cats have been implicated as definitive hosts and transmitters of this disease by certain authors including Hutchinson, et al., (1971), Sheffield and Melton (1970) and Frenkel, et al.,(1970). However, epidemiological studies which have investigated the association of positive Toxoplasma skin test, and serology with exposure to household cats have been quite inconclusive.

McCulloch, et al., (1963) in a skin test survey of veterinary and medical students found a statistical association of positive reacters and moderate or marked contact with cats. They also found a relationship between positive skin tests and contact with swine, horses, sheep, cattle, chickens, and turkeys. Whereas Price (1969) in a study on an urban community, found no association with cats alone, but did find a greater frequency of positive titers among individuals who owned either a cat or dog, and amongst those who

handled pet food and owned dirty kitchens.

Peterson et al., (1972) also studied the age-standardized Toxoplasma prevalence rate for 235 subjects who had possessed a pet cat, and found it equalled 20.9% compared to 9.3% of 78 respondents denying possession. Corresponding data for pet dog possession, farm living, and meat cooking preference yielded differences that were not statistically significant. This study was done on adult health clinic patients in Seattle, Washington.

Fisher and Reid, (1973) investigated animal contacts among blood donors and found no significant association with possession of cats nor with duration of contact.

Investigators such as Schnurrenberger, et al., (1964) and Walton, et al., (1966) have also attempted to correlate the incidence of detectable toxoplasmosis with animal contact, and found that antibodies were present more frequently in persons who are in contact with animals and animal products.

Epidemiology:

Feldman and Miller, (1956) conducted dye test surveys amongst 10 human and 24 animal populations. In their study, only titers of 1:16 (original serum dilution) or more were considered to be positive. They were also able to show that all humans do not

react in the same way to the production of antibodies to Toxoplasma.

The subject of age prevalence levels of Toxoplasmosis have been studied for ages using the different diagnostic tests available. High infection rates in childhood, which are attributed to the abundance of cats, and playing habits of young children, were reported from villages in North Iran by Ghorbani, et al., (1978) who used the Indirect Fluorescent Antibody Technique (IFAT). The antibody positive rates rose from 17.3% in the second year of life to 59.7% for a 5 - 9 years age group.

In order to obtain information on Toxoplasma infection risks at different ages, Van der Veen and Polak (1980) examined sera from 1661 persons in various age groups, for Toxoplasma antibodies by the Indirect Immuno-fluorescence Test. The age trend of sero-positivity was interpreted as evidence of continuous risks of infection with Toxoplasma during the first ten years of life until the age of 50 years.

The epidemiology of Toxoplasmosis in Kuwait was determined by Behbehani and Al-Karmi, (1980), and they found the incidence to be high amongst the Kuwaitis, and to be directly dependent on the initial condition of the patient. In general agreement with the work of others, such as Mahajan, et al., (1975), there was no obvious difference between the proportion of females as compared with males with antibodies to T. gondii.

Extreme effects on sero-conversion were observed by Desmonts, et al., (1965) in a tuberculosis department of a hospital near Paris, where under cooked meat was served to children as a normal diet constituent. Of 644 children without antibodies, at the time of admission, 204 became seropositive during the course of their hospital stay. This corresponds to a monthly infection risk of 5.9% as calculated from the formula of Van Der Veen and Polak (1980).

Mackie, et al., (1971) reported in their study which agrees with previous reports of Burkinshaw, et al., (1953), Cook and Derrick (1961), Fair (1959) and Hongo, et al., (1964); that the incidence of toxoplasmosis was not more frequent in the mentally retarded than in normal populations. The IFAT and the IHAT were used to run this experiment. Due to the nature of the test, the surface antigens of T. gondii were tested in the IFAT, while intracellular antigens were measured in the IHAT Siim (1968).

In a conference on the subject of prevention of mental retardation through control of infectious diseases, Frenkel (1966) stated that "more rapid diagnosis and more effective chemotherapy applied to infants with generalized toxoplasmosis should make it possible to reduce the proportion of those sustaining cerebral damage and blindness, resulting in mental deficiency".

Congenital Toxoplasmosis:

Wilson et al., (1980) studied the development of adverse sequelae in children born with congenital Toxoplasma infection, and concluded that those born with subclinical infections subsequently develop clinically evident disease. In the United States, one in one thousand live births suffer congenital infection, caused by toxoplasmosis, although this incidence is lower than that of congenital infection with cytomegalovirus, it is equal to or greater than that of congenital rubellar infection.

Alford, et al., (1969) did prospective studies to determine the clinical nature of the chronic intrauterine infections, including toxoplasmosis. During the course of these investigations, 10 infants with proven congenital toxoplasmosis were detected out of 7,500 screened. The incidence of intrauterine toxoplasmosis as defined by this approach in their population then averaged 1 case per 750 deliveries for the 2½ year interval studied. They further went on to show that of these 10, 1 with an overt congenital infection, involving hydrocephaly and infected CNS fluid, died after 4 months. Another with serious chorioretinitis and infected Central Nervous System (CNS) improved after treatment, but a follow-up showed that Intelligent

Quotient (IQ) was not well developed. The other 8 responded well with treatment of Spiramycin and Pyrimethamine.

Kimball, et al., (1971) in their study on congenital toxoplasmosis showed that the incidence is simply an index of the rate of acquisition of the infection by the pregnant mothers. There was no evidence that women of different ages or geographic areas vary in the rate at which they transmit the infection to their offspring. The age group showing the highest rate of antibody acquisition differed in all the studies mentioned above. According to Broadbent, et al.,(1981), the lowest incidence of congenital toxoplasmosis would be expected in children born to women aged 26 - 30 years (40% of the study population) and the highest incidence in those born to women aged 36 - 40 (67%). Ruoss and Bourne (1972) found their highest sero-conversion rate in the 20 - 25 years age group and a stable low rate in the two groups 30 - 35 and 36 - 40 years. In this latter case, the pregnant population that was studied was much younger than in the former case.

Desmonts and Couvreur (1974) dealt with the out-come of 180 pregnancies, (abortions excluded) complicated by primary maternal toxoplasmosis. Definite evidence of congenital infection was obtained in 59 cases and

possible evidence in 11 cases. Thus the risk of fetal infection was about 40%. The ratio of subclinical to clinical infection of the child was 2:1.

Experimental Toxoplasmosis:

Cowen and Wolf (1950) experimented on congenital Toxoplasmosis, using mice model, and attempted using the vagina as a portal of entry of the parasite. The result of their work shows the incidence and general characteristics of such an infection in the female and found out that there was no significant difference in the effectiveness of the 3 strains of Toxoplasma employed. The incidence of toxoplasmosis in pregnant animals (82%) was approximately 3 times, and the incidence in 'mated' animals without pregnancy (46%) was almost twice the rate of infection in virgin mice(27%).

Cooney, et al.,(1958) had studied the complement Fixation Test with peritoneal exudate antigen, and concluded that antigen prepared from the above source has gained disfavour for use in complement fixation tests, because they are more anti-complementary than antigens prepared from chorioallantoic membranes from infected chick embryos, and because satisfactory control antigens are not available. The disadvantages include the fact that the lower reactivity of peritoneal

Exudate (PE) antigen as compared to Chorioallantoic Membrane (CAM) antigen can be attributed to a later rise in titer, and an earlier disappearance of reactivity to PE antigen; as this has the practical advantage of permitting a rise in titer to be shown more frequently when PE antigen is used.

Chordi, et al., (1964) studied the specificity of the indirect haemagglutination test for Toxoplasmosis, and the differences in test reactivities were determined by testing 1020 sera by the methylene Blue Dye (MBD) test, and the Indirect Haemagglutination Test (IHA). The results obtained show that the agreement between the two tests was increased from 80% to 93% correlation, with the utilisation of the haemagglutinations inhibition reaction to separate antigens or the one-step reaction to eliminate non-specific reactions. They also found out that the presence of soluble antigens, the production of precipitates by heparin and clotting of fibrin, and the quality of accessory factors that modify or interfere with the MBD test are factors that do not affect the IHA test.

Huldt (1967) carried out a study on the host-parasite interaction in reticulo-endothelial and lymphatic tissues of sero-positive animals, in order to elucidate some features of the complex defence

mechanism in toxoplasmosis. Some of the main findings show that considerable proliferation of monocytes both in the spleen and the peripheral blood was demonstrated in all experimental groups. Likewise, a significant increase in large lymphocytes in imprints from lymph nodes and in lymph was demonstrated in all groups.

Pande, et al., (1961) did a comprehensive investigation on the immunochemistry of this protozoan species, and isolated and studied a polysaccharide fraction, with special reference to its immunobiological properties. The RH strain of T. gondii was used and the chemical analysis showed some unusual features, as the nitrogen content varied from 0.8% to 1.2%, and phosphorus from 0.2 - 0.4%. Studies on its topographic distribution on intact cells of T. gondii indicated that it is at least a partial surface antigen.

Huldt, et al., (1973) also studied the effect of Toxoplasma infection in mice, during the neonatal period, and in comparison with matched controls, growth was significantly retarded in infected animals. Thymus-weights were also markedly reduced, in relation to body weights. They also reported that parasites were never found in thymic lymphocytes in sections or imprints. In this study, Toxoplasma of low virulence was used, and each animal was infected with

about 3 - 5 cysts within 24 hours, after birth. In mice infected as adults, a marked effect on thymus weight and immune responses were observed. At 3 weeks after infection, a significant reduction in thymus was recorded. They finally concluded that Toxoplasma infection affects both the anatomy and function of the thymus. The mechanism of the effect is obscure, however, it seems that adrenal activity might be a contributing factor. This was later proved wrong, when experiments of adrenalectomy did not eliminate the effect.

Immunosuppression in Toxoplasma infection has been studied by different authors, including Buxton et al., (1980), who studied the effect in mice infected with louping-ill virus. Buxton and his associates used the MT strain of Toxoplasma, (an avirulent cyst-producing strain), and inoculation was intraperitoneal. The result of their study showed that the onset of viraemia was delayed in mice that received virus 7 or 14 days later, but thereafter more dually infected mice were viraemic than control mice. They however, went on to show that mice which received virus 7 days after T. gondii had a cumulative mortality of 42.5% compared with 37.5% in mice given virus alone. They therefore concluded that T. gondii is capable of producing 2 independent mechanisms, and proposed that one might possibly be mediated though the

stimulation of interferon production by the Toxoplasma.

In another study in Scotland, on the perinatal aspect of the disease, Williams (1977) collected hospital data of a prospective survey of antenatal women attending the Royal Maternity Hospital in Glasgow, and used an automated complement fixation technique (CF), as well as a direct Haemagglutination test (HA) for comparative purposes, on the survey. The comparative results on approximately 9,500 sera, involving 4160 women show that 53% of the 65 sera were detected by both the CF test and the HA test. 9 were missed by the CF test, and 3 by the HA test.

The most frequently used serological tests described for the detection of antibodies to T. gondii include the Indirect Immunofluorescence (IIF) (Kelen et al, (1962), Passive Haemagglutination (Jacobs and Lunde, 1957), and the dye test (Sabin and Feldman 1948). Recently, some other workers have deviced and described new tests for the detection and measurement of Toxoplasma antibodies. They include Voller et al., (1976), who deviced a microplate enzyme labelled anti-globulin followed by assay of the enzyme reaction with its substrate. The test is easy to carry out on a large scale and there is a positive correlation between the results and dye test and haemagglutination test titre.

Another new method recently deviced is the micro-titre Radio-immunoassay (RIA) for Toxoplasma gondii antibody deviced by Finlayson (1980). The RIA described was developed in an attempt to find a test which was specific for one Immunoglobulin (Ig) class of antibody and which could be used to handle large numbers of samples more readily than those mentioned. In this case, the test sera are reacted with antigen sensitized wells and any attached antibody is shown by the addition of a radioisotope - labelled antiglobulin.

Also, Desmonts and Remington (1980) deviced a new method for increasing the sensitivity and specificity of the agglutination test, and a method for suppressing non-specific agglutination by the use of a buffer containing 2 - mercaptoethanol (2ME). Peculiar to the preparation of this antigen is the fact that the RH strain of Toxoplasma is cultivated along with mouse TG180 sarcoma cells in the peritoneal cavities of mice. This produces a yield that is approximately 10 times more than can be obtained when the conventional method of inoculating Toxoplasma alone is used.

In another study a stable haemagglutination antigen for detecting Toxoplasma antibodies, which will remain stable for at least 1 year at 50% has been described by Thorburn and Williams, (1972). In doing this, they used peritoneal exudate from cotton rats, as opposed

to mice (which are usually used) and the cells were sensitized with pyruvic aldehyde and suspended in Sorenson's buffered saline pH 6.0. The haemagglutination tests were performed in microtitre wells, and standardized against the W.H.O. reference serum. The Dye Tests were performed according to the method used in the Public-Health Laboratory Service (PHLS) Toxoplasma Reference Laboratory in Leeds. From this research, they concluded that the correlation of Haemagglutination and Dye Test results may rest to some extent on the relative concentrations of the Immunoglobulin class of Toxoplasma antibody present in the serum.

With all this done, there is still need to contribute to our knowledge of Toxoplasmosis in our environment, to fill up the loopholes created by the scattered areas of study, some of which have been mentioned in this text.

CHAPTER III

PREVALENCE OF TOXOPLASMA ANTIBODIES IN THE OPEN POPULATION

INTRODUCTION:

Toxoplasma gondii is being studied intensively because of its importance as a cause of human disease. Toxoplasmosis is apparently extremely common in man, and also in many domestic animals. As Jacobs *et al*, (1957) said, "there is a sea of *Toxoplasma* infection around us". However, toxoplasmosis is far less common. Most infections are inapparent, and the disease itself appears only under special circumstances, many of which are still unknown".

The prevalence of antibodies vary widely in man in different geographic locations. For example, according to Jacobs *et al* (1957), there is relatively less infection in California than in the East United States. In Brazil, Walls and Kagan (1967) found that the prevalence of antibodies among military recruits was high, in low plain areas and low in the mountainous areas.

These statements were later confirmed by Feldman, (1968), and Zardi *et al*.(1980), who have indicated that toxoplasmosis is affected by climate.

The role of toxoplasmosis as a world-wide zoonosis has been established, but its epidemiology on a world-

wide basis has not yet been elucidated.

Several attempts to estimate the rate of acquisition of Toxoplasma antibodies by humans have been reported (Feldman and Miller, 1956), but additional studies of acquisition rates in different areas will help to clarify some of the questions concerning the mode of infection.

Seroepidemiologic screening surveys have shown that seropositivity ranged from 2 - 93% world-wide, depending upon the population surveyed. In Nigeria, the overall seropositivity rates have not yet been determined, as the few investigations carried out have been restricted to a segment of the country.

The first report in Nigeria on Toxoplasma antibodies was on the inhabitants of the Niger Delta region by Ludlam (1965) who reported 64% of the 62 cases he studied as positive.

The prevalence of Toxoplasma antibodies in healthy blood donors was found by Ogunba and Thomas (1979) to be 20.6% in Ibadan area. They used the Latex slide Agglutination Method and the Indirect Fluorescent Antibody Tests. In patients with eye diseases, and in choriocarcinoma patients, the prevalence increased to 44.39% and 45.9% respectively. Olurin et al.,(1971) in another study of the disease in Ibadan showed a high prevalence of Toxoplasma antibodies in patients with chorioretinitis.

Despite the fact that various investigators have studied Toxoplasmosis in tropical Africa, including the Delta region and Ibadan areas of Nigeria, no report has been published on the severity of this infection in other parts of Nigeria including Lagos.

This survey reports on the prevalence of Toxoplasma antibodies in the Lagos metropolis in various age groups and this is correlated with susceptibility to infection. It also reports on the incidence of primary infection.

MATERIALS AND METHODS

Serum Specimen:

5 ml of venous blood was collected with sterile disposable needles and syringes from each of 775 health clinic patients, selected at random. The patients reported to some hospitals located in 4 major areas of Lagos City. These areas are Yaba (189 persons), Ikeja (197 persons), Surulere (225 persons) and Lagos Island (164 persons).

Patients were chosen from amongst those who reported for routine check-up, and were found fit. The blood sample was stored in the cold room at $-4^{\circ}C$ over-night, after which it was centrifuged at 6,000 r.p.m. for 30 minutes to complete separation of the serum. Serum was collected with sterile pasteur Pipettes and stored in bijou bottles at $-21^{\circ}C$. They were inactivated before use in a water bath at $56^{\circ}C$ for 30 minutes.

Serological Tests:

The Wellcome haemagglutination test kit was used in this study. It is often referred to as the Indirect or Passive Haemagglutination test for toxoplasmosis, based on the work of Jacobs and Lunde (1957). The procedure used in the test is that supplied by the manufacturers (see Appendix). Toxoplasma Haemagglutination Test

(Toxo-HAT) uses a suspension of formalin tanned, turkey erythrocytes coated with a sonicate of T. gondii and freeze-dried. This test is readily standardized against the WHO International Standard, as it contains a positive control and a negative control sera.

The tests were performed in polystyrene microtitre plates and an Oxford Micropipetting diluter which was equipped with disposable tips to eliminate mixing of the samples was used. Serial dilutions were made, and the test and control sera were added. The plates were then covered, and allowed to rest on the desk. The results were read after one hour and compared with the standard positive and negative control wells, which were repeated on every microtitre plate used, to eliminate errors that might be introduced from the environment. The microtitre plates were kept and cross-checked after 24 hours. The tests were repeated, and the results agreed with each other, and were similar.

K-Values (Infection Risk):

The K-value indicates the risk of non-immune (sero-negative) persons acquiring infection, and this is used as a measure for the incidence of primary infections.

The infection risk is derived from the prevalence of antibodies in successive age groups, from the equation (after Van der Veen and Polak 1980).

$$K = \left(\frac{\log_e Po - \log_e Pl}{t}\right) \times 100$$

where Po = the percentage of negative sera in the younger age group.

Pl = Percentage of negative sera in the older age group.

e = Base of natural logarithms.

t = Interval (years) between median ages of both age groups.

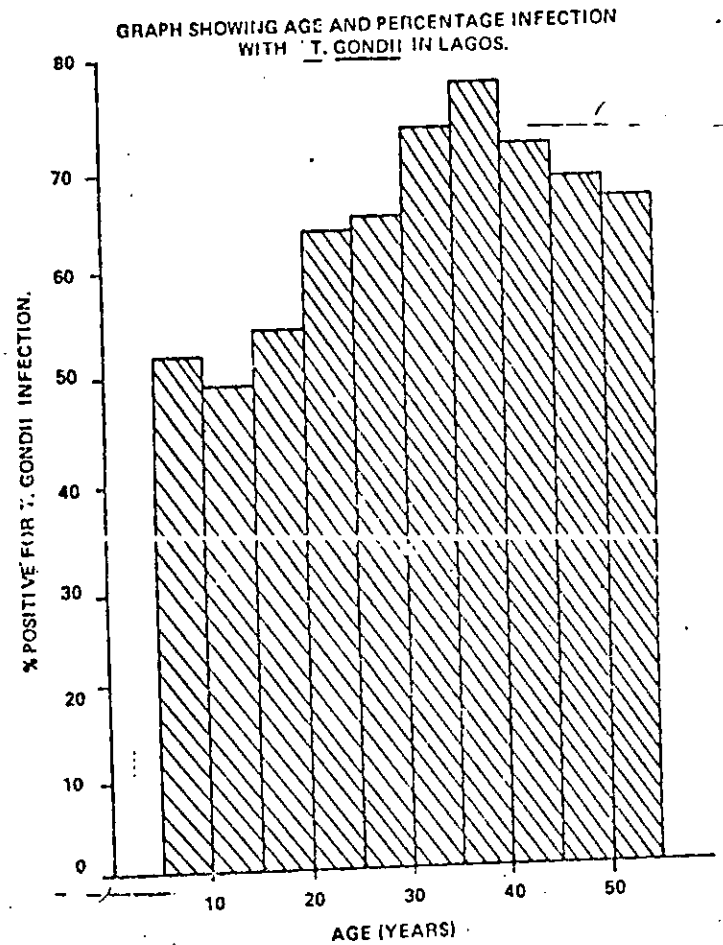
RESULTS

Of 775 specimen tested for Toxoplasma antibodies, 490 (63.2%) were positive, at titres ranging from 1:16 to 1:1024 dilutions. A majority of the positive cases were between the 1:16 and 1:64 serum dilution levels; which is referred to as the latent phase of infection, whereas, only one had a titer of 1:1024 dilution (acute phase). There were a few cases of 1:256 serum dilution levels, which had no complaints. The results in the different age groups are shown in Table I: From the table, it could be seen that specimens were collected from persons between the ages of 6 years and 55 years. The lowest number studied was (18) in the 51-55 years age group, whereas, the highest number studied was (157) in the 26-30 years age group. That notwithstanding, the highest percentage positive was seen in the 36-40 years age group, while the lowest percentage positive was recorded in the 11-15 years age group. (Figure I).

TABLE I: SHOWING THE PREVALENCE OF INFECTION WITH *T. GONDII* IN LAGOS IN DIFFERENT AGE GROUPS, USING THE INDIRECT HAEMAGGLUTINATION TEST.

Age (Range) (in Years)	No. in Group	Percentage of Study Population	No. Positive	Percentage Positive
6 - 10	55	7.096	28	50.9
11 - 15	87	11.225	42	48.2
16 - 20	91	11.741	49	53.8
21 - 25	140	18.06	90	64.2
26 - 30	157	20.258	102	64.9
31 - 35	88	11.354	65	73.8
36 - 40	51	6.580	40	78.4
41 - 45	50	6.451	36	72
46 - 50	38	4.903	26	68.4
51 - 55	18	2.322	12	66.6
TOTAL	775	100	490	63.2

FIGURE I:



KEY: Age here represents Age group at intervals, not specific ages.

Figure I shows the profile of the infection rate in the different age groups with the minimum of 48.2% in the 11-15 years age group, and the maximum of 78.4% in the 36-40 years age group. These results show that the infection rate increases with age, after an initial drop, at age 11-15 till it reached a peak, at 41-45 years and starts to drop again after 45 years of age. No age group within this study population is completely free of the antibody for Toxoplasma gondii.

Of the 4 different areas of study shown in Table II, the highest percentage was recovered in the Surulere area (69.8%), while the lowest was 56.8% in the Ikeja area. From the total number positive, we find that the greatest number is seen in the 26-30 years age group; whereas the lowest is seen in the 51-55 years age group. In Surulere area, the peak lies in the 26-30 years age group, whereas in Yaba, it falls in the 21-25 years age group. Both the Ikeja and Lagos Island patterns are similar to the Surulere pattern, thereby leaving the Yaba area out on its own.

FIGURE II:

Graph of k-- values against age(years) showing the relationship between annual conversion rate within each age group.

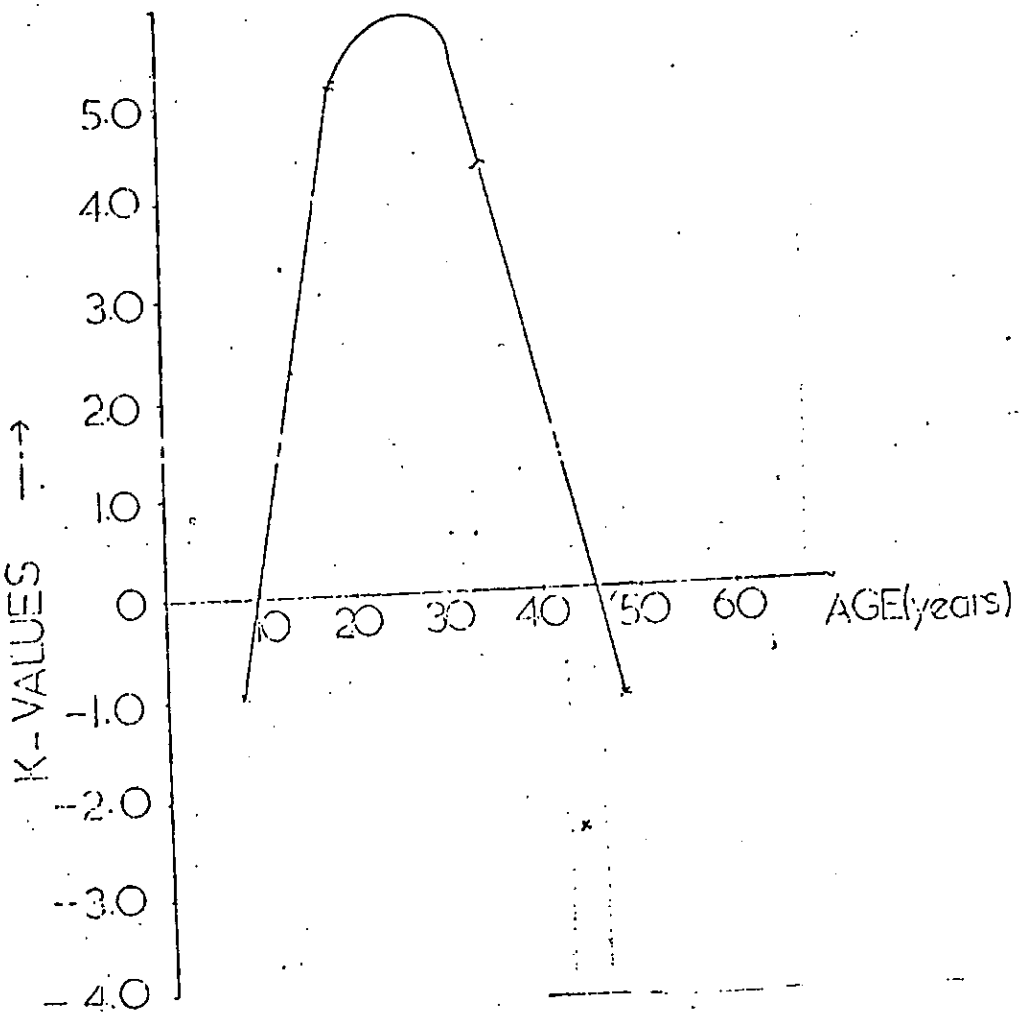


TABLE II : PREVALENCE IN THE DIFFERENT STUDY AREAS, SHOWN IN THE DIFFERENT AGE-GROUPS.

Age Groups (in Years)	Number Positive				
	Surulere	Yaba	Ikeja	Lagos Island	Total
6 - 10	10	7	5	6	28
11 - 15	16	12	5	9	42
16 - 20	18	12	9	10	49
21 - 25	24	30	16	20	90
26 - 30	31	19	29	23	102
31 - 35	22	17	16	10	65
36 - 40	12	7	15	6	40
41 - 45	12	7	8	9	36
46 - 50	7	6	8	5	26
51 - 55	5	2	1	4	12
Total Positive	157	119	112	102	490
Total in Group	225	189	197	164	775
Percentage Positive	69.77	62.96	56.85	62.195	63.2
Percentage of the Study Population	29.32	24.31	25.41	21.16	100

Table III and Figure II show the calculated infection risk (K-values), after Van Der Veen and Polak (1980). The relationship between the recorded K-values and the percentage negative sera in each pair of age groups, using their median as average age is directly proportional to each other. This is shown if the results are considered in pairs. Between the first pair of readings, it is seen that there is a rise in the percentage negative from the 6-10 years age group to the 11-15 years age group; from 49.1% to 51.72% over 5 years interval; and the K-values also show a rise from -1.04 to 2.28 respectively. This pattern holds throughout the whole Table, and depicts the number of sero-negative people left in each age group that can be sero-converted.

TABLE III: K-VALUES

SHOWING THE CALCULATED INFECTION RISK (K-VALUES)

(after Van Der Veen and M. F. Polak, 1980).

Age Group (in Years)	Percentage Negative Sera	Interval (Years) Between Median Ages	K-Values
6 - 10	2.62	5 years	-1.04
11 - 15	5.55	5 years	2.28
16 - 20	10.45	5 years	5.13
21 - 25	0.7	5 years	0.4
26 - 30	8.9	5 years	5.88
31 - 35	5.1	5 years	*4.35
36 - 40	-7.0	5 years	-5.75
41 - 45	-3.57	5 years	-2.4
46 - 50	-2.73	5 years	-1.060
51 - 55			

DISCUSSION:

The overall seropositivity which is 63.2% in Lagos metropolis is much higher than reports from other parts of Nigeria, with the exception of 95.4% prevalence reported by Olurin et al. (1971) in Ibadan.

The trend in Nigeria is seen to compare closely with that of other Countries as shown in the work by Feldman (1968) in Europe, Zardi et al.(1980) in Somalia, and Walton et al.(1966) in Central America and Guatemala. They showed that the percentage infection is higher in a more humid atmosphere. In Nigeria, the Niger Delta region where Ludlam (1965) recorded 64% of the 62 cases he studied as positive is obviously much more humid than Lagos State, and the latter much more humid, when compared to the Ibadan area where Ogunba and Thomas (1979) recorded 20.6% and 45.9% in patients with eye infections and choriocarcinoma patients.

The results of the Indirect Haemagglutination tests show that there is a definite source of infection and re-infection in our environment; hence the display of seropositivity in each age group. The method of this reinfection has not yet been established in this environment, and this leaves room for further research. The most important mode of infection which comes to mind, and which is yet to be investigated, but thought to be a risk of re-infectivity is through ingestion of oocysts from

fresh vegetables (in salads), such as lettuce and spinach, since the incidence of stray cats in Lagos is very high, and these roam around our farms and gardens at night. The established mode of infection so far reported is through the ingestion of under-cooked infected meat.

The low infectivity recorded in the 6-10 years age group might be due to loss of the antibodies acquired prenatally. The progressive increase in the prevalence of the antibody after the 6-10 years drop may therefore be as a consequence of acquired infection, which reaches a peak at the 36-40 years age group. The low infectivity recorded for the older group may either, be due to the higher standard of personal hygiene (since reinfection occurs orally), or it may be that the infections in those initially infected are going into latency, and thus, leading to a drop in the infection rate.

The pattern of the K-values calculated can be interpreted to mean that, from childhood up to the age of 30 years, there is a very high infection risk, which means that in each age group, the sero-negative people stand a high chance of sero-conversion. This is due to the fact that the percentage negative in the younger age group is usually always more than in the older age group (with few exceptions). After the age of 30 years, the rate of sero-conversion is less, and the number of sero-negative people left in each age group becomes

fewer, as they pass into an older age group, hence the negative K-values. This trend is particularly seen in the 36-40 to 41-45 years age group. The results obtained so far agree with the fact that age is a contributory factor to the subclinical infections of Toxoplasma in man. Other factors incluce virulence of the strain, as well as the susceptibility of the individual host (Smydth (ed.) 1981).

Considering suspected cases reported in Lagos University Teaching Hospital (LUTH) within the last 11 months between June 1984 to April 1985, a fairly high percentage of positive cases (53.42%) have been recorded. One case presented with a congenital bilateral cataract, while a number of others presented with eye complications. There was also a very significantly high titre of 1:1024 in a 36 years old female who reported to the hospital. Unfortunately, there was no follow up, even in such a delicate case. Most of the infants who had positive titres of between 1:64 and 1:256 presented with cases of congenital jaundice, for which they were being treated.

CHAPTER IV

PREVALENCE OF TOXOPLASMA ANTIBODIES IN PREGNANT WOMEN

INTRODUCTION:

Transplacental transmission from the mother to her offspring is perhaps the commonest cause of toxoplasmosis.

Congenital toxoplasmosis was first suspected by Wolf and Cowen (1937) in New York City, where they recognized the organism in brains of children dying of neonatal encephalitis and drew attention to placental transmission. Although many of the fundamental biological features of toxoplasmosis are today well known, the practical problem of prevention of its most serious consequence disabling congenital disease remains unsolved. The reasons vary in different communities, as Remington and Desmonts (1976) reported, but are concerned with detection of the chief mode of spread, assessment of incidence, delineation of vulnerable ante-natal women in latently infected population, and assessment of the risks of therapeutics.

Finding the solution to these problems have led to the study, of the prevalence of Toxoplasma antibodies in pregnant women in the Lagos metropolis, and to correlate the antibody levels with the pregnancy outcome.

Megafu and Ugbuegbulam (1981) suggested that some cases of recurrent abortions in Nigerian (Ibo) women

may be due to infection of toxoplasmosis. However, Southern (1972) and Kimball et al., (1971) in their different studies, in different countries, did not find any aetiological relationship between toxoplasmosis infection and recurrent abortion.

The difficulty in identifying infected neonates due to variation in its clinical manifestations have been demonstrated repeatedly by Eichenwald, et al., (1952), Feldman (1953) and Sabin (1959); and this led Eichenwald (1959) to state that "congenital toxoplasmosis is a disease with an extraordinarily wide range of manifestations, so wide in fact, that it must be considered in the differential diagnosis of nearly all types of obscure illnesses occurring during early infancy". And as was stressed by Couvreur (1971), even if a new born appears normal on clinical and routine ancillary examination, the risk of actual, though sub-clinical, toxoplasmosis is close to 50%.

MATERIALS AND METHODS

When first seen, the patients were in various stages of pregnancy. 2 - 5 ml of blood was collected from pregnant women most of them at their first visit to the antenatal clinic which falls mainly within the first 4 months of pregnancy. The blood was collected from the ante-cubital vein with sterile needles and stored in sterile - disposable bottles, which were brought into the laboratory in ice. Each patient from whom blood was collected was interviewed, and a questionnaire (see Appendix) was completed on her behalf with regards to age, state of pregnancy, former miscarriage or abortion, presence or contact with cats or dogs in their home environment, as well as previous farming experience. The expected date of delivery was noted, and reports of backaches and persistent headaches were also noted. The questionnaires were completed and the case notes of the patients studied to obtain past information on their history of child bearing, especially with special regards to those infections that correlate closely to toxoplasmosis. The blood samples were kept in the cold room at $4^{o}C$ overnight, and centrifuged the following day at a speed of 6,000 r.p.m. The clear serum was removed with a sterile Pasteur pipette and inactivated in a water bath for 30 minutes at $56^{o}C$. Inactivation was to

destroy complement, where it was present. The inactivated sera were then used to run the haemagglutination test using the method of Jacobs and Lunde (1957). The tests were done in duplicates. The second blood sample was collected from each of the patients just before delivery. Products of Conception (POC) were provided from only 34 women and these were used in attempts to isolate T. gondii. These POC included cord blood from patients during delivery as well as samples of placental tissues.

Cord Blood:

Cord blood specimen were collected from 34 patients in the labour ward after delivery and put into sterile bottles and kept in the fridge till they were brought into the laboratory for separation of the sera The sera were also inactivated before they were used to run the Indirect Haemagglutination Test as previously mentioned. All the sera were stored at -21°C till they were used.

Placental Tissue Specimen:

This was also collected from the same 34 patients during delivery. These tissue specimens were cut into two half-square inch pieces, and each was put in a separate bottle. One bottle contained a preservative and fixative, i.e. 10% formol saline; whereas the other

contained just antibiotic saline. The former was for histological sectioning, while the latter was for trituration and feeding to mice.

The placental tissue put in 10% formol saline was fixed and processed histologically and stained with Haematoxylin and Eosin. They were then mounted permanently on microscope slides with Canada Balsam. The full procedure is included in the appendix.

The placental tissues stored in antibiotic saline were placed in a petri dish, and cut into small pieces. Then it was teased out into very tiny bits using fine forceps and mixed with ground laboratory chow and fed to mice, without any other food. The mice were observed for changes in their fur for up to 20 days. Positivity was confirmed by death of the mouse, and presence of viable trophozoites in its peritoneum.

The data from the questionnaire was compiled and related to the results got from the haemagglutination titration. The percentage of the results were then calculated.

Titers are reported as reciprocals of the last dilution which was positive.

RESULTS

The haemagglutination titration performed on 452 venous blood samples gave 272 or 60.18% positive cases. The results obtained are shown on Table IV. 24 of the cord blood specimen collected were positive and gave 70.6%, while one of the placental tissue collected, processed histologically and stained with Haematoxylin and Eosin showed cysts or groups of parasites (bradyzoites) when examined microscopically (Plate I). The other half of the placental tissue which was triturated and fed to mice did not contain any infective stages of the parasite as the mice did not show any sign of positive Toxoplasma infection.

Table V and Figure III show the results of the 2-fold dilution titers which reveals that only one patient had a 1:1024 titer, in the 26-30 years age group. This 27 years old lady had a spontaneous abortion at 5 months pregnancy, and lost the foetus. It was not possible to follow up on her case, as she failed to show up at the health centre after she was discharged initially. From the Table, it is also possible to see that, on the whole, only 20 patients, that is 7.35% of all the positive cases, and 4.4% of all examined cases had a titer of 1:256 and above, thus, indicating active infection, (where there is a rise in titer).

TABLE IV: PREVALENCE OF TOXOPLASMA ANTIBODIES AND INFECTION RISK (K-Values) IN ANTENATAL PATIENTS

Age Group	Number in Group	Number Positive	Percentage Positive	Percentage Negative	K-Values
16 - 20	140	78	55.71	44.29	1.4
21 - 25	164	96	58.53	41.47	1.2
26 - 30	98	60	61.22	38.78	9.2
31 - 35	24	18	75.48	24.52	88.9
36 - 40	16	16	100	0	
40 +	6	0	-	-	
Unknown	4	4	100	0	
Total	452	272	60.176	39.824	3.570
			app. 60.18%	39.82	3.6

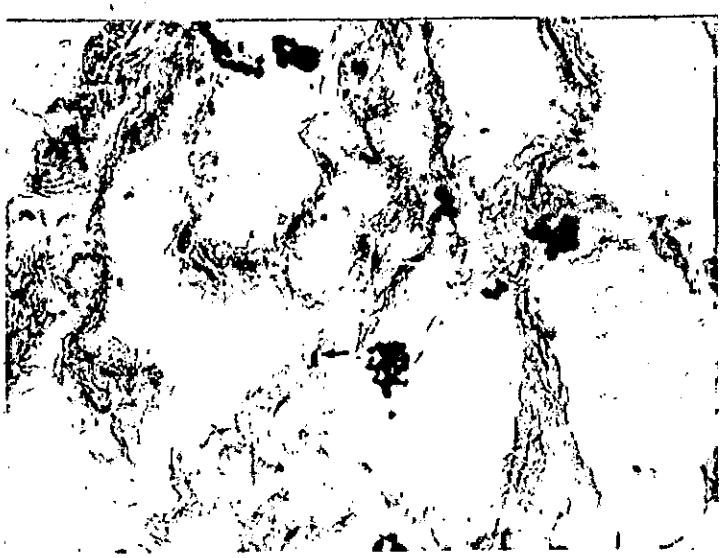


PLATE I : Placental Tissue showing Toxoplasma parasite from a pregnant woman with a 1:256 titre before and at birth x 400

TABLE V : SHOWING TITRE ATTAINED IN 2-FOLD DILUTION INDIRECT HAEMAGGLUTINATION TEST ON SERA OF SOME PREGNANT WOMEN IN LAGOS METROPOLIS

Age Group	Number Positive	Titre						
		1:16	1:32	1:64	1:128	1:256	1:512	1:1024
16 - 20	78	38	10	12	16	1	1	-
21 - 25	96	42	31	18	2	2	1	-
26 - 30	60	19	17	9	3	11	-	1
31 - 35	18	9	2	2	4	-	1	-
36 - 40	16	6	4	4	-	1	1	-
40 +	0	-	-	-	-	-	-	-
Unknown	4	3	-	-	1	-	-	-
Total	272	117	64	45	26	15	4	1

FIGURE III: Graph showing the degree of sero-positivity in different age groups.

Analysis of the questionnaires that were completed (Table VI) show that 64.71% of the positive haemagglutination cases have had frequent contact with cats and dogs in the recent past. Also, of the positive cases, 25% of the patients have had previous (up to 3 years back) farming experience. 16.9% had lost one or more children, while 11.03% have undergone spontaneous abortion before the present pregnancy. None of the offsprings of the seropositive mothers was born with any observable congenital effect before they were discharged from the hospital.

On calculating the K-values or infection risk amongst these women, it was found that the lowest of 1.2% was in the age group 21-25 to 26-30 years (Table IV) while the highest conversion rate was in the 31-35 to 36-40 years age group. Also, a significant drop of 0.2% was noticed from the first to the second age groups.

TABLE VI: SHOWING A SUMMARY OF THE QUESTIONNAIRES COMPLETED BY THE SUBJECTS IN THE EXPERIMENT

Subject Matter	Total Number Positive in Group	Percentage Positive	Percentage of the Study Population
Contact with cats/dogs	176	64.71	39
Previous/present farming experience	68	25	15
Infant Mortality rate	46	16.9	10.1
Abortion (Spontaneous)	15	11.03	3.3
Waist or Back-ache	118	43.38	26

DISCUSSION

Identifying those pregnant women who seroconvert during pregnancy, and treating them before their expectant date of delivery to prevent intrauterine infections is most appropriate in this environment, since congenital toxoplasmosis may lead to malformations in the unborn child, if the infection is not prevented.

One method of preventing congenital transfer of toxoplasmosis (by testing for infection in-utero) is screening of all antenatal patients. This procedure is highly recommended in our environment; as the number of malformed congenitally infected children that are seen around in the country, may be reduced.

A possible hypothesis may be found in the work of Fair (1958a) and Perkins (1961) who proposed that the majority of cases of adult retinochoroiditis due to T. gondii are congenital in origin. If this hypothesis is correct, T. gondii infection is transmitted to fetuses that show no clinical symptoms during infancy. These infected infants form the pool of individuals who may develop retinochoroditis with onset of reinfection in later life.

This hypothesis agrees with the results in this study as, despite the seropositive mothers and seropositive cord blood samples; none of the neonates were born with any observable congenital malformation.

This though, does not exclude the fact that the infection has been congenitally transferred.

Alford et al., (1969) found more asymptomatic than symptomatic congenital toxoplasmosis, thus demonstrating that such a relatively large pool of congenitally infected patients do indeed exist. This can be related to those examined expectant mothers who do not show any symptoms of the infection, even after been seroconverted during the period of their pregnancy and attaining a 1:256 titre at the period of delivery. In such cases, there is the likelihood that the infection would already have been transferred to the offspring, who is born without any clinical symptoms.

The incidence of Toxoplasma antibodies in antenatal patients in this study in Lagos is 60.18% and is higher than in London, where Broadbent, et al, (1981) recorded 23.3% positive cases.

In Paris, Desmonts, et al.,(1965) showed that 0.8% of persons acquire toxoplasmosis during pregnancy and that 40% of the patients transmit it to their offspring.

Table IV shows the age distribution and the occurrence of antibody positive titres in prenatal women studied in Lagos. The incidence of those with antibody increased with age from 16 - 40 years of age.

This followed the same trend with an apparent reduction after 40 years of age as in the study by Broadbent (1981). An explanation for the reduction after 40 years may be due to the fact that by that age, most women have given up child bearing, and thus, the number studied in the over 40 years age group is always small, when compared to the younger age groups. By this age, menopause begins to set in for several women, and fewer females are available for study.

Considering the annual conversion rate, there was a drop in the 21 - 25 years age group, which later increased at a very fast rate till it reached 88.9% in the 36 - 40 years age group. This shows that the number of seronegative people left in each age group are at the highest risk of infection as we go up the age ladder. and therefore, the risk of infection is greatest in the 36 - 40 years age group, as opposed to 1.2% in the 21 - 25 years age group.

In the work by Kimball et al, (1971), it was confirmed that 65% of the patients lacking the protection of Toxoplasma antibodies were at risk. They also suggested that habitual abortion due to chronic toxoplasmosis is uncommon.

In this environment, the result obtained from the test agrees with the work of Kimball et al., (1971) in

Europe as it is very obvious that most of the women with titres below 1:256, who neither seroconvert during the period of their pregnancy (i.e. over the study period) nor showed an increase in titre, have chronic Toxoplasma infection, and this, in real fact, did not lead to any form of abortion. They had normal babies at birth, and both mother and baby were discharged after a short while.

In this study it is shown that the presence of Toxoplasma antibody has no significant relationship with farming or gardening in our environment, as only about 25% of the seropositive women engage in these. Here seropositivity was associated only with presence of antibody in the cord blood at birth, and also ownership or close contact with cats and dogs.

In spite of what appeared to be a rapid acquisition of toxoplasmosis, during the obstetrics years, only 7 patients showed definite serologic evidence of having acquired toxoplasmosis during the study period, with a rise in titre from 1:16 to a maximum of 1: 256 in one of the patients.

The risk of toxoplasmosis in the child-bearing population in Paris is high, being 0.55% per month (Desmonts and Couvreur, 1968). Thus the chances of infection in this group are approximately 5% for a pregnancy of 9 months.

The average infection risk in women of child-bearing age in our environment was calculated to be 1.325% per annum. Despite that, no significant difference occurred either in frequency of total abnormal outcomes or of abortions in relation to antibody level.

CHAPTER V

PREVALENCE OF TOXOPLASMA ANTIBODIES AND OOCYSTS IN STRAY CATS IN DIFFERENT AREAS OF LAGOS

Resarch on transmission of Toxoplasmosis employing cats was initiated by Hutchinson et al (1971), and they provided conclusive evidence that T. gondii is in fact a coccidian undergoing schizogonic cycles and gametogony in the intestinal epithelial cells of the cat.

The trophozoite is a proliferative stage of Toxoplasma which seems to be capable of infecting, and multiplying within nucleated cells. Cells parasitized subsequently rupture and the trophozoites escape to continue their intracellular multiplication elsewhere.

According to Hutchinson et al (1971), early workers were forced to attempt to explain the transmission and the life cycle of Toxoplasma in terms of the two stages known to exist, which were the pseudocysts and the tissue cysts. However, for reasons reviewed by Hutchinson et al. (1969), it became apparent that unknown stages of Toxoplasma existed. These were the faecal cysts or oocysts found in cats faeces as later confirmed by observations of Sheffield and Melton (1970), as well as a host of other researchers.

Other workers who have also implicated cats as definitive hosts and transmitters of this disease include Hutchinson et al, (1971), Frenkel, et al.,(1970) as well as Miller, et al.,(1972).

On the other hand, some workers who have tried to associate Toxoplasma infection with exposure to household cats have been quite inconclusive. These workers include McCulloch, et al., (1963), who not only found positive Toxoplasma tests in a statistical association with marked contact with cats, but also found same with contact with sheep, swine, horses and cattle. Price (1969) in a study with an urban community found no such association with cats alone. Vaage and Midfuedt (1977) in a skin test survey of Norwegian naval recruits found no differences in the frequency of positive tests between those who had cats in their homes and those who did not.

This conflicting epidemiological evidence suggests that if cats are the definitive hosts, some factors must strongly influence the transmission of this disease to humans. This conflicting evidence prompted this study to see if transmission of T. gondii to man in our environment is associated with the presence of cats in our homes or surroundings or otherwise.

Materials and Methods:-

(a) Collection of Cats:

Stray cats were randomly collected from the 4 different study areas of Lagos metropolis and these were brought into the Animal House and caged individually. These areas are Surulere, Yaba, Ikeja and Lagos Island. The faeces of all cats were examined prior to use in any of the experiments. The faecal samples were collected and analysed using the Formol-Ether Concentration method after Ritchie (1948) to detect the presence of any oocyst in the specimens.

(b) Isolation and Separation of Oocysts:

Cat faeces was collected in stool cartons and brought into the laboratory. About 3 gms of the faeces were mixed with 10cc of physiological saline (0.85% NaCl). The suspension was then strained through 2 layers of surgical gauze, and then poured into a 10cc centrifuge tube. The suspension was centrifuged at 1800 revolutions per minute (rpm) for 3 minutes, and the supernatant poured off. Physiological saline was then added to the sediment at the bottom of the

centrifuge tube up to 10cc, and this was properly stirred. The suspension was then centrifuged, the supernate decanted, and the particulate matter resuspended repeatedly until the supernate was clear. After an additional decantation, the sediment was mixed with 7cc of 10% formalin. This was to reduce the distortion of protozoan cysts, and for fixation. The mixture was allowed to stand for 5 minutes after which 3cc of ether was added and the suspension was vigorously shaken for about 2 minutes. The mixture was centrifuged again at 1800 r.p.m. for 3 minutes, and the supernatant was poured off. A thin film of the sediment was then placed on a slide with a drop of iodine stain. The preparation was mounted with a cover slip and examined under the microscope. The suspected cysts were measured for confirmation.

(c) Bleeding of the Cats for Serology:

The cats were weighed and their weights were recorded. They were fed for 7 days before they were bled and checked for Toxoplasma seropositivity. Blood was collected from the thigh vein into sterile bottles; after the cats were anaesthesized by

intraperitoneal injection of pentobarbitone sodium (60mg per ml) using sterile needles. The amount of anaesthesia used depended on the weight of each cat. About 0.5 ml was given per kilogram body weight. The cats were then left for about 30 minutes for the anaesthesia to take effect, before the bleeding was done. The hair covering the inner region of the thigh was scrapped off, and a tourniquet was tied around the upper region of the knee. A sterile 21G needle and syringe was used to draw blood from the femoral vein after the area had been swabbed with methylated spirit. Only 0.5 ml to 1 ml of blood was removed from each cat. The cats were then returned to their labelled cages after they had regained consciousness.---

The blood was left to clot, and the serum was separated and inactivated. The serum was then used to run the Indirect Haemagglutination Test, after Jacobs and Lunde (1957). Titres of 1:16 and above were considered as positive.

(d) Infection of Cats:

The cats found to be seronegative to Toxoplasma serology test were fed acutely-ill Toxoplasma-infected mice, after being starved for a period of

24 hours. (These are mice infected intraperitoneally 4-5 days previous with 0.2 ml of 1:19 dilution of pure peritoneal fluid).

These cats were then kept in their labelled cages in the animal house, and they were checked upon daily. They were fed adequate food and water, and beginning from the 9th day, after infection; their faeces were collected and examined for the presence of oocysts.

The Formalin-Ether Sedimentation technique (previously mentioned) was used, and the deposit was stored in sterile bijou bottles. Pasteur pipettes were used to put a drop of the sediment on slides for observation microscopically. All preparations were examined for Toxoplasma oocysts. Photomicrographs were taken of the parasites. Where cysts suspected to be those of Toxoplasma were observed, identification was done using the outline after Siim, (1968) and Levine (1973). The cysts observed were measured, using a calibrated micrometer eye piece.

(e) Infection of Mice:

After 14 days storage at room temperature, aliquots from each of the separation were washed several times and

fed to a group of 4 clean mice in each cage. 4 mice were set aside as controls. This procedure was followed for all faeces separated. In doing this, aliquots from the remaining deposit were washed several times with sterile physiological saline, and centrifuged after each wash. The supernatant was then discarded, while more saline was added. After 3 thorough washings, the deposit was mixed with ground laboratory chow in petri dishes and was fed to young mice (4-6 weeks old), which had been starved overnight. It was ensured that every bit of the food was eaten off the petri dish before more food was given to the mice. The control group were fed only normal laboratory chow mixed with sterile saline. All the other cages were used for each of the cat faeces that was treated.

Positivity of the infection was determined by death of the mice, and confirmation of infection was by direct microscopic identification of Toxoplasma trophozoites from the peritoneal fluid of the mice. Positive or negative results were then related to the presence or absence of oocysts in the cat. The remaining deposit was mixed with 2cc formol-saline and stored in bijou bottles.

(f) Killing of Cats:

After the 20th day on which the cats were infected, they were killed with chloroform, and immediately their intestine, as well as their liver, lungs, spleen, brain and heart were removed. They were fixed in formol saline for histological processing. Portions approximately 1 cm cube were removed and fixed for a minimum of 24 hours, before the processing continued. The sections were stained with Haematoxylin and Eosin and mounted on slides using Canada Balsam. These sections were examined microscopically, and the areas showing the parasites in tissue were photographed.

The method of processing the tissue is fully stated in the appendix.

RESULTS:

The cats collected and used in this study were from the same areas from which human blood samples were collected for the previous serological tests. 23 cats were studied on the whole. This number excludes those cats that died **unexpectedly** from one form of infection or the other, before the end of the experimentation period.

On initial examination of their faeces, using the formol-ether sedimentation method; no oocysts were observed;

thus indicating the absence of active Toxoplasma infection in the cats.

The average weight of the cats was 1.24kg. The smallest weighed 0.84 kg., while the largest weighed 2.01 kg. It was not possible to tell the ages of the cats, as most of them were stray cats.

On bleeding and performing the Indirect Haemagglutination test, after Jacobs and Lunde (1957), 4 cats (numbers 9, 15, 19 and 23) showed seropositivity. (See Table VII). Two of these 4 cats (Numbers 15 and 19) were from the Surulere area while one was from Yaba, (No. 9) and the fourth (No. 23) was from the Lagos Island area. None of the 6 cats from Ikeja area showed any seropositivity. The exact titres of the result of these cats blood ranged from 1:16 to 1:128.

After infection, 9 of the 23 cats died before the 9th day, and faecal samples were collected from the remaining 14 cats. Cat (number 4) and Cat (number 16) showed cysts that were taken to be oocysts of Toxoplasma, following the characterization after Siim (1968) and Levine (1973).

The cysts had the characteristic nature of Toxoplasma oocysts, each containing 2 sporocyst, and each sporocyst, containing 4 sporozoites. These were measured and were 12μ by 10.6μ in diameter.

TABLE VII: SHOWING DATA ON CATS COLLECTED FROM THE 4 DIFFERENT STUDY AREAS

Cat (Number)	Weight (kg)	Reciprocal of Titre ToxoIHA sero-positivity	Result of Toxo-Infection	Area of Collection
1.	0.84	Negative	-	Ikeja
2.	1.2	"	Died	
3.	1.93	"	-	(6)
4.	1.11	"	Positive	
5.	0.92	"	-	
6.	1.16	"	Died	
7.	1.7	Negative		Yaba
8.	1.42	"	Died	
9.	0.88	Positive 1:16	-	(5)
10.	0.91	Negative	-	
11.	1.22	"	Died	
12.	1.47	Negative	Died	Surulere
13.	0.99	"		
14.	1.33	"	Died	
15.	0.91	Positive 1:128	-	(8)
16.	0.84	Negative	Positive	
17.	1.27	"	Died	
18.	1.61	"	Died	
19.	2.01	Positive 1:32	-	
20.	1.7	Negative	-	Lagos-Island (4)
21.	1.39	"	Died	
22.	0.98	"	-	
23.	0.87	Positive 1:32	-	
Total	Average 1.246	4	2	23

After the cats were killed, the histologically processed tissue showed cysts in the intestinal epithelial cell layers, as well as in the liver. These are shown in Plates II and III.

Mice infected with washed cats faecal sediments showed signs of positive Toxoplasma infection in 2 cages. These cages were those infected with sediments from cats number 4 and number 16.

PLATE II: Section through infected cat intestinal tissue showing *Toxoplasma* parasites.

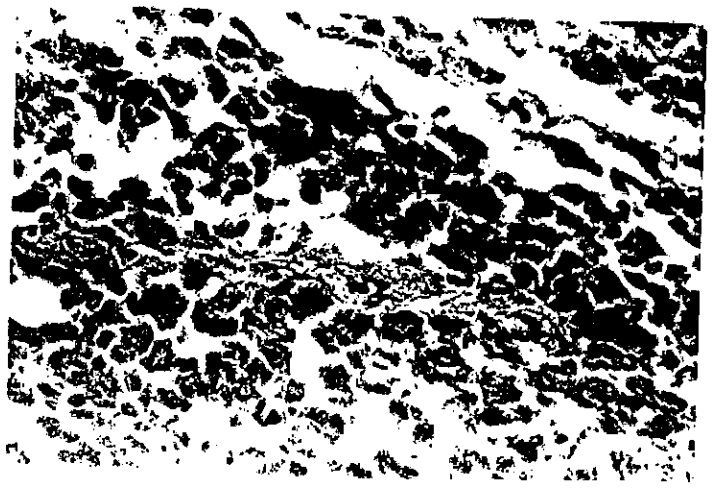


Plate III: Section through infected cat liver showing *Toxoplasma gondii* parasites in the tissues.

DISCUSSION

The association of cats and toxoplasmosis, with the acquisition of positive antibody **litre**, as employed by Hutchinson et al(1971)is not directly related to all cases of Toxoplasma infection in the Lagos environment.

Of the 23 cats studied from different areas of Lagos, only 4 had seroconverted, when examined. This gives a percentage of 17.391%(approximately 17.4%.) This is relatively low, considering the high percent of persons (over 60%) with seropositive antibody titre in the Lagos metropolis, as previously discussed. Of the 4 cats that were brought into the laboratory with seropositive **titre**, one each was from Yaba and Lagos Island area, while 2 were from the Surulere area. Cat number 15, which gave the highest titre of 1:128 was from the Surulere area, while the **cat, number 9 gave the lowest** titre of 1:16. These cats are stray cats, and are therefore expected to carry a very high infection risk, but the result of this experiment has proved this otherwise, with regards to Toxoplasmosis. The sexes of the cats were not taken into consideration; so, both male and female cats were used in the experiment.

The infected tissues which were prepared histologically and examined microscopically showed some stages of development of the coccidian parasite. These slides were sections of the intestine of the cat as well as

sections from its liver. A photomicrograph of the latter is included in the text as Plate III. From the section, through the intestine, the infection appears to be concentrated in the cells of the tips of the villi.

The epidemiological data obtained from this experiment on cats, when compared to that obtained on the study of the open population, does not show any positive correlation between cats and man. The correlation of these results are uncertain. In the light of this conflicting data, several questions must be asked, concerning the role of cats in this disease, or our present methods of looking at this association. The extreme possibilities may be that cats are not the definitive host, in this environment. Other animals may harbour the oocyst. On the other hand, a much more intensive survey of house to house cats as well as occupants may be necessary to give a definite epidemiological pattern.

So far, it is concluded that the result in this area of study (Lagos), agrees with that of McCulloch et al., (1963). Also Price (1969) found no such direct association with cats alone, as reported in literature.

This therefore opens a new area of study with regards to the transmission of Toxoplasma amongst humans in our environment.

Another point to note lies in the fact that the ages of the cats were not known. It might have been possible to get a positive correlation using younger cats, which are more likely to pass oocysts in their stool, before the infection becomes latent.

CHAPTER VI

EFFECT OF TOXOPLASMA INFECTION ON PREGNANCY, USING MICE MODELS.

Introduction:

Since the original description of congenital toxoplasmosis by Wolf et al., (1939), chorioretinitis, hydrocephaly, and intracranial calcification have been accepted among clinicians as the "classical triad" of symptoms of intrauterine toxoplasmosis (Eichenwald, 1959). Unless these findings appear in neonates, congenital toxoplasmosis is usually not considered as a cause of disease in spite of the fact that variability in clinical manifestations have been demonstrated repeatedly (Feldman 1953 , Hedenstrom 1957 , and Miller et al 1967).

Toxoplasma infections in pregnant women have been said to initiate premature labour, even though disease in the infected fetus is minimal. Whether this process can occur earlier in pregnancy is an important consideration, regarding the ability of Toxoplasma to produce abortion in women who appear to be normal (Alford, et al., 1973).

Beverley (1969) reported that infections with virulent strains, early in pregnancy (but after 28 days) will cause fetal death and abortion or miscarriage. Maternal infections in the first month are unlikely to be transmitted across the zone of intense proteolytic digestion, which is taking place in the endometrium. If infection occurs later, the foetus can be still-born, or born severely deformed.

Toxoplasma gondii has been isolated from the products of conception, and women have aborted, following conversion from seronegativity to seropositivity during pregnancy (Beverley (1969). However, the controversy that reinfection of the fetus results in subsequent pregnancies, and subsequent abortions, led to the performance of this experiment to verify if the situation is as reported in man; using mice models.

Jones et al(1966) showed that acute infection during pregnancy will frequently lead to spontaneous abortion, whereas Kimball et al., (1971) and Southern (1972) did not find any aetiological relationship.

In Nigeria, Megafu and Ugbuegbulam (1981) did some work in the East, and concluded that the causes of habitual abortions are multifactorial, and include factors such as chromosome abnormalities, hormone (progesterone) deficiency incompetent internal cervix os, etc. In their study, they found that patients with recurrent abortions have a higher titer of Toxoplasma antibodies, than in the general population.

Materials and Methods:

The gestation period for the randomly bred Swiss White mice (albino mice) available in the animal house, and which was used for this experiment is 21 days. One hundred 4 weeks old mice (50 male and 50 female) weighing between 20 and 25 gms were allowed to mate overnight, and pregnancy was checked by the appearance of sperms on the vagina, using a light microscope to examine a smear. The female mice were then picked out, and grouped in tens.

The pregnant mice at different thirds of pregnancy were infected under aseptic conditions with 0.1 ml (diluted 1:1000) peritoneal exudate, containing an estimated amount of Toxoplasma trophozoites, and these were observed throughout the gestational period. The mice were kept in separate cages, and a fourth cage of uninfected pregnant mice, was kept as control. They were all given enough food and water all through the period of study. Each mouse was infected with 7-8 parasites in 0.1ml of fluid.

Mice infected 3-5 days previous were sacrificed, and the peritoneal fluid was collected and diluted with sterile saline in a 1:100 ratio. This was adequate for the counts, using a Neuber-Levy counting chamber. The counts were repeated 3 times over, to avoid any mistakes, and the average of the 3 readings was taken as final. This was further diluted to give a 1:1000 dilution value, so that each 0.1 ml contained approximately 4-6 parasites.

Each group of 10 mice was divided into two groups of 5 each. For the first third period of pregnancy, 5 mice were infected intraperitoneally on day 1, and another 5 on day 4. For the second third period day 8 and day 11 were chosen respectively; whereas, day 15 and day 18 were chosen for the third period. Each mouse in each of the 5 groups was marked, and numbered 1 - 5, for identification purposes. The mice were observed daily, and the fetus and/or suckling mice were collected and checked as soon as they were expelled dead or alive by the female mouse. The period and date they were expelled were recorded, and their ages were determined. They were then opened up, and their peritoneum flushed out onto a petri dish with a few drops of sterile saline (depending on the size, about 0.5 mls to 1 ml of sterile saline was used). 1 to 2 drops of the peritoneal washing was put onto a clean slide and this was observed under the compound microscope, for the presence of Toxoplasma trophozoites. The fluid in the petri dish was also observed using a dissecting microscope. Also, some of the female adult mice that remained alive were monitored for a further 5 days before they were sacrificed and checked for infection. Apart from their peritoneal washings; their spleen, liver, lungs, intestine, as well as their brains were removed for histological processing, and stained with Haematoxylin

and Eosin. These organs were removed, and immediately dropped into a sterile bottle containing 10% formol saline, for preservation as well as fixation.

After the 27th day, the female mice left alive were put back into cages with male mice for mating, and these were then observed further. All inoculations with parasites were done intraperitoneally.

Results:

The day of confirmation of mating is hereby referred to as Day 0, in this experiment. Each mouse was inoculated with 4-6 parasites only in 0.1 ml of saline. Of the 50 female mice allowed to mate, only 11 did not show any evidence of mating, as the smear made from their vagina did not show presence of any sperms, and no enlargement of the abdomen was observed later. Of the other 39 left, 3 died immediately after inoculation, thus leaving 36 used in the experiment.

Of the 5 mice infected on day 1, only the 4th did not pick up the infection. 3 others expelled their incompletely formed fetus between day 9 and day 17, and lived on, while mouse No. 2 died on the 10th day, after infection. Peritoneal washings of the expelled fetus, as well as that of mouse 2 (removed after it was opened up), produced infection in clean mice into which it was inoculated (see Table VIII).

TABLE VIII:

THE EFFECT OF TOXOPLASMA INFECTION ON PREGNANCY, USING MICE MODELS.

Cage Number	Day of Infection (after mating)	Mice Number				
		1	2	3	4	5
A	1	FE17	D 10	FE 9	N I	FE 12
B	4	FE14	FE10	FED11	FED15	N I
C	8	FE13 D 17	FED 17	D 15	D15	D 15
D	11	N I	FED 19	FED 20	FED 20	N I
E	15	FE D16	FE20 FN I	FE21 D 27	FN I ND	FN I ND
F	18	ND FN I	ND FN I	ND FN I	ND D29	ND D22

KEY TO TABLE:

FE = Fetus Expelled (aborted)
D = Died
ND = Normal Delivery
FNI = Fetus not infected
NI = Adult not infected.

Of those infected on the 4th day after mating, mouse No. 5 did not show any sign of the infection, and had 4 suckling mice at the end of the gestation period. When 2 of these were opened up, washings from their peritoneal cavities inoculated into clean mice did not cause any infection in these mice, thereby proving the infection as negative. On the other hand, mice 1 and 2 expelled incompletely formed fetus on days 14 and 10 respectively, while mice 3 and 4 aborted and died on day 11 and 15, after expelling 4 and 2 fetuses respectively. Peritoneal washings from these all caused infection in clean mice when inoculated into them.

Of those infected on day 8, Mouse 1 expelled its fetus on day 13 and died on day 17, whereas Mouse 2 aborted on day 17, and died on the same day. Mouse 3 died on day 15, without abortion. Mice 4 and 5 also died on day 15 without any abortion, i.e. 7 days after infection. Peritoneal washings of parent mice 3, 4 and 5 produced infection in clean mice, when inoculated.

On day 11, which is the last within the 2nd third period Mice 1 and 5 did not pick up the infection, whereas Mice 2, 3 and 4 expelled their fetus, and died on days 19 and 20 respectively. Their aborted fetuses however, produced infection in clean mice, after peritoneal washings were inoculated into same.

On the 15th day after pregnancy, i.e. in the last third of the gestational period, only mouse number 1 expelled its fetus and died the following day, after infection. The fetus lived for 6 hours before it died. When examined, the fetus did not show any sign of infection. All the others in the group had normal delivery on days 20 to 21, with their fetus not infected, and not producing infection in other clean mice. Mouse number 3 died later on day 27. All the mice infected on Day 18 did not pick up the infection. Mice 1, 2, and 3 had fetus that were not infected, while Mice 4 and 5 died on days 29 and 22 respectively, after normal delivery.

All the mice infected in the 7th cage, i.e. the control group, had their suckling mice at 20 and 21 days, and had between 4 and 6 suckling mice each. None of these produced infection when their peritoneal washings were inoculated into clean mice.

Discussion:

Of the 50 female mice chosen for use in the experiment, only 36 became pregnant. They were then divided into their respective groups and the 7th group was the control group of uninfected pregnant mice. All the 6 mice in the control group gave birth to normal suckling mice on the 20th and 21st days of gestation. Most of the mice that died in the experimental group did not show prior signs of infection characteristic of Toxoplasma infection, such as sluggishness and ruffled coats. No case of convulsion was noticed either; not even in cases, where peritoneal washings gave rise to infection when inoculated into clean laboratory mice. Death occurred in adult mice beginning from day 11 to day 29, and not earlier. The first mouse that died was Mouse Number 3 in cage B. This died 7 days after infection, and peritoneal washings were infective to clean mice. This shows that the infection is very easily and quickly transferred from mother to fetus if primary infection occurs within the latter half of the first third period of pregnancy.

All the animals that died, even without exhibiting signs of disease, within the first and second third period of pregnancy were found to be infected. This, though, was not the case in cages E and F, which contained mice infected within the last third period of pregnancy.

None of these animals transferred the infection to their offspring, not even in cases where the adult mice died of Toxoplasma infection.

The occurrence of external signs did not in itself afford a reliable indication of the true incidence of infection in this experiment, in-as-much as post-mortem examination often revealed the presence of Toxoplasma in the peritoneal washings of apparently normal animals.

On taking a look at the table of results (TableVIII). it is seen that of the 30 experimental mice infected, 17 or 56.67% expelled their fetus at one stage of their development or another, whereas only 7(23.3%) of the lot had normal delivery. About 20% also reproduced fetuses that were not infected, as their peritoneal washings did not cause infection in clean mice, into which they were inoculated. All 6 mice used as control had 4-6 normal suckling mice each. When they were allowed to mate a second time, they had normal sucklings on the 21st day, as previously recorded. Further infection was not shown to have taken place.

Considering the results in the different thirds of pregnancy, it is seen that within the first third period 70% of all the mice aborted between days 9 and 17 after infection; whereas within the second third

only 50% of the mice expelled their fetus, and 3 of these were almost at term on days 19 and 20. The above result contradicts the work of Desmonts and Couvreur (1974) in France who found that 61% of babies born of infected mothers escape infection and, about 26%, although infected, remain healthy.

CHAPTER VII

IN VITRO STUDIES OF TOXOPLASMA GONDII IN DIFFERENT FLUID MEDIA.

Introduction:

Toxoplasmosis, like malaria and syphilis, is an infection in which an individual may harbour the offending organism in his blood stream during asymptomatic periods (Feldman and Miller (1956). In addition, some patients are virtually asymptomatic, even though isolation of Toxoplasma gondii from their lymph nodes indicates recent acquisition and dissemination of the infection, (Jones et al., 1966).

In many hosts, it is well known that toxoplasmosis is accompanied by the development of both circulating antibodies and delayed sensitivity.

Studies on the transmission of T. gondii, have in general been concerned with the proliferative form of the parasite. Eichenwald (1959) succeeded in producing infection in newborn mice through the milk of acutely infected mothers, the proliferative forms may have survived in the stomach because of the low content of digestive enzymes in the newborn. However, milk of acutely infected mothers have not always produced

infection as Cowen and Wolf (1960) reported. Since a number of authors, including Roth et al., (1971) and Raisanen (1978) have reported transmission of Toxoplasma and acquisition by humans, through blood transfusions, it is important to devote attention to the ability of the Toxoplasma trophozoites to survive in various fluid conditions. The present report shows the results of tests on the survival of trophozoites when stored and subjected to different temperatures and different ranges of pH in different fluid media.

It is hoped that a favourable result in this experiment will ease the labourious maintenance of T. gondii parasite in the laboratory. The result may also help to eliminate transfer of the infection through blood transfusion, as the method of blood storage can be changed.

At present, the blood for transfusion is kept at $4^{o}C$ for two weeks, and if not used in 21 days, is disqualified from use.

Materials and Methods:

The RH strain of Toxoplasma gondii was used in this experiment.

Toxoplasma gondii trophozoites were obtained from the peritoneal exudate of mice previously infected 5-7 days. A drop of the fluid is observed wet under the

microscope to ensure the viability of the parasites. Counts were made of the parasites per unit volume, using the Neubaeur-Levy Counting technique.

Preparation of Fluid Media:

The different fluid media used were prepared and their pH measured with a pH meter. These included

1.	Whole Human Blood	pH 7.4
2.	Fetal Bovine Serum	pH 7
3.	Horse Serum	pH 7.6
4.	Ringer's Solution	pH 6.3
5.	Distilled Water	pH 7
6.	Normal Saline	pH 7

These different fluids were collected and prepared sterile, and 15 mls of each was measured, and put into sterile Universal Bottles.

Approximately 10,000 trophozoites from undiluted peritoneal fluid was put into each tube, and the dates were recorded and temperature taken. A set of tubes was kept in the fridge at 4°C, another in the hot room at 36°C, and the 3rd on the laboratory desk top at 28°C. After every 5 days interval, 0.2 ml was removed from each tube and used to infect a clean mouse intraperitoneally, under aseptic conditions.

To prove that it was the Toxoplasma trophozoites in the suspension that was killing the animals, control experiment was set up, in which just the clean fluid medium was stored side by side with the Toxoplasma suspension, and this was also injected into the peritoneum of other clean mice used as control animals.

Presence of the living organisms were indicated by death of the mice inoculated with the suspension, and by demonstration of T. gondii in the peritoneal fluid of the dead animals.

Results:

Of the 3 different temperatures used, $4^{o}C$ was found to be most adequate, as the organisms survived longest at this temperature.

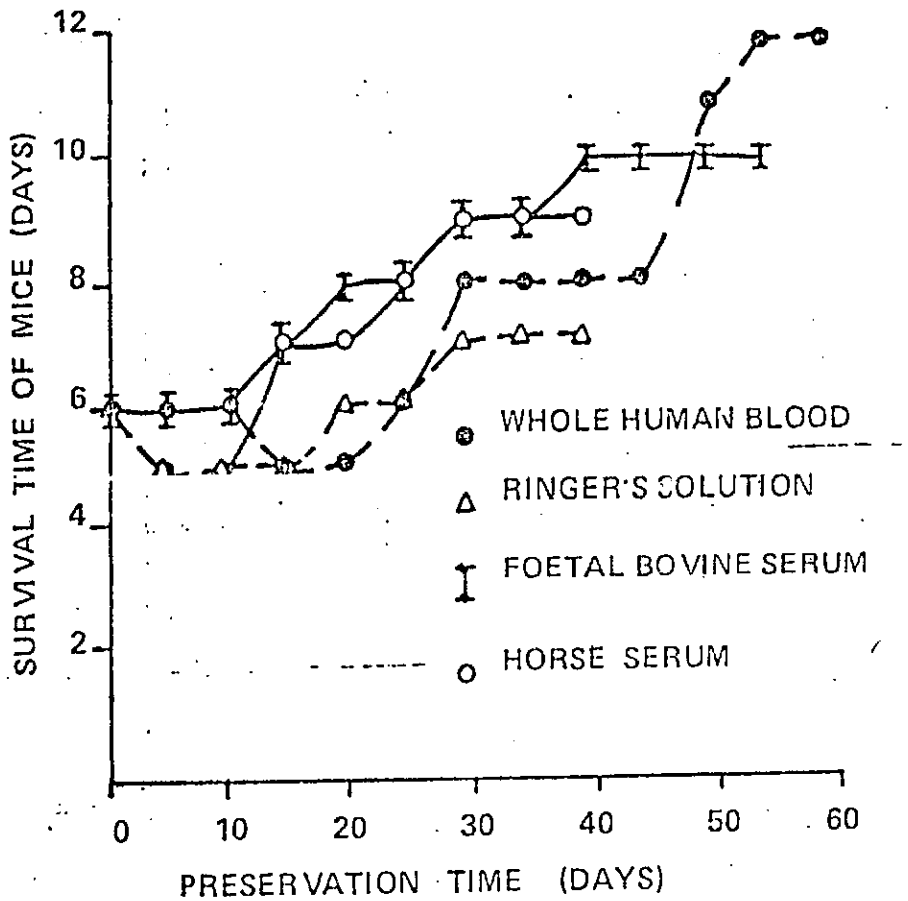
Toxoplasmosis was transmitted by the blood of intraperitoneally infected mice throughout the 56 days of the experiment. After intraperitoneal infection of mice with the suspensions stored for 1 day, the mice lived for 5-7 days (approximately 6 days). (Table IX.) This is the approximate period for which mice infected with freshly harvested Toxoplasma from peritoneal exudate survives, whereas mice in the control group stayed alive for over 30 days.

TABLE IX: SURVIVAL PERIOD OF INFECTED MICE (DAYS)

Distilled water	Normal Saline	Survival Time in Days of Mice Infected with *T. gondii* Trophozoites				Length of Storage Period of Suspension at 4°C (in days)
		Whole Human Blood	Fetal Bovine Serum	Ringer's Solution	Horse Serum	
6		6	6	5	6	5
6		6	5	5	5	10
8		5	6	5	5	15
-		5	7	6	7	20
-		6	8	6	7	25
-		8	8	7	8	30
-		8	9	7	9	35
-	9	8	9	7	9	40
-	9	8	10	-	9	45
-	11	11	10	-	-	50
-	12	12	10	-	-	55
-	-	-	-	-	-	60

FIGURE IV:

SURVIVAL PERIOD OF MICE INFECTED INTRAPERITONEALLY WITH *T. GONDII* STORED IN DIFFERENT FLUID MEDIA FOR DIFFERENT TIME, INTERVALS.



After storage for 30 days, the mice lived for up to 7-9 days (average of 8 days), and after 55 days, it lived for up to 12 days in whole human blood, and for only 10 days in fetal bovine serum. (See Figure IV).

The survival time of the mice became gradually longer with storage period. By the 56th day, the survival period was 12-13 days. The control group remained for over 30 days, showing no infection.

When the maximum length of survival period is compared with the pH of the solutions used, it is seen that (with the exception of Distilled Water) maximum survival rate was obtained around pH 7±0.4, which is about neutral; whereas, about ±0.6 gave a much reduced survival period. (Table X.) The reason for this is still to be investigated biochemically. Normal saline, which was used as a control procedure with pH 7 gave results very similar to that of whole human blood; whereas, distilled water gave a completely different set of results. The biochemical contents of the different fluid media might show which contains the more useful components, needed for survival and most important viability.

TABLE X: Showing pH, and maximum length of Survival of the trophozoites in the different fluid media at $4^{o}C$ (in days).

Fluid Medium	pH	Maximum Length of Survival at $4^{o}C$ (in days)
Whole Human Blood	7.4	56
Fetal Bovine Serum	7	56
Horse Serum	7.6	45
Ringer's Solution	6.3	40
Normal Saline	7	57
Distilled H_2O	7	15

DISCUSSION:

The gradual increase in length of survival of the infected mice, with increase in the preservation period of Toxoplasma trophozoites can be attributed to two main reasons and these are, that the virulence of the trophozoites decrease in vitro with storage, as opposed to in vivo, thereby allowing the mice to live longer than the average 6 days. On the other hand, the number of the parasites in the suspension were decreasing gradually, as they were dying off; thereby, allowing the mice to live longer. The rate at which these 2 factors are operating is left to be determined, as immunological factors would be mediated in the infected mice, with the length of the infection. It is also possible, that in vitro storage in fluid reduces the virulence of the parasite. This latter phenomenon is however, not noticed in tissue culture reports of studies on Toxoplasma gondii by authors including Ogunba (1977) and Abbas (1967).

Miller et al., (1969) showed that in the early stages of Toxoplasma infection, parasitaemia is common, even in people who are clinically asymptomatic, and this can last for up to 14 months, before immunity sets in completely. The degree of parasitaemia which

develops has been shown by Huldt (1963) to be similar in both clinical and asymptomatic toxoplasmosis. Obviously, the number of circulating parasites is not an accurate indicator of the severity of this disease.

Huldt (1963) also showed that in the beginning of the infection, parasitaemia remains strongly evident, only until the circulating antibodies start forming. Thereafter, the infection is intracellular. The degree of intracellular parasitaemia decreases gradually after the cell-associated immunity has developed.

CHAPTER VIII

GEL FILTRATION PATTERN OF TOXOPLASMA GONDII LABORATORY PREPARED ANTIGEN

INTRODUCTION:

Protozoa are made up of a Karyotic cell type consisting of a cytoplasmic mass, a limiting plasma membrane; one or more nuclei, and other organelles.

Toxoplasma gondii is an intracellular protozoan parasite, and it is the etiological agent of the world wide human and animal toxoplasmosis. This organism evokes both humoral and cell-mediated immunological responses in its host The nature of antigen, towards which these responses are directed remain elusive.

Lunde and Jacobs (1959) did some work on Toxoplasma, and suggested that the antigen is a protein with thermolabile properties. Although lots of authors have written on the detection of antibodies by serologic means, in patients infected with T. gondii yet little is known concerning the antigenic composition of the parasite and the antigen employed in the sero-diagnostic tests. The origin of the antigen (parasite or host) responsible for these bands have not been ascertained neither have they been analysed.

Chordi et al. (1964b) studied the antigenic analysis of 4 antigen preparations of T. gondii as determined by the agar-double-diffusion methods in tubes by Ouchterlony agar plate methods and by immuno-electrophoresis and found that the Toxoplasma Complement Fixation antigen demonstrated 9 components as against 1 line of precipitation found by other workers using the same antigen with immune rabbit serum with a dye test titer of 1:50,000. Chordi et al.,(1964b) in their work also found that human sera with T.gondii haemagglutination titers of less than 1:800 did not produce parasitic bands in the Ouchterlony reactions. It is apparent that these sera did not have sufficient antibody to develop precipitin lines, but had sufficient amount to show titers in a reaction as sensitive as the haemagglutination test. The large discrepancy in their result is believed to be due to different quality of immune sera tested and the differences in the technique of immunoelectrophoresis which have not been completely standardized. These variations are expected to occur amongst individual animals or species used to produce the antisera.

Pande et al.,(1961) did a comprehensive investigation on the immunochemistry of this protozoan

species, and isolated and studied a polysaccharide fraction, with special reference to its immunobiological properties. The RH strain of T. gondii was used and the chemical analysis showed some unusual features, as the nitrogen content varied from 0.8% to 1.2%, and phosphorus from 0.2 - 0.4%. Studies on its topographic distribution on intact cells of T. gondii indicated that it is at least a partial surface antigen.

Little is known about the biochemical determinants of virulence and antigenicity in protozoa. So far, just few reports of immunochemical studies on T. gondii have been documented. Hook and Faber (1957) have shown that cytoplasm-modifying and complement-fixing antibodies were related to the protein, but not carbohydrate fractions, obtained from sonically fragmented cells. Lunde and Jacobs (1959) concluded that the Toxoplasma haemagglutination test antigen was a protein contained in the crude lysate of washed parasites; but no attempts were made to isolate and identify the soluble antigens in a relatively pure state.

This study thus attempts to identify the soluble proteins in the laboratory prepared Toxoplasma gondii antigen, after the method by Voller et al., (1976); and to identify them, using molecular standards to determine their molecular weights, and also to find out if they will function separately as whole antigen in the haemagglutination test.

MATERIALS AND METHODS

(a) Preparation of Toxoplasma gondii Antigen:

The antigen used in this experiment was prepared from the RH strain of Toxoplasma gondii, derived from the peritoneal fluid of previously infected mice, using the method of Voller et al. (1976).

Toxoplasma gondii trophozoites obtained from the peritoneal exudate of previously infected mice was checked under a light microscope to be sure there were no contaminations by either blood cells or other host cells. It was also ensured that the parasites were no longer within the host cells. Positive samples from 20 mice were pooled, frozen at -20°C and thawed, alternatively, several times, till it was ready for use. The thawed sample was sonicated (or disintegrated) at 4°C for 2 minutes at 4 bursts of 5 seconds each, in the Labsonic 1510 ultrasonic Oscillator, with an intervening resting period of 30 seconds between each burst. Throughout the disintegration process, a constant temperature of 4°C was maintained by keeping the sonication tube embedded in ice chips; so that the heat produced by the machine would not destroy the protein nature of the sample. The cell debris was later sedimented by centrifugation at 10,000 r.p.m. for 30 minutes in the cold room. The supernatant,

which constitutes the 'antigen' was decanted and stored at -20°C, until required.

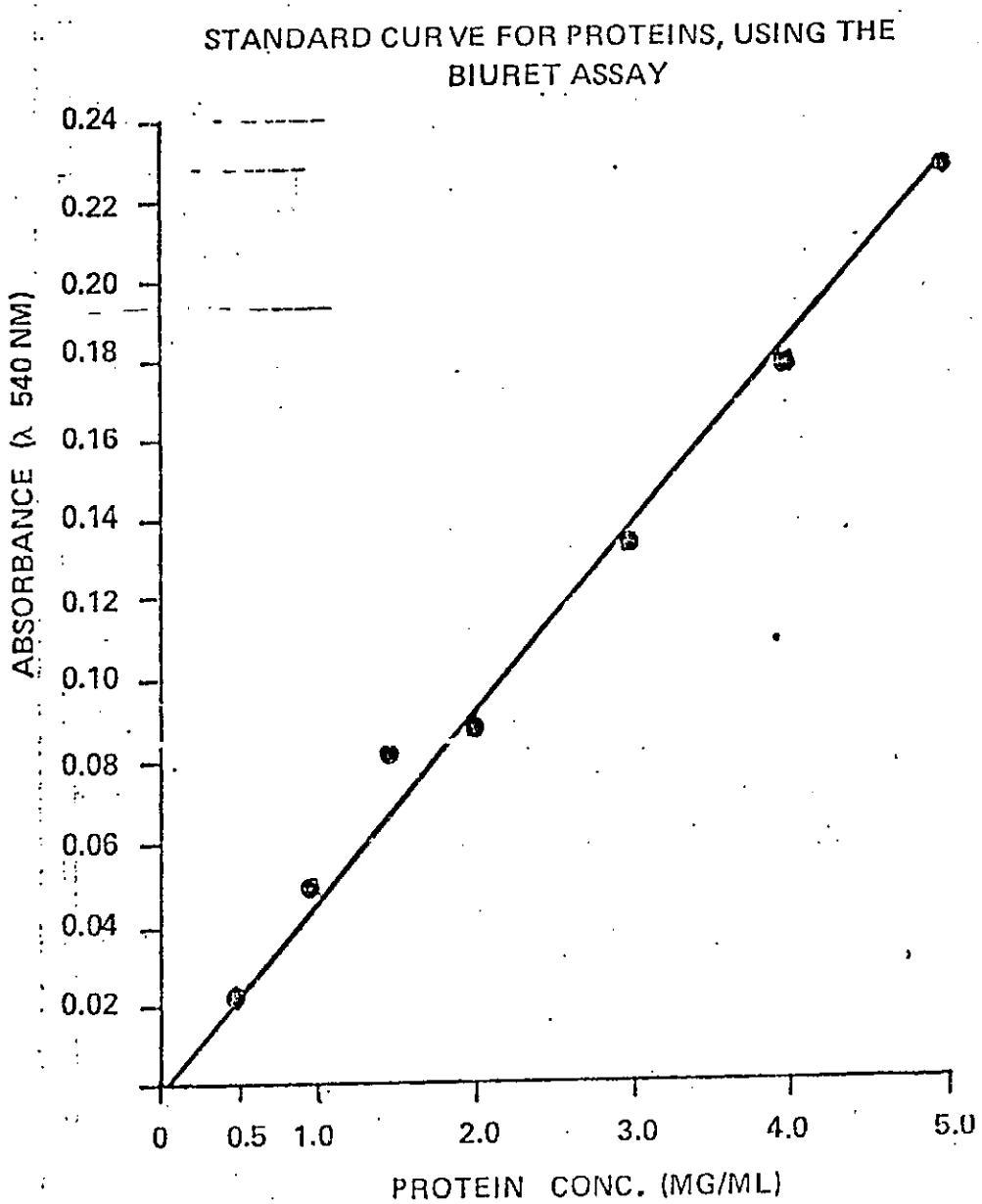
(b) Protein assay:

The protein content of the antigen was routinely assayed by the Biuret method, using Bovine Serum albumin as standard, to cover the range in mg/ml. With this, a calibration curve of extinction at 540 nanometers against protein concentration is drawn, and by extrapolation, the sample protein content of the antigen was determined (See Page 105).

The basic or Biuret reagent (commonly called Royal Blue) is prepared from $CuSO_4.5H_2O$ (Copper sulphate), and sodium potassium tartrate ($NaKC_4O_6.4H_2O$) to which is added 10% NaOH (W/v) potassium Iodide and glass distilled water.

The Biuret assay involves mixing different concentrations of the Royal Blue and glass distilled water, plus the basic protein, which is 5 ml of a 1mg/ml concentration of Basic albumin. The mixture, containing different dilutions of the basic protein is mixed thoroughly and incubated at 37°C for 20 minutes in a water bath. The absorbance is then determined at 540nm using the Unicam SP-1800 ultraviolet spectrophotometer (PYE UNICAM).

FIGURE IVB:


STANDARD CURVE FOR PROTEINS, USING THE BIURET ASSAY
ABSORBANCE (λ 540 NM)
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0.5
1.0
2.0
3.0
4.0
5.0
PROTEIN CONC. (MG/ML)

A graph of extinction at 540nm against albumin concentration was prepared, and this was the standard curve from which the amount of protein in 1 ml of the antigen was extrapolated (See Page 105, Figure IVB).

(c) Packing of the Column, and its Equilibration:

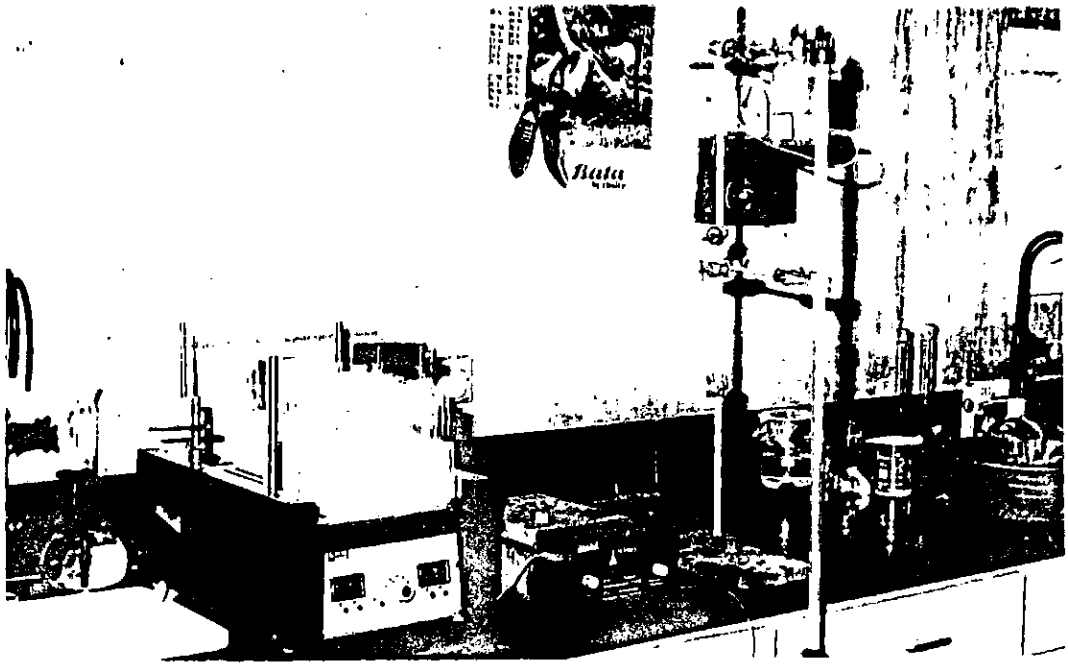
Sephadex G.200 (Pharmacia, Fine Chemicals) was the gel of choice, and this was selected due to the fact that compounds with the widest range of molecular weights can be fractionated on this gel. Sephadex G.200 will cover a molecular weight range of 5,000-600,000 daltons.

A column of 90cm by 1.5cm was packed with Sephadex G.200. The bed volume of the gel is 30-40 ml/g. That means that 1 gm of the gel will swell to 30-40 mls. Thus, 2 gms of the dry powdered gel was weighed and suspended in excess buffer (0.05m Tris - HCl $_{p}$H 7.5). A few drops of 0.01m sodium azide (NaN_3) an antimicrobial agent were added. This was left overnight, and allowed to swell. The resulting suspension was deaerated in a water bath at 37^{o}C for about 1 hour to get rid of any trapped air bubbles. The column was then packed gradually, to avoid breaking of the delicate beads of the gel. The overhead pressure was measured, and kept at a maximum of 15 cm. A peristaltic pump

(Model Desaga STA Multipurpose peristaltic pump 131900) was attached to the buffer supply end, and this was to produce a constant overhead pressure on to the column. The NaN_3 added to the buffer and gel was to prevent the growth of micro-organisms that could tamper with the flow rate of the column (See Picture I on Page 108).

(d) Determination of the Void Volume (Vo):

After equilibration of the column by passing a large volume of the buffer (0.05 M Tris-HCl pH 7.5) through the column, the flow-rate of the column was determined, till it became constant at 16 mls per hour. To determine the Void Volume, blue dextran a high molecular weight protein (M.wt. = 1,000,000) was layered on the column and allowed to be absorbed into the gel. This dye was then eluted with the buffer (0.05 M Tris HCl; pH 7.5), until the final traces of the dye were collected, and the total volume which had eluted the dye from the column was recorded. This was the Void Volume or Vo for the column. The Vo is the elution volume of molecules which are only distributed in the mobile phase, because they are larger than the largest pores in the gel.



PICTURE I:

The Gel Filtration set up used to separate T. gondii antigen using Sephadex G200. Notice the peristaltic pump as well as the automatic Fraction Collector, incorporated in the system.

(e) Fractionation of the Sample:

5 ml of the sample (the antigen) was applied onto the column, allowed to be absorbed, and eluted with the buffer. 4 ml fractions were collected into test tubes, using the LKB automatic fraction collector, model Number 7000.

The fractions were then analysed for protein at an Optical Density of 280nm, using the Unicam SP-1800-Ultraviolet Spectrophotometer. The readings are shown in Table XI. The elution profile, ~~i.e. the graph~~ of OD at 280 nm against Fraction Number was drawn (Figure V). The 3 major peaks observed were marked out and designated FI, FII and FIII. Tubes under these major peaks were pooled together and freeze-dried using the Edwards, High Vacuum, PIRANI 11 freeze-drier. Model No EF03 at a voltage of 220-240V.

(f) Determination of the Elution Volumes (Ve) of Standard Proteins:

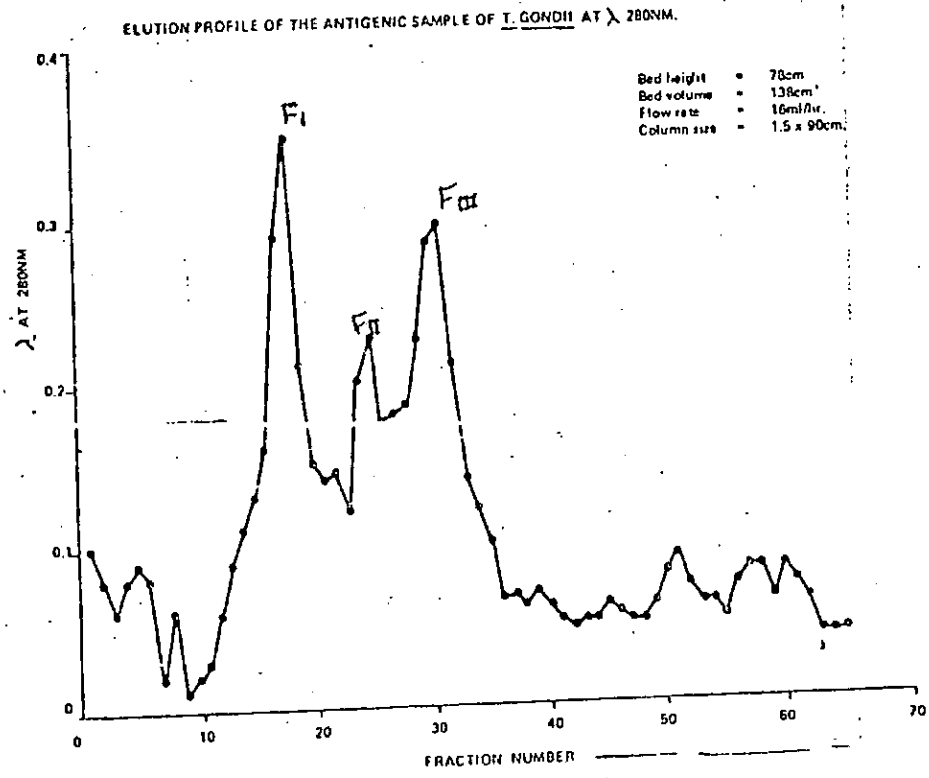
The standards used were proteins with known molecular weights, and those chosen included.

1.	Catalase	M.wt. 60,000
2.	Pepsin	M.wt. 35,000
3.	Myoglobin	M.wt. 17,000
4.	Cytochrome C	m.wt. 13,000

FRACTIONATION OF THE SAMPLE ON SEPHADEX G-200

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	0.1	26.	.175	51.	.088
2.	.08	27.	.18	52.	.068
3.	.06	28.	.184	53.	.058
4.	.08	29.	.225	54.	.06
5.	.092	30.	.285	55.	.048
6.	.08	31.	.295	56.	.066
7.	.022	32.	.21	57.	.082
8.	.06	33.	.14	58.	.082
9.	.01	34.	.115	59.	.06
10.	.02	35.	0.1	60.	.078
11.	.03	36.	.064	61.	.07
12.	.06	37.	.064	62.	.06
13.	.086	38.	.058	63.	.04
14.	0.11	39.	.07	64.	.042
15.	.13	40.	.058	65.	.04
16.	.16	41.	.05	66.	.046
17.	.29	42.	.044	67.	.056
18.	.352	43.	.05	68.	.068
19.	.205	44.	.048	69.	.042
20.	.152	45.	.056	70.	.036
21.	.14	46.	.054	71.	.034
22.	.145	47.	.052	72.	.038
23.	.118	48.	.05	73.	.042
24.	0.2	49.	.062	74.	.032
25.	.225	50.	.082	75.	.03

FIGURE V:


ELUTION PROFILE OF THE ANTIGENIC SAMPLE OF T. GONDII AT λ 280NM.
Bed height = 78cm
Bed volume = 138cm³
Flow rate = 16ml/hr.
Column size = 1.5 x 90cm.
F I
F II
F III
λ AT 280NM
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
FRACTION NUMBER

Preparation of the standards included measuring 1 mg/ml of each of these proteins in an equivalent 5 ml of buffer (Tris-HCl. buffer pH 7.5). After the standards were prepared, 5 ml from each was applied onto the column, left to be absorbed, and each was eluted with the buffer. 4 ml fractions were collected. The fraction collection of any particular standard was stopped when the absorbance or optical density readings remained almost constant in the ensuring 5-10 tubes. The optical density readings for the standards are shown in Tables XII to XV. Buffer was used to wash the column, before another standard was applied. From the Optical Density (OD) readings the elution volume (Ve) was determined. The Ve is the total volume of eluent collected till the highest point of the peak is attained. The graph of Ve for all the standards is shown on Figure VI.

(g) Determination of the Elution Volumes (Ve) for FI, FII, and FIII:

The freeze-dried sample was dissolved in buffer (0.05 M Tris-HCl pH 7.5) and 5 ml portion was applied onto the column, and elution was carried out under the same conditions as for the standard protein. The Elution profile for each fraction was then computed on the same graph, and the elution volume (Ve) deter-

TABLE XII: Optical Density Readings for Fractions of Catalase eluted from the Column.

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	.036	26.	.385
2.	.05	27.	.3
3.	.034	28.	.21
4.	.028	29.	.15
5.	.038	30.	.155
6.	.038	31.	.185
7.	.06	32.	.235
8.	.142	33.	.285
9.	.26	34.	.295
10.	.225	35.	.395
11.	.132	36.	.39
12.	.142	37.	.35
13.	.12	38.	.31
14.	.14	39.	.26
15.	.126	40.	.24
16.	.154	41.	.2
17.	.18	42.	.21
18.	.22	43.	.22
19.	.275	44.	.146
20.	.32	45.	.134
21.	.385	46.	.13
22.	.445	47.	.08
23.	.51	48.	.046
24.	.52	49.	.034
25.	.49	50.	.032

$V_e = 96\ cm^3$

TABLE XIII: Optical Density Readings for Fractions of Pepsin eluted from the Column.

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	.066	26.	.26
2.	.06	27.	.4
3.	.068	28.	.165
4.	.068	29.	.15
5.	.082	30.	.1
6.	.01	31.	.12
7.	.09	32.	0.1
8.	.09	33.	.102
9.	.08	34.	.126
10.	.098	35.	.104
11.	.09	36.	.118
12.	.016	37.	.09
13.	.09	38.	.092
14.	.082	39.	.18
15.	.074	40.	.16
16.	.09	41.	.12
17.	.088	42.	.154
18.	.086	43.	.225
19.	.108	44.	.152
20.	.08	45.	.11
21.	.082	46.	.116
22.	.076	47.	.1
23.	.072	48.	.11
24.	.154	49.	.009
25.	.27	50.	.092

$Ve = 108cm^3$

TABLE XIV: Optical Density Readings for Fractions of Myoglobin eluted from the Column.

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	.02	26.	.086
2.	.028	27.	.11
3.	..002	28.	.12
4.	.002	29.	.124
5.	.02	30.	.146
6.	.024	31.	.18
7.	.026	32.	.175
8.	.026	33.	.145
9.	.012	34.	.124
10.	.008	35.	.108
11.	.008	36.	.074
12.	.008	37.	.076
13.	.008	38.	.074
14.	.016	39.	.071
15.	.014	40.	.061
16.	.016	41.	.054
17.	.020	42.	.052
18.	.026	43.	.04
19.	.022	44.	.044
20.	.032	45.	.041
21.	.022	46.	.038
22.	.041	47.	.038
23.	.043	48.	.037
24.	.056	49.	.034
25.	.074	50.	.024

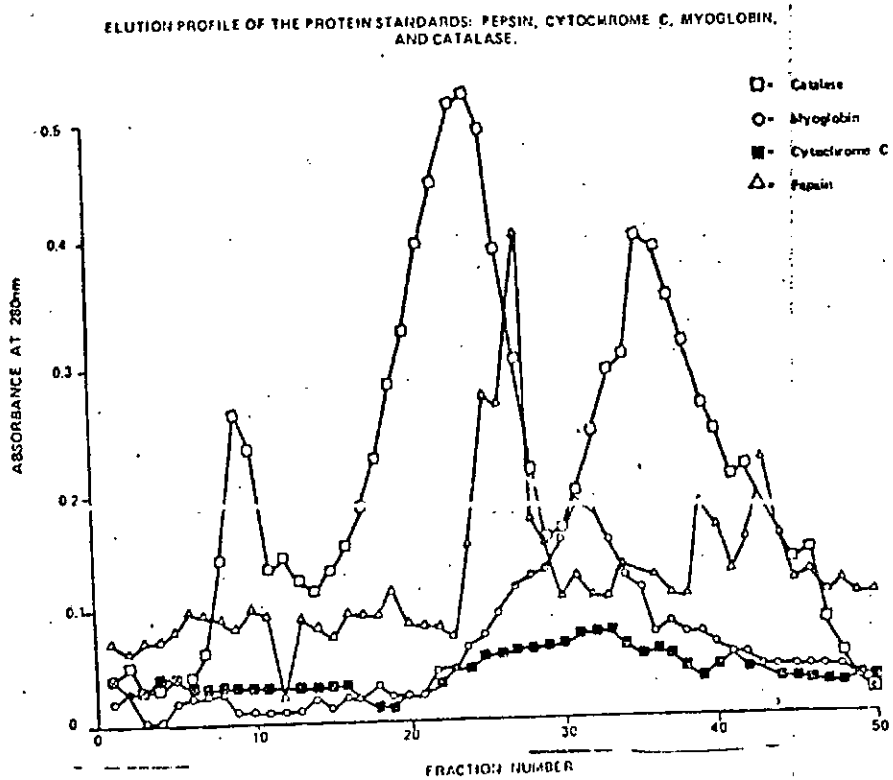
$Ve = 125cm^3$

TABLE XV: Optical Density Readings for Fractions of Cytochrome C eluted from the Column.

Tube Number	Optical Density Readings (Nanometes)	Tube Number	Optical Density Readings (Nanometers)
1.	.04	26.	.054
2.	.034	27.	.056
3.	.026	28.	.058
4.	.038	29.	.061
5.	.04	30.	.063
6.	.032	31.	.065
7.	.03	32.	.068
8.	.028	33.	.072
9.	.028	34.	.06
10.	.028	35.	.05
11.	.026	36.	.055
12.	.032	37.	.05 1
13.	.03	38.	.04
14.	.028	39.	.033
15.	.03	40.	.04
16.	.032	41.	.048
17.	.02	42.	.042
18.	.014	43.	.041
19.	.014	44.	.03
20.	.018	45.	.032
21.	.02	46.	.028
22.	.03	47.	.026
23.	.04	48.	.026
24.	.042	49.	.03
25.	.052	50.	.024

$Ve = 132\ cm^3$

FIGURE VI:


ELUTION PROFILE OF THE PROTEIN STANDARDS: PEPSIN, CYTOCHROME C, MYOGLOBIN, AND CATALASE.
Catalase
Myoglobin
Cytochrome C
Pepsin
ABSORBANCE AT 280nm
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
FRACTION NUMBER

mined for each fraction. The readings are shown in Tables XVI to XIX. The spectrophotometer used was initially standardized and zeroed with the respective buffer (See Picture II on Page 122).

(h) Determination of the Molecular Weights of FI, FII, and FIII:

After all Ve had been determined, a calibration curve was computed plotting a graph of $\frac{Ve}{Vo}$ against the log of the molecular weights of the standard proteins, and by extrapolation, the molecular weights of FI, FII and FIII, were determined (Figure VII).

These different fractions were used to run the Indirect Haemagglutination Test (IHA), using titers of 1:128, 1:256 and 1: 512 seropositive human sera and the results observed are discussed.

Also, agar-gel diffusion technique, in which agarose was poured on a glass slide and holes bored to hold the antigen, surrounded by the positive sera was carried out, and the observations are recorded.

TABLE XVI: Optical Density Readings for FI (FRACTION I)

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	.026	26.	.058
2.	.022	27.	.062
3.	0.0	28.	.056
4.	.01	29.	.042
5.	0	30.	.04
6.	.044	31.	.042
7.	0	32.	.042
8.	0	33.	.034
9.	.014	34.	.032
10.	.004	35.	.036
11.	.04	36.	.036
12.	.036	37.	.032
13.	.034	38.	.03
14.	.044	39.	.042
15.	.046	40.	.036
16.	.049	41.	.032
17.	.05	42.	.022
18.	.072	43.	.026
19.	.096	44.	.024
20.	.083	45.	.022
21.	.072	46.	.014
22.	.066	47.	.04
23.	.054	48.	.004
24.	.05	49.	.002
25.	.05	50.	.002

$Ve = 75cm^3$

TABLE XVII: Optical Density Readings for F2 (FRACTION II)

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	.078	26.	.126*
2.	.114	27.	.106
3.	.066	28.	.09
4.	.062	29.	.08
5.	.04	30.	.74
6.	.04	31.	.054
7.	.056	32.	.036
8.	.064	33.	.03
9.	.094	34.	.026
10.	.09	35.	.054
11.	.088	36.	.068
12.	.066	37.	.05
13.	.066	38.	.03
14.	.066	39.	.014
15.	.054	40.	.032
16.	.058	41.	.046
17.	.064	42.	.042
18.	.026	43.	.03
19.	.052	44.	.03
20.	.052	45.	.046
21.	.05	46.	.02
22.	.06	47.	.052
23.	.096	48.	.014
24.	.119	49.	.036
25.	.124	50.	.034

Ve = 104 cm^3

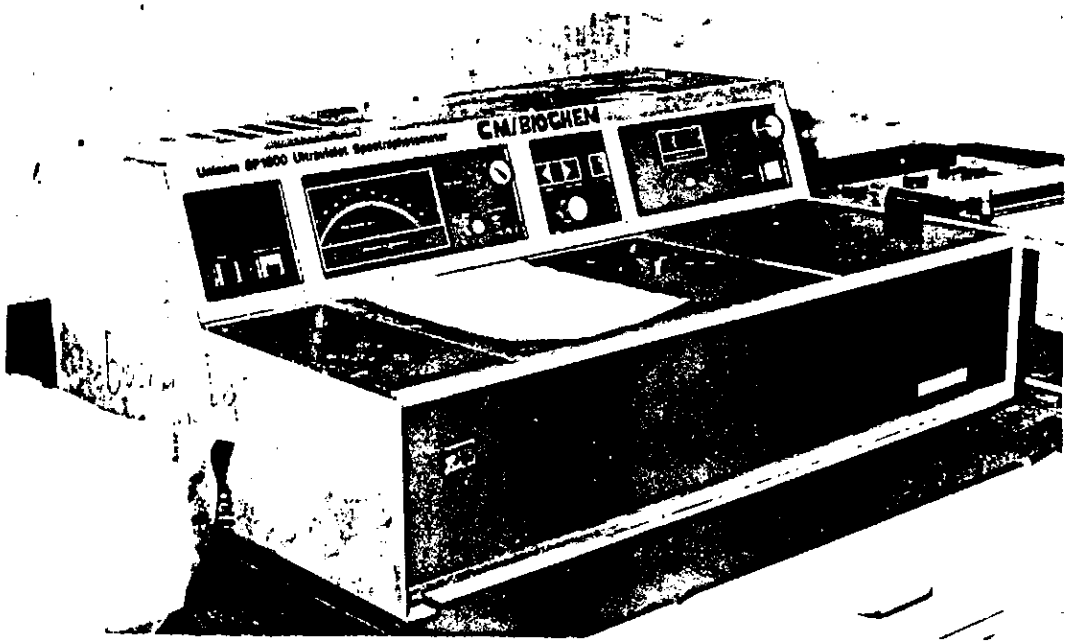
TABLE XVIII: Optical Density Readings for F3 (FRACTION III)

Tube Number	Optical Density Readings	Tube Number	Optical Density Readings
1.	.012	26.	.14
2.	.035	27.	.114
3.	.018	28.	.16
4.	.018	29.	.18
5.	.011	30.	.18
6.	.026	31.	.25
7.	.039	32.	.31
8.	.032	33.	.33*
9.	.012	34.	.25
10.	.014	35.	.128
11.	.015	36.	.106
12.	.012	37.	.14
13.	.012	38.	.185
14.	.014	39.	.151
15.	.088	40.	.19
16.	.078	41.	.19
17.	.12	42.	.175
18.	.1	43.	.172
19.	.12	44.	.2
20.	.13	45.	.16
21.	.132	46.	.14
22.	.125	47.	.114
23.	.124	48.	.12
24.	.12	49.	.12
25.	.12	50.	.104

$Ve = 133.12 cm^3$

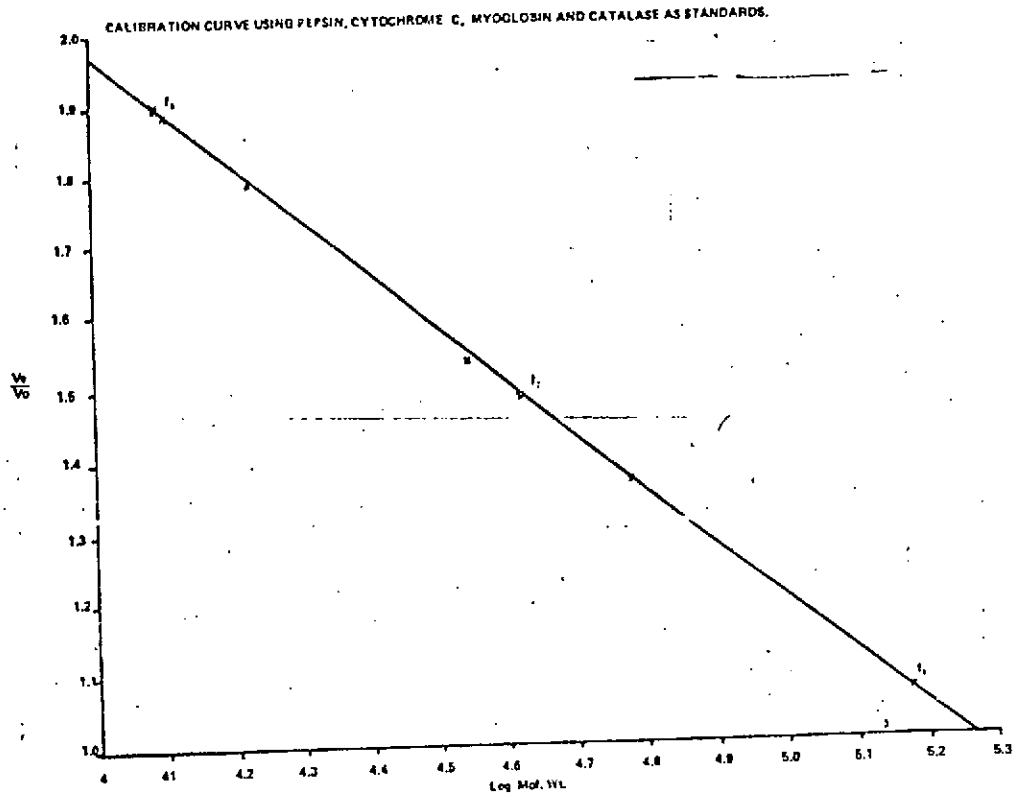
TABLE XIX: Showing the Elution Volume (Ve), Ve/vo and Log of the Molecular Weights of all the Fractions as well as the Standards, Fractionated on Sephadex G200 Column.

Vo = 70cm^3	Molecular Weight	Log of Mol. Weight	Ve/vo
Ve Cytc = 132cm^3	13,000	4.11	1.89
Ve Myo = 125cm^3	17,000	4.23	1.785
Ve Pep = 108cm^3	35,000	4.54	1.54
Ve Cat = 96 cm^3	60,000	4.78	1.37
Ve F_1 = 75 cm^3	186,209	5.27	1.07
Ve F_2 = 104 cm^3	41,687	4.62	1.49
Ve F_3 = 133.12	12,445	4.095	1.90



PICTURE II: The Unicam SP 1800 Ultraviolet Spectrophotometer (PYE UNICAM) used for measuring the amount of Protein (absorbance) in the fractions.

FIGURE VII:


CALIBRATION CURVE USING PEPSIN, CYTOCHROME C, MYOGLOBIN AND CATALASE AS STANDARDS.
Ve/Vo
Log Mol. Wt.
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
4
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
5.0
5.1
5.2
5.3

RESULTS:

The antigen prepared after the method of Voller et al., (1976) was a colourless fluid, that was stored at -20°C, till it was fractionated.

The protein content of the antigen, assayed by the Biuret method and read at an OD of 540nm was 3.6 mg/ml., and this was diluted 4 times with distilled water before it was fractionated. The standard curve is shown on Page 105. The other protein contents were determined, using the Deuterium lamp, of the spectrophotometer at 280nm.

The flow-rate of the column was determined to be 16ml per hour, and 4 ml fractions were collected. The Void Volume (Vo), using Blue Dextran 2000 (Molecular weight 1,000,000) was determined as 70cm^3.

Fractionation of the sample resulted in 3 definite peaks, and these were designated FI, FII and FIII, for the 1st, the 2nd and the 3rd peaks respectively, (Fig. V). The Optical Density Readings for these 3 peaks are shown on Tables XVI-XVIII. The tubes around the different peaks were pooled and concentrated by freeze-drying. They were then applied onto the column again, one at a time, to determine their elution volume (Ve). The Ve for the different fractions as well as the standards are shown on Table XIX.

The Calibration curve (Figure VII) was drawn with the $\frac{Ve}{Vo}$ of the standard proteins, against the log of their molecular weights, and by extrapolation the log of the molecular weights of the 3 fractions from the antigen were read off. The molecular weight of FI was found to be about 186, 209 daltons. FII was 41, 687 daltons, while FIII was 12,445 daltons (Table XIX).

These fractions were used to run the Indirect Haemagglutination test separately, using human positive sera of titre 1: 128, 1:256, and 1:512 obtained from the hospital laboratory, but none of these functioned separately as a complete antigen, against human positive sera.

Other tests carried out, using the agar-gel diffusion technique did not produce any positive results, as no precipitation lines were observed.

DISCUSSION:

The protein content of the antigen which was derived was 3.6 mg/ml, and since this was further diluted, before it was fractionated on the chromatography column, it becomes apparent that the protein concentration in the fractions is relatively low, to exhibit any positive reaction in the Haemagglutination test (HAT), in which whole organisms are used when the standard kits are commercially prepared, to sensitize the red blood cells used; after the method of Jacobs and Lunde (1957). Thus, none of the 3 fractions, FI, FII or FIII could detect haemagglutination antibodies either against their homologous or heterologous antisera.

Practically, it has been reported by Alikhan and Macrovitch (1970) and Lunde and Diamond (1969), that in the case of mono xenic and axenic amoebic antigens, the optimal protein concentrations of about 40.0 and 77.0ug/ml are required for the sensitization of erythrocytes to be employed for IHA test.

This variability in antibody responses may be due to the fact that these serological tests detect different types of antibodies (Maddison et al., (1965). Therefore, the failure of the fractions FI, FII, and FIII to demonstrate any antibodies may be due to its low molecular weight or the protein concentration employed in these tests.

The main observation made in the present study indicates that none of the 3 fractions is a major component of the Toxoplasmic antigen, which is immunologically active, and exerts a response similar to that elicited by the whole antigen. It is virtually 100 percent of the Toxoplasmic antigen protein that is functionally active.

This result agrees with that obtained by Fujita et al.(1970), in their study on Toxoplasma sero-reactions with antibody fragments. They found that the affinity of monovalent antibody is lower than that of the original bivalent one. This comparison they showed by reacting their whole antigen with the dye test (DT), the Complement Fixation test (CFT), and the Haemagglutination test (HAT), and it elicited positive reactions in every one of these tests; whereas, their 5s fragment or bivalent antibody fragment elicited positive reactions in HAT, and negative reactions in CFT and DT. , The 3.5s fragment or monovalent antibody fragment failed to elicit positive reactions in any of these tests. Their inference, as well as that reported in this paper is compatible with the observation of Greenbury et al. (1965); that antibody bound bivalently to an erythrocyte exhibits considerably higher affinity than mono valently bound antibody.

CHAPTER IX

GENERAL DISCUSSION:

The aims of the series of investigations reported in this thesis are:

(i) to determine the age prevalence levels of *Toxoplasma* antibodies in the general population, as well as in pregnant women, in Lagos Metropolis, and to correlate *Toxoplasma* seropositivity with susceptibility to the infection.

(ii) to investigate the prevalence of Toxoplasmosis in cats in Lagos, and thereby determine the sources and modes of infection in the environment, as well as transmission mechanisms.

(iii) to study the effect of experimental *Toxoplasma* infection in pregnancy, with reference to recurrent abortions, using mice models.

(iv) to develop a less tedious and less expensive method of maintenance of *T. gondii* (RH strain) in the laboratory, by investigating in vitro susceptibility of the trophozoites in different fluid media and,

(v) to separate by gel filtration technique, the different components resulting from laboratory

prepared Toxoplasma gondii antigen after the method of Voller et al., (1976) and to test the antigenicity of each fraction separately.

The experiments reported in Chapter III show the overall seropositivity which is 63.2% in Lagos metropolis to be much higher than previous reports from other parts of Nigeria, with the exception of 95.4% prevalence recorded by Olurin et al. (1971). The results obtained in these studies in Nigeria is very similar to those reported by Walton et al. (1966) in their study in Central America and Guatemala. From the data collected on the prevalence of Toxoplasma antibodies in Nigeria so far, we find that the results agree with the fact that this infection varies with a variation in climate.

The age prevalence levels of Toxoplasma antibodies in Lagos show the highest percentage positive in the 36-40 years age group, in the general survey; whilst the lowest was in the 11-15 years age group. This overall rate of 63.2% obtained using the Indirect Haemagglutination test shows that there is a definite source of reinfection in the environment, thus suggesting a constant transmission cycle, through which the populace gets infected, by contamination of food or drink. This acquired infection develops with age, from about 8 years, on the average, to about

38 years, the median age in the group with the highest seropositive percentage in the tests. The K-values or infection risk (i.e. the risk of sero-negative people acquiring the infection) show that from childhood, up to the age of 30 years, there is a very high infection risk, which means that there is a very high chance of sero-conversion within each of these age groups.

The prevalence of the infection in pregnant women follows virtually the same trend of sero-positivity and sero-conversion as portrayed in the general population. Here, seropositivity rate was 60.18% in 452 pregnant women observed. The few cord blood specimen analysed by the Indirect Haemagglutination test gave over 70% positive cases, and placental tissue showing the organism was also portrayed. Here, the highest percentage positive was seen in the 31-35 years age group; whereas the greatest number studied was in the 21-25 years age group. This particular age group consisted of most of the women in their child-bearing age, and this group of women fall into a younger age group here in Nigeria, as opposed to other countries where it falls within the 26-30 years age group. The K-values or infection risk, in this case is low in this age group, as the number of non-pregnant women are very few, when compared with those pregnant. Questionnaires completed by these women, with relevance to their

activities and background behaviour showed that most have had frequent contact with cats and dogs. Only 11.03% have undergone any form of spontaneous abortion, while the infant mortality rate was determined to be 16.9% amongst the positive cases. From all indications, and with particular reference to the result of this survey here in Lagos, it is important to identify and treat pregnant women who sero-convert during pregnancy, or who have a very high titre at the period of early gestation, to avoid abortion. This agrees with the results of Kimball et al.(1971) and Southern (1972), as no direct relationship was seen between presence and acquisition of Toxoplasma antibodies, and recurrent abortion. Most of the women in this study (up to 60%) had seropositive titres, but only one with 1:1024 titre had a miscarriage.

Alford et al. (1969) found more asymptomatic than symptomatic congenital toxoplasmosis, thus demonstrating that such relatively large pool of congenitally infected persons do exist. This can directly be related to the situation in our environment, as most of the people who showed seropositivity to toxoplasmosis were asymptomatic to this or any other infection. The incidence of women with antibodies to Toxoplasma increases with age from 16 years to 40 years of age. This follows the same

trend, with an apparent reduction after 40 years as reported by Broadbent et al (1981).

Thus, routine antenatal care is highly recommended in our environment, with relevance to testing for infection in-utero, by performing serological tests for the detection of toxoplasmosis on all pregnant women who report for antenatal care in the clinics. This will help reduce the amount of insignificant infections such as retinochoroiditis which may not be manifested in the newly born child, but become established in later life.

The prevalence of toxoplasmosis in cats in Lagos involved collecting stray cats from the 4 study areas, where humans were tested for seropositivity. On initial examination of the cats' faeces, no oocysts were observed from any of them, whereas, serological tests on these cats showed that 4 were seropositive to the Indirect Haemagglutination test. Since these cats were not producing oocysts, at the period of collection, it showed that they did not have active infection, despite the seropositivity. Thus, it is concluded that the association of cats and toxoplasmosis, with the acquisition of positive antibody titer, as employed by Hutchinson et al (1971) is not directly related to all cases of Toxoplasma infection in the Lagos environment. Only 17% of the cats examined were seropositive. This is relatively low, considering the

above 60% obtained in humans in the same environment. The relationship here shows that though some cats produce oocysts, after infection, there are other major sources of transmitting and transferring these oocysts to man in Lagos, since this survey has shown that about 83% of stray cats are not producing oocysts. The results of the transmission pattern here in Lagos does not agree with studies reported by McCulloch et al. (1963), who found positive association with cats and Toxoplasma tests, but agrees with reports by Price (1969), who found no such association with cats alone. The survey on pregnant women, where a high percentage of the sero-positive mothers showed recent association with cats and dogs, in their environment, does not correlate with this result on transmission, and only shows that there are other intermediate sources of infection to man in this environment, as opposed to the cats. However, experience with the cat population is not enough, and a wider variety of domesticated animals as well as house pests such as cockroaches and rats (which abound here in the tropics) must be taken into consideration, for precise identification of the means of transmission of this infection directly to man.

In the study to show the effect of Toxoplasma infection on pregnancy, using mice models, the results

obtained were similar to those reported by Beverley (1969), that infection, early in pregnancy, (but after 28 days) in humans, will cause fetal death and abortion. Here, the first 28 days is within the first half of the first trimester of pregnancy, whereas, the first 4 days of infection with regards to the mouse also covers the first half of the first third of the gestational period. This study shows that the infection is transferred fastest to the fetus, if it takes place in the latter half of the first trimester of pregnancy, as this effect is seen in the results recorded. Also, infection was transferred within the 2nd trimester of the gestational period. Of the mice infected experimentally, 56.67% expelled their fetus at one stage of their development, while only 23.3% had normal delivery. The rest died, together with their fetus, while the others did not pick up the infection. These included the remaining 20% not accounted for. On allowing the mice to mate a second time over, they did not show recurrent abortion, but had normal suckling mice at the appropriate period.

In vitro maintenance of the RH strain of *Toxoplasma gondii* in fluid medium survived best at $4^{o}C$, therefore, all reports were made on growth at this particular temperature. *Toxoplasma* trophozoites were still viable, after being maintained in whole human blood, and fetal bovine serum for up to 56 days.

The gradual increase in the length of survival of the mice (in days) into which these organisms were inoculated, intraperitoneally gives rise to the fact that one of two mechanisms were taking place; That is, either the viability of the organisms are decreasing or the number of living organisms have decreased in the medium, as they have died off slowly. The rate at which these 2 factors are operating is left to be determined, as immunological factors would be mediated in the infected mice, with the length of the infection.

Since Toxoplasma trophozoites can live for so long in whole human blood and fetal bovine serum, we can use this method for maintenance of the parasite, after cell counts are done daily, and the virulence of the organism tested. It is believed that with both obstacles out of the way, it will be cheaper and less tedious to maintain Toxoplasma parasites in the laboratory, instead of the 4-6 days intraperitoneal transfer from mice to mice, which is the accepted mode of maintenance.

The result of the work on Toxoplasma done by Jacobs and Lunde (1957) led them to suggest that the Toxoplasma antigen is a protein with thermolabile properties, and although lots of authors have written on the detection of antibodies by serologic means, in patients infected with T. gondii, yet little is known

concerning the antigenic composition of the parasite and the antigen employed in the serodiagnostic tests.

In this study, the proteins present in the antigen produced from the parasite after the method of Voller et al.(1976) was identified, using Molecular Weight Standards, to determine the molecular weights of the fractions derived from the separation of the antigen by gel filtration method. Sephadex G200 was the gel used, as this covers a very wide range of molecular weights. Column chromatography analysis of the antigen gave 3 fractions and these were labelled FI, FII and FIII. The elution volumes of each fraction, determined from their elution profile was used to determine $\frac{Ve}{Vo}$ and this was used to extrapolate the molecular weights of these fractions from the standard elution profile. The results of molecular weights derived in this study are quite reliable, as Andrews (1965) had proved the correlation between elution volume and molecular weight of proteins, investigated by gel filtration method on Sephadex G200 column, at pH 7.5. The fact that none of these fractions performed singly as an antigen in the Indirect Haemagglutination test shows that they are all components of the antigen, and cannot perform the antigenic activity, when broken down. This can be supported by the work of Chordi et al (1964b)

who also found that human sera with T. gondii Haemagglutination titers of less than 1:800 did not produce parasitic bands in the Ouchterlony reactions. It is apparent that these sera, like in this report, did not have sufficient amount of antibody to develop precipitates. It does follow that none of these fractions is a main component of the antigen which is immunologically active and exerts no response similar to that elicited by the whole antigen. This aspect of the work requires further evaluation and precise investigation.

In conclusion, the age prevalence levels of Toxoplasma antibodies in the general Lagos population as well as in pregnant women have been determined. Also, Toxoplasma seropositivity has been correlated with susceptibility to infection. The sources and modes of infection in the Lagos environment, as far as transmission mechanisms are concerned have been shown to be otherwise, than by cats. There appears to be certain intermediate agents, which are yet to be determined. Toxoplasma infection in relation to recurrent abortions have been shown to have no adverse effect on mice, used as the model in this study. Expulsion of the fetus, was however shown to occur, if infection took place mainly in the first, and partly in the 2nd third of the gestational period.

Toxoplasma trophozoites have been shown to survive in whole human blood, as well as in fetal bovine serum for up to 56 days at 4°C, and still cause infection if inoculated into clean mice intraperitoneally. The Toxoplasma antigen prepared after the method of Voller et al (1976) has been shown to consist of 3 main protein fractions with molecular weights 186,209 daltons, 41,687 daltons and 12,445 daltons respectively. None of these can however function as the whole antigen itself in the Indirect Haemagglutination test with human seropositive serum.

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APPENDIX

Quantitative Estimation of *Toxoplasma gondii* trophozoites using the Neubaer - Levy counting chamber. To estimate the number of parasites per cubic milimeter of peritoneal fluid, using the improved Neubaer's counting chamber, it was ensured that the peritoneal fluid used contained individual trophozoites that were already released from the host cells in which they multiplied. A 1:100 dilution of peritoneal fluid was very adequate for the counts. All 4 squares were counted 3 times over and the average was used in the calculation.

Calculation:

Depth = 0.1mm

Area = $\frac{1}{400}mm^2$ (for eadh 1:10 dilution, we multiply by 100)

$\therefore$ Vol. = $0.1 \times \frac{1}{400} = \frac{1}{10} \times \frac{1}{400} = \frac{1}{4000}mm^3$

$\frac{1}{4000}$ x Dil factor x Average of 64 squares (in number of parasites)

i.e. $\frac{1}{4000} \times \frac{1000}{10} \times 3134 = \frac{3134}{40} = 78.35$

Number of parasites per mm^3 of Peritoneal fluid = 78 $\pm$ 10 trophozoites. Using a 1:20 dilution factor, which is usually used for transfer and maintenance of the parasites in the laboratory, the number of parasites per mm^3 of peritoneal fluid is equivalent to 157 $\pm$ 20 parasites.

Bleeding of Cats

The cats were caged, and labelled. They were anaesthesized by intraperitoneal **injection** of pentobarbitone sodium. They were left for 30 minutes, for the anaesthesia to take effect. The quantity of anaesthesia given was related to the weight of the cat. 30-40g per body weight of the anaesthesia was used on the cats.

A 21G needle and a 2ml syringe **were** used to draw blood. The hair covering the inner layer of the thigh was scrapped off, and a tournique was tied around the upper region of the knee, before blood was drawn from the femoral vein. Only 2mls of blood was removed from each cat. The blood was separated and the serum was used to run the Indirect Haemagglutination test.

Calculation of K-values or Infection Risk using Van Der Veen's equation:

$$\left\{\frac{(\text{Log}_e P_o - \text{Log}_e P_1)}{(\text{Age Interval } (t))}\right\} \times 100$$

e.g. $$\left\{\frac{N_o \times 2.3026 - N_1}{5(t)}\right\} \times 2.3026$$

where $N_o = 44.29$, $N_1 = 41.47$

$= 1.6463 \times 2.3026 - 1.6177 \times 2.3026$

$= 3.79 - 3.72$

$= \frac{(0.07)}{5} \times 100$

$= 0.014 \times 100 = 1.4$

$K = 1.4$

QUESTIONNAIRE: TOXOPLASMA GONDII RESEARCH

1. Name:.....Hospital No:.....
2. Age:.....
3. Sex:.....
4. Occupation:.....
5. Address:.....
6. Marital Status:.....
7. (a) Full past obstetrics history:.....
   No. of spontaneous abortions:.....
   No. of still-births:.....
   (b) Past Medical history:.....
8. Any history of cervical Lymphadenopathy?.....
9. Soil Contact: (a) Gardening:.....(b) Farming..:.....
10. Presence of Household Pets: (a) Dogs .....(b) Cats.....
11. Type of Specimen: (a) Cord Blood:.....(b) Venous blood:.....(c) Products of conception:.....
12. Premature or Normal birth:.....
13. Present complaint:.....
14. Clinical findings or remarks:.....
15. Siblings:
    i) Name:.....
    ii) Sex:.....
    iii) Age.....
    iv) Any Congenital Anomalies?
    (a) Jaundice:.....(b) Hydrops Fetalis:.....
    (c) Chorioretinitis:.....(d) Hydrocephaly:.....
    v) Clinical findings:.....
    vi) Type of Specimen:
    (a) Venous Blood:.....(b) Cord Blood:.....
    (c) Faeces:.....

Histological Preparation of Tissue Sections

To examine the tissues that were collected from pregnant women as well as cats and mice, they had to be prepared and sectioned, thin enough to transmit light. To process such tissue sections, the following procedure had to be followed.

Tissues (e.g. placental tissue, cat liver, lungs, heart, spleen, as well as those of mice inclusive) of about 5 mm in thickness were fixed in 10% formal saline for at least 24 hours, to preserve the cells as life-like as possible. The tissues were then dehydrated through a graded series of alcohol, beginning from 70%, through to 100% ethanol, to remove all traces of water, and prevent the distortion of the tissue. After dehydration, they were cleared in chloroform to remove all traces of the alcohol, then they were infiltrated with molten paraffin wax in the oven, at $60^{o}C$; $2^{o}C$ higher than the melting point of the wax ($58^{o}C$). The tissues were given 3 changes in the molten paraffin wax, then blocking was done, from which sections were cut.

Sectioning was done on a microtome at 10 microns, and they were floated out on a water bath and mounted on slides. They were left on an oven to dry and were deparaffinized in xylene, hydrated through 100%, 90% and 70% alcohol, passed through water, and then stained with Haematoxylin and Eosin. They were then again dehydrated through 70% to 90% to 100% ethanol, and cleared in xylene, before they

were mounted with Canada Balsam and coverslips were applied over to prevent them from dust particles. When they were dry, they were observed under the microscope, before photomicrographs were taken of the parasites in the different tissues sectioned.

Preparation of Tris-HCl Buffer pH 7.5

(Tris = (Hydroxymethyl) Amino Methane)

HCl Molecular weight of Tris = 121.1

For 0.1ml Tris

$$\frac{0.1 \text{ ml}}{121.1} = \text{Moles} = \frac{\text{Mass}}{\text{M.wt}}$$

$$\text{Moles} = \frac{0.1 \times m}{121.1}$$

$\therefore$ M = 0.1 x 121.1 = 12.11g/l

$\therefore$ For 0.05m, we measure 6.055g/litre

For 0.1m HCl

86ml — 1000ml = 1m

8.6ml - 1000ml = 0.1m

$\therefore$ 4.3ml - 1000ml = 0.05m.

Therefore to prepare 0.05m Tris-HCl pH 7.5 6.055g of Tris was weighed and dissolved in 1 litre of distilled water. Then 4.3mls of Conc. Hydrochloric acid (HCl) was added to 1 litre of distilled water.

For Tris - HCl pH 7.5,we added 400ml of the prepared 0.05m HCl to 500ml Tris solution, and then made up to 1 litre with distilled water in a round bottomed flask. The resulting mixture, gave a solution of pH 7.5, when it was checked with a pH m64 Research pH meter.

This buffer was then used to elute the proteins from the antigen fractionated on Sephadex G200 column.

Preparation of Biuret Reagent for Protein Assay

Materials:

(1) Protein standard (5mg albumin/ml). This was prepared fresh.

(2) Biuret reagents:

Copper sulphate ($CuSO_4.5H_2O$)

Sodium potassium tartrate ($NakC_4O_6.4H_2O$)

Sodium hydroxide NaOH 10% ($^{w}/v$)

Potassium iodide.

Preparation of Biuret reagent (Royal Blue)

(a) 1.5g $CuSO_4.5H_2O$ was mixed with 6.00g $NakC_4O_6.4H_2O$, and stirred with a glass bar in a 1000ml volumetric flask.

(b) 500ml glass distilled water was then added to the flask and the solids.

(c) While the contents of the flasks were stirred vigorously, 300ml of 10% NaOH ($^{w}/v$) was added to the mixture.

(d) Then the stirrer was removed and the volume was made up to 1 litre with glass distilled water.

(e) The contents were thoroughly mixed and transferred to a plastic bottle. This mixture termed 'Royal Blue' can be stored indefinitely. However, if a black precipitate is observed, the solution is dissolved and a fresh one prepared.

BSA (Bovine Serum Albumin)

BSA	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.1
gdw	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.0
Biuret Reagent	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0

Biuret Assay:

The required quantity of the Biuret reagent (4 mls) was added to the stated quantity of protein solution diluted with distilled water, and both were mixed thoroughly, and incubated in the water bath at 37°C for 20 minutes. This was allowed to cool, and the extinction was read at 540nm, using the Unicam SP1800 ultraviolet spectrophotometer (PYE UNICAM). The readings were taken, after the spectrophotometer was standardized, and zeroed with the Tris-HCl buffer pH 7.5 in the cuvettes of both the reference beam and the sample beam. A graph of absorbance at 540nm was then drawn against protein concentration (in mg).

The graph is shown on Page 105 of the Text.

Result of Biuret Assay for Standard Curve

PROTEIN CONC. (mg/ml)	TUBE NUMBER	VOLUME OF BSA	ABSORBANCE AT 540 nm		
			1	2	3
0.00	1	0.00 =	Blank		
0.5mg/ml	2	0.1	0.024	.038	.023
1mg/ml	3	0.2	.048	.044	.048
5mg/ml	Sample (4)	0.5	.08	.082	.082
2	5	0.4	.09	.088	.088
3	6	0.6	.134	.136	.132
4	7	0.8	.178	.178	.178
5mg/ml	8	1	0.25	.23	.22

Sodium Azide NaN_3 (antimicrobial agent)

$NaN_3 = 23 + (14 \times 3) = 23 + 42 = 65.$

$Mw = 65$

$65g/litre = 1m$

$0.01m = 0.65gm/dm^3$

0.65gm in 1 litre of Distilled H_2O = 0.01m NaN_3

1ml of 0.01m NaN_3 was added to 1 litre of the buffer to keep out microbial growth.

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