A SEROLOGICAL SURVEY OF THE PREVALENCE OF ANTIBODIES TO TOXOPLASMA GONDII IN ANIMALS AND IN MAN IN KENYA

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1984.
DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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SUMMARY

Toxoplasmosis is a widespread zoonotic disease, caused by a protozoan parasite, *Toxoplasma gondii*. Toxoplasma infections have been recorded all over the world in most of the domesticated and wild animals, birds and man. Man can acquire the disease by ingestion of sporocysts from infected cats or from the ingestion of inadequately cocked meat of infected food animals.

Toxoplasmosis is a relatively uncommon clinical disease, although some of its manifestations are serious. Congenital toxoplasmosis is a medically and economically important disease, in both human beings and animals. In younger animals, toxoplasmosis may be fatal, causing considerable economic losses in sheep and goats.

Only limited studies on *Toxoplasma* infection have been carried out in domestic animals and man in Africa. The prevalence of *Toxoplasma* infection in Kenya is not known with certainty, and only a few records have been found concerning the prevalence of the infection in animals and man in this country.

The main objective of the work described in this thesis was to provide some data on the prevalence of *Toxoplasma gondii* infection in some animal species and in man in Kenya.
The Sabin and Feldman dye test (DT) has been considered the most reliable method for the detection of antibodies to Toxoplasma, and is still used as a reference test for comparison with other methods. In recent years, an enzyme immunoassay (ELISA) has been adapted as an adequate and more easily performed alternative method.

The ELISA was used in this study for the detection of antibodies to Toxoplasma in random serum samples from goats, sheep and man. Antigen preparation consisted of peritoneal fluid from mice infected experimentally with live virulent tachyzoites of the RH strain of T. gondii.

It was anticipated that the use of a crude non-purified antigen preparation which contained mouse serum components, especially mouse immunoglobulins, might give rise to false positive reactions in ELISA. Immunodiffusion and immunoelectrophoresis experiments revealed that anti-sera produced against the IgG of one species would react with immunoglobulins of other species, including mouse immunoglobulins. Since mouse immunoglobulins present in the Toxoplasma antigen preparation would adsorb to the plastic plates used in the ELISA, it was found that anti-sera to goat, sheep and human IgG had to be absorbed with mouse serum.
before species specific enzyme - conjugates lacking antibody activity mouse immunoglobulins could be prepared. In most cases the IgG fraction obtained by DEAE - cellulose chromatography of an absorbed antiserum was used for the preparation of enzyme conjugates, but purified antibodies were also occasionally used to obtain conjugates with high antibody activity and specificity. In addition, normal mouse serum was added to the conjugates to abolish all antibody activity to mouse immunoglobulins.

Although the procedures outlined above resulted in a considerable decrease in false positive reactions - a conclusion based on the absence of reactions by these conjugates when tested on plates coated with dilution of normal mouse peritoneal fluid or mouse serum - two additional factors were considered crucial to achieving maximum specificity for Toxoplasma antibodies. Firstly, the mouse immunoglobulins adsorbed to the plates might possess antibody activity to the immunoglobulins of the species whose serum was being tested in ELISA. Secondly, the test serum might possess antibody activity to mouse immunoglobulins adsorbed to the plates. In both cases,
false positive reactions would be observed. It was found that addition of normal mouse serum to the diluent used for the test serum samples almost completely abolished these reactions.

Further experiments were carried out to define optimal conditions for the ELISA. Particular attention was paid to the diluents used for conjugates and serum samples, as well as to the diluent used for the coating of the plates with crude antigen. The diluents found to be optimal in this study differ considerably from those described in the literature.

Using this ELISA system a comparative study was performed using serum samples with known DT titres. A total of 13 human sera and 11 goat sera were found to give ELISA values which correlated with the DT titres, 92% and 100% respectively, while the correlation was only 44% for 9 serum samples from sheep.

The possibility of cross-reactions due to infections with parasites possessing antigen(s) in common with *T. gondii* was not extensively examined although an obvious need exists for such investigations. The only relevant experiment performed was to investigate if hydatid cyst and *Cysticercus bovis* antigens were capable of reducing the ELISA values in 9 human serum samples.
with known DT titres. The results showed that no cross-
reactions occurred between Toxoplasma antigens and those of
hydatid cyst fluid and C. bovis, as no reduction in ELISA
values were found. Hydatidosis, a common condition of small
stock in Kenya should therefore not give rise to false
positive results in ELISA.

When this ELISA system was used in a survey for antibodies
to T. gondii in random serum samples from 155 goats, 158 sheep
and 214 human blood donors, prevalence rates of 21%, 56% and
43% respectively were found. While some caution must be
exercised in the interpretation of the prevalence rate found
in sheep, due to the low correlation between the DT and ELISA,
the results obtained with goat and human serum samples should
provide a reasonably accurate reflection of Toxoplasma infection
in these populations. The results are largely in accordance
with prevalence rates found in other parts of the world.

The high prevalence rates of Toxoplasma antibodies in
goats and sheep indicate that these animals may play an
important role in the epidemiology of Toxoplasma infections.
To identify the epidemiological factors of importance for maintaining the widespread distribution of the parasite, it is suggested that further investigations should be carried out on other common slaughter animals, domesticated animals and wildlife in Kenya.
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Dedicated to my wife, Rebeccah and our three children

Sarah Ullah, Milkah and David.
INTRODUCTION

More than seventy years have elapsed since Nicolle and Manceaux (1908) described the protozoan parasite, Toxoplasma gondii. Extensive studies, especially during the past three decades, have shown that Toxoplasma infection is a widespread zoonosis. Toxoplasma has been demonstrated all over the world in virtually all the domesticated and wild animals, birds and man. Numerous epidemiological studies have been carried out in most parts of the world.

Some reports have clearly shown a relationship between cats and the prevalence of Toxoplasma infection in man and other non-feline animals. The discovery that cats are the definitive hosts for T. gondii (Hutchinson et al., 1970, 1971), and the recent public awareness of the potential transfer of the infection from infected cats to their owners, have stimulated more interest and caused concern among veterinarians and the public health workers.

It now seems clear that Toxoplasma infection can be
traced directly to man's use of animals as pets or for food.

The potential role of meat animals serving as a source of infection with *T. gondii* has been well established since Jacobs and Melton (1957) developed a method to recover the parasite from meat samples. Several workers have shown that common food animals (sheep, cattle, pigs and goats) may have a degree of latent infection, and harbour *Toxoplasma* cysts in their tissues, which could serve as an important source of human infections (Work, 1967; Katsube *et al.*, 1975; Waldeland, 1976a). Ingestion of inadequately cooked meat (Durfee *et al.*, 1976) and unpasteurized milk are documented modes of transmission for human toxoplasmosis (Frenkel, 1973a).

Although toxoplasmosis is a relatively uncommon clinical disease, some of its manifestations are serious, especially in human foetuses; neonates, and children. Congenital toxoplasmosis is a medically and economically important condition, both in human beings and animals. In the U.S.A. an estimated 3,000 babies are born with the disease each
year (French, 1970). It has been calculated that of these 3,000 babies, 5 to 15% die, 8 to 10% suffer marked brain and ocular diseases, 10 to 13% have moderate to marked visual damage, 58 to 72% are clinically normal at birth but some of them develop active retinochoroiditis in childhood or young adulthood (French, 1970).

The total annual cost incurred by the neonatal toxoplasmosis cases in the U.S.A. has been estimated at 31-40 million U.S. dollars for hospitalization, institutionalization, and special public health education (Frenkel, 1973).

Whereas toxoplasmosis is generally clinically inapparent in adult meat animals, it has been shown to be fatal to younger animals, causing economic losses in sheep and goats. About 80,000 to 100,000 lambs are lost in Britain due to toxoplasmosis annually (Beverley, 1974).

Only limited studies on Toxoplasma infection have been carried out in domestic animals and man in Africa. Mas Bakal et al., (1968) performed a limited pilot study on the disease using the classical dye test. They established that
Toxoplasma infection actually exists in the Kenyan human population, and suggested further follow-up investigations. Olubayo and Mwongela (1978), and Olubayo and Karstad (1981) found two cases of fatal toxoplasmosis in wildlife in Kenya.

The prevalence of Toxoplasma infection in Kenya is not known with certainty. From the literature review, it has been recognized that a scarcity of Toxoplasma infection records exist regarding the work done on the prevalence of the infection in slaughter animals and man in Kenya.

The traditional serological tests that have been used for detecting Toxoplasma antibodies in animals and man are: the methylene blue dye test (Sabin and Feldman, 1948), indirect fluorescent antibody test (Goldman, 1957a, and b), and indirect haemagglutination test (Jacobs and Lunde, 1957). However, these methods have been found to pose some practical and technical difficulties.

Recently, Enzyme-linked immunosorbent assay (ELISA) has been found to be suitable for detecting antibodies in
human toxoplasmosis (Lin et al., 1980), and in goats (Berdal et al., 1983). Several reports indicate that ELISA is sensitive, specific, reproducible and can be used to analyse a large number of sera.

Hence, an indirect method of ELISA was adapted for use in this study for seroepidemiological survey of Toxoplasma antibodies in animals and man in Kenya.

It was considered that a study of the prevalence of Toxoplasma infection in Kenya would be of considerable interest. To understand further the epidemiological features of toxoplasmosis in Kenyan livestock, it was necessary to investigate toxoplasmosis in domestic animals which man uses as food.

It was therefore, the objective of the work described in this thesis to provide some of the missing epidemiological data on the possible existence of Toxoplasma infection in domestic animals and man in Kenya.
2. LITERATURE REVIEW

A. 1. AETIOLOGICAL AGENT.

Toxoplasmosis is caused by a coccidian parasite, **Toxoplasma gondii**, first described by Nicolle and Manceaux (1908) in the gondis (small Northern African rodent) which were maintained as laboratory animals at the Pasteur Institute in Tunis. The parasite belongs to the subphylum Apicomplexia (Levine, 1973) and classified in the family Eimeriidae. Other members of the coccidian group are the genera Eimeria, Isospora, Cystoisospora, Besnoitia, Hammondia, Frenkelia, Sarcocystis and Cryptosporidium. **Toxoplasma** and Cryptosporidium are the only coccidia known to have a zoonotic life-cycle, (Jacobs and Frenkel, 1982). **Toxoplasma gondii** is obligately intracellular and is indiscriminate in the types of host cell it will parasitize. There is only one recognized species of **Toxoplasma**, but Levine and Nye (1976) have recently described a new species **T. panae** from frogs, based solely on morphology.
A. 2. **LIFE CYCLE**

In 1965, Hutchison demonstrated a resistant form of *Toxoplasma* in cat faeces, and this formed a strong basis for the establishment of the life cycle of *Toxoplasma*. Hutchison et al., (1970, 1971) later demonstrated that *Toxoplasma* cysts, on ingestion by susceptible cats, went through a typical coccidian cycle in the epithelium of the small intestine.

Several workers have shown that the final hosts of *T. gondii* are the domestic cat and a number of other members of the family Felidae (Dubey, et al., 1970; Mckenna and Charleston, 1980; Frenkel and Ruiz, 1981; Fayer, 1981). Its predilection site in these definitive hosts is the small intestine, where it develops through a sequence of asexual and sexual stages. The final stages of development of *T. gondii* in the intestine of the felines are the resistant oocysts (Yilmaz and Hopkins, 1972; Ruiz et al., 1973; Frenkel et al., 1970, 1975). It has been shown that the resistant oocysts develop further into sporocysts which are infective to nearly all warm-blooded animals and man (Dubey et al., 1972; Hartley and Munday, 1974; Frenkel et al.,...
1975; Frenkel and Ruiz, 1981). Reptiles and amphibia have also been found infected (Jacobs and Frenkel, 1982).

A. 3. DISTRIBUTION OF TOXOPLASMA INFECTION

Toxoplasma gondii has an unusually wide host range for a protozoan parasite, causing infections in wild and domestic animals, birds and man (Franti et al., 1976; Fayer, 1981; Frenkel and Ruiz 1981). The parasite is widespread geographically, and has been studied in many parts of the world (Siim et al., 1963; Jones, 1973, Jacobs; 1973; Turner, 1976).

A. 3.1. Toxoplasma infection in man

Toxoplasmosis in man has been widely studied (Eichenwald, 1960; Kean et al., 1969; Kimball et al.; 1971a; Jacobs, 1973, Beverly, 1974, Stray-Pedersen, 1979; Williams, et al., 1981; Fayer, 1981). Toxoplasma infection is usually asymptomatic in adult humans and is often so in children.

Surveys from the tropical areas indicate that Toxoplasma infections exists among the indigenous people. Gibson and Coleman (1958) studied the prevalence of T. gondii infections in Guatemala and Costa Rica and found the overall prevalence was 94.0% in 100 persons and 50% in 30 Guatemala military recruits. Rawal (1959) found about 30% positive reactors in Bombay a titre of 8, and the rates were almost similar
in vegetarians and in meat eaters. Toxoplasma antibodies have been detected in human populations in Nigeria (Ludlam, 1965). As many as 83.3% of adult Nigerians around the Niger delta were found to have Toxoplasma antibodies. Fulton et al., (1966) tested sera from healthy Greeks, Liberians and East Africans for Toxoplasma antibodies. They found that 137/228 Greeks, 84/133 Liberians, 2/13 Bondei infants, 3/7 Bondei adults, 3/9 Masai infants and 20/43 Baganda adults gave dye test positive results. Mas Bakal et al., (1968) performed a pilot study of toxoplasmosis in Kenya and established a prevalence of 56% positive dye test among 106 human sera examined. In Brazil, Lamb and Feldman (1968) observed that more positive Toxoplasma reactors were found in military recruits from urban than from rural backgrounds; 57% and 48% respectively. Ludlam et al., (1969) found the prevalence of T. gondii to be 6% in the Chinese in Hong Kong. Suzuki et al., (1971) found the prevalence of T. gondii to be 16.5% in Japanese. Infected and inapparently healthy vegetarians (43.7%) including a Nigerian (Behymer et al., 1973) have been found to have T. gondii antibody levels. Wallace et al., (1974)
tested very primitive people in New Guinea and found the prevalence of the antibody against Toxoplasma to be between 14% and 34%. Wallace (1976) found the prevalence of Toxoplasma antibodies to be high, ranging between 84% and 100% in the adult population on the Pacific Islands. In South Africa, the prevalence of antibodies to Toxoplasma gondii was found to be 37% (Turner, 1976). Durfee et al., (1976) performed a seroepidemiological study of toxoplasmosis in Indonesia in 1050 persons and found that the prevalence of Toxoplasma antibodies was 9.7% to 51% in man. Omland et al., (1977) found an overall prevalence of Toxoplasma antibodies of about 60% at a titre of 4, and 45% at a titre of 10 in the native population of Liberia. Recently, Williams et al., (1981) carried out a prospective study in the West of Scotland in pregnant women, and found that the incidence of congenital toxoplasmosis was at least 0.5 per 1000 births.

An epidemic of acute toxoplasmosis was reported in five medical students who had eaten under cooked hamburgers (Kean et al., 1969). Acute toxoplasmosis has also been found in Brazil, Canada, Norway and U.S.A. (Stray-Pedersen, 1979; Fayer, 1981). During the acute primary infection
**Toxoplasma** localizes in nearly all the organs of the body and may cause the following changes: encephalitis, placentitis, lymphadenitis, and skeletal myositis. These inflammatory changes may cause severe or lethal illness, characterised by fever, malaise, pneumonia, splenomegaly, and lymphadenopathy. Women who acquire the infection during pregnancy may transmit it to their foetus *in utero* although they themselves are asymptomatic, resulting in congenital toxoplasmosis (Kimball et al., 1971a; Williams et al., 1981). Congenital toxoplasmosis may cause abortions, premature or stillbirths, or mental retardation in children (Langer, 1963; Kimball et al., 1971b; Terragna 1975; Beverley, 1974; Fayer, 1981). Acute *Toxoplasma* infections are usually terminated by the development of immunity. In Europe, congenital human toxoplasmosis ranges in frequency from 1 to 6% of the new born populations, and is probably the most serious form of *Toxoplasma* infection (Fayer, 1981).

A. 3.2 *Toxoplasma* infection in food animals.

A. 3.2.1 Swine

The first report of toxoplasmosis in swine was
published in U.S.A. (Farrel, et al., 1952). Harding et al.,
(1961) described an outbreak of toxoplasmosis in swine
in England. Three piglets had died and post-mortem
examination showed fibrinous pneumonia, focal necrotizing
inflammation in the liver, spleen, kidney and lymph nodes.
Jacobs, et al., (1960) isolated Toxoplasma from 24% of swine.
In Denmark, Work (1967) found the prevalence of Toxoplasma
antibodies in swine to be 35.2%. Catar et al., (1969)
reported the isolation of T. gondii from 11 of 15 (73.5%)
pools of diaphragms from 75 pigs in Czechoslovakia.
Katsube, et al., (1975) found that 10% of the apparently
healthy swine population in Japan had latent Toxoplasma
infection.

A. 3.2.2. Sheep and goats

Wickham and Carre (1950), described toxoplasmosis
in sheep in Australia, and in 1959, Osborne reported
abortion due to Toxoplasma in two flocks of ewes in
Australia. Jacobs et al., (1960) described the presence
of Toxoplasma in the flesh of 9.3% sheep. Beverly and
Watson (1961) isolated Toxoplasma from foetuses and
membranes of ovine abortions in England. Work (1967), working in Denmark, made a serological and parasitological survey of toxoplasmosis in domestic animals and found that 58% of sheep had Toxoplasma antibodies. Franti et al., (1975) found 13.0% of sheep tested were seropositive. In Norway, Waldeland (1976a) found that 10-15% of the lamb carcasses and 25-37% of the carcasses from mature sheep had T. gondii in their muscles, detectable by the peptic digestion method. In an investigation on the prevalence of Toxoplasma in sheep in Norway, he also found serological evidence of the infection in about 46% of the mature ewes and in about 2% of the lambs. Franti et al., (1976) found that about 15% of domestic sheep were infected with T. gondii in Northern America.

Durfee et al., (1976) found that the prevalence of Toxoplasma antibodies was 61% in goats in Indonesia. Chhobra et al., (1981) found that 34% of 371 apparently healthy dairy goats had Toxoplasma antibodies in India, and 4.9% of the animals tested had recent infection. Berdal et al. (1983) detected
Toxoplasma antibodies in goats in Norway using ELISA.

Toxoplasma in sheep and goats seems to run a subclinical course in the majority of cases. But in some countries Toxoplasma infection is the cause of considerable morbidity and mortality in domestic animals, especially in sheep and goats where abortions, stillbirths and weak lambs and kids are common manifestations. For example, Beverley and Watson (1961) working in England, Hartley and Kater (1964), in New Zealand, Plant et al., (1974) in Australia, and Chhabra et al., (1981) in India have reported reproductive losses due to ovine and caprine abortions and neonatal deaths in lambs. Toxoplasma infection in sheep and other food animals may also assume a chronic form with formation of cysts in muscular tissue without obvious clinical symptoms (Jacobs et al., 1960; Work, 1967; Waldeland, 1976 a,).

A. 3.2.3. Cattle

Spontaneous toxoplasmosis in cattle has been reported only once by Sanger et al., (1953). Toxoplasma in cattle seems to be much less frequent in contrast to other
food animals. Jacobs et al. (1960) isolated Toxoplasma from cattle and found that 1.7% of cattle were infected with Toxoplasma. Work (1967) found that 11.4% of cattle in Denmark had Toxoplasma antibodies. Catar et al. (1969) isolated T. gondii from 9.4% cattle in Czechoslovakia. Sogandares-Bernal et al. (1975) used a dye test to examine beef and dairy cattle in the northwest U.S.A. and found a lower prevalence of antibodies in beef cattle (27-37%) than in dairy cattle (62%). Makinde and Ozeh (1981) carried out a serological survey of T. gondii in Nigerian cattle and reported 65.2% cattle were seropositive for T. gondii.

Cattle generally have asymptomatic Toxoplasma infections as a cause of weak calves, but no abortions have been reported (Stalheim et al., 1980).

A. 3.2.4 Wildlife

Ratchiffe and Worth (1951) reported a case of toxoplasmosis in rock hyrax in Africa. Franti et al. (1975) performed a survey for Toxoplasma gondii antibodies
in deer and other wildlife and found that 20% of the deer tested were seropositive, 50% of the wild carnivores (badgers, bobcats, coyotes, foxes, racoons, and skunks) were seropositive. All the bobcats tested were seropositive, and of 120 native birds tested 5% were seropositive. They also found a seropositive rate of 2% among squirrels, 8% among jackrabbits and 6% among brush rabbits. Franti et al. (1976) found the prevalence of Toxoplasma infection in wild animals as being 10.8%. of the 10.3% wild animals surveyed, 68% bobcats, 28.0% coyotes, 27% grey foxes and 48% racoons had antibodies against \textit{T. gondii}. Toxoplasmosis has been reported in Kenya in a colobus monkey by Olubayo and Mwangela (1978); and tree hyrax (Olubayo and Karstad, 1981). Roher et al. (1981) reported a case of acute fatal toxoplasmosis in squirrels. Two squirrels had died suddenly and on post mortem examination, they had nonsuppurative encephalitis and interstitial pneumonitis, with typical \textit{T. gondii} cysts and tachyzoites in many body tissues.

Franti et al. (1975) found the prevalence of
Toxoplasma infection in domestic cats as 14%, and Behymer et al. (1973) reported the prevalence infection in cats as being 14%, and 15% in dogs. Durfee et al. (1976) tested 69 cats for Toxoplasma antibodies, and found that 41% cats were seropositive.

Jacobs et al. (1952) performed a survey of Toxoplasma infection of pigeons in Washington D.C., and found 12.5% positive. Surveys conducted on chicken in U.S.A. have indicated a high prevalence of infection (Jacobs and Melton, 1966). Franti et al. (1975) tested 120 native birds and found 6 (5%) positive. Franti et al. (1976) found the prevalence of T. gondii antibodies among birds as being 3.5%. Chicken infected with T. gondii rarely develop illness. However, a few instances of encephalitis have been reported (Jacobs and Melton, 1966).

A. 4. IMMUNITY TO TOXOPLASMA INFECTION

Beattie (1963) and Remington and Krahenbuhl (1976) have extensively reviewed immunity to Toxoplasma infection, which appears to be largely humoral in nature (Cox, 1968; Jacobs, 1973). During an infection specific antibodies are produced,
and the detection and quantitation of these antibodies have been the aim of most diagnostic tests. In adults, the acute disease is rare, but antibodies are commonly found in the population, indicating that exposure to *T. gondii* is quite common and that most infections are subclinical.

Protective antibodies against *Toxoplasma* pass via the placenta and the milk to the new born rats (Lewis and Markell, 1958). Fleck (1963) reported that human antibodies to *T. gondii* which react in the methylene blue dye test (DT) (Sabin and Feldman, 1948) are directed against a heavy antigen obtained by high-speed centrifugation. Lunde and Jacobs (1967) suggested that the DT antibodies have a high molecular weight and Staib et al. (1966) found that this antibody was inactivated by 2-mercaptoethanol suggesting that it is, in part at least an immunoglobulin M (IgM). In man, Remington and Miller (1966) found antibodies of the IgG class in the cord blood of mothers with chronic toxoplasmosis and also concluded that while antibodies of the IgM class predominate in acute toxoplasmosis, antibodies of the IgG class are more commonly found in the
chronic disease. The overall situation in toxoplasmosis is that circulating antibodies are probably protective against reinfection, but cannot eliminate the cystic stages of the parasite. Matsubayashi and Akao (1966) tested the functions of the Toxoplasma cysts. They labelled antibodies to Toxoplasma with ferritin and later located it on the surface of the free Toxoplasma organisms, whereas in the cysts, the ferritin-conjugated antibodies did not reach the parasites, but remained inside the vacuole containing them.

More recently, Handman et al. (1980); Naot et al., (1983) and Sharma et al. (1983) have demonstrated that human IgG and IgM antibodies are produced during acute primary infection with T. gondii.

A. 5. **TOXOPLASMA GONDII ANTIGENS USED IN IMMUNODIAGNOSIS.**

Protozoal parasites are antigenically complex and their antigens ill-defined. The parasitic antigens consist of a variety of proteins and polysaccharides. Despite the large number of studies of the antibody response to infection with *Toxoplasma gondii* in humans and animals, relatively little is known about the antigens which elicit the antibody response.
to this intracellular parasite. Since the parasite is obligately intracellular, it is difficult to obtain antigenic preparations of *Toxoplasma* free of host material. The organism has never been successfully cultured in a cell-free medium to provide material for antigenic studies.

In 1942, Warren and Sabin used an extract from infected rabbit brain as an intact parasite antigen. Sabin and Feldman (1948) used isolated intact parasite antigen for use in their methylene blue dye test. Steen and Kass (1951) first described the preparation of a soluble antigen from infected mouse peritoneal exudate for a complement fixation test for toxoplasmosis.

Studies performed by Handman *et al.*, (1980); have demonstrated that *T. gondii* tachyzoites have four major membrane surface proteins that are recognized by sera obtained from mice chronically infected with *T. gondii*. Mechanical fragmentation of a whole parasite by either lysis-in-water, sonication or freeze-thawing, also yield soluble cytoplasmic antigens (Steen and Kass, 1951; Voller *et al.*, 1976; Hughes and Barfour, 1981; Sharma *et al.*, 1983). In their studies, Handman *et al.*, (1980) also
found that three different *Toxoplasma* strains (Virulent RH human strain, moderately virulent C56, and avirulent C37 chicken strains) possessed identical membrane antigens, despite their variability in virulence. In the case of *Toxoplasma*, virulence appears to be correlated with generation time, but does not seem to correlate with the antigenic pattern of the parasite membrane (Kaufman et al., 1958, 1959).

Hughes and Barfour (1981) investigated the basic antigenic structure of *T. gondii* and defined a total of 11 antigenic components using saponin and octyl glucoside for solubilization. They showed that antigen 3 is a glycoprotein, antigen 7 is a lipopolysaccharide and antigens 1, 2, 4, 5, 6 and 8 to 11 are proteins, and the majority of the 11 antigens have a molecular weight of $10^5$ to $1.5 \times 10^5$ daltons. They also found that all the 11 distinct antigens were recognized in a soluble antigen preparation of *T. gondii* by a serum from an individual with natural *Toxoplasma* infection. Their work was in agreement with the previous work by Chordi et al. (1964) who showed that freeze-thawed and hypotonic lysate antigens both contain several
Toxoplasma components (at least 7) with which human anti-

Toxoplasma sera will react.

In addition to the protein components, T. gondii
polysaccharides have been reported to be antigenic and
reactions between the polysaccharides extracted from T. gondii
and human antibodies from infected individual have been
observed by the same research workers (Pande et al., 1961;
Handman et al., 1980). Mineo et al., 1980) reported that
human IgM antibodies react preferentially with polysaccharides
present in T. gondii, whereas IgG antibodies recognize
primarily the protein antigens.

The most recent studies on the immune response to
T. gondii antigens have been those of Handman et al. (1980).
Hughes and Barfour (1981), Naot et al. (1983) and
Sharma et al. (1983), which have revealed that both
IgM and IgG antibodies are produced to both protein and
carbohydrate components of the invading parasite. The
observations by Handman et al. (1980) were substantiated
by Sharma et al. (1983) who also found that IgG antibodies
recognise 4 major polypeptide species corresponding to molecular weights of 43,000, 32,000, 27,000, and 21,000 daltons. IgG antibodies appear to react with all the antigens recognized by IgM antibodies.

A. 6. IMMUNODIAGNOSTIC PROCEDURES WHICH HAVE BEEN APPLIED TO T. GONDII INFECTIONS.

The diagnostic procedures used in the diagnosis of toxoplasmosis consist of the following:- serology, isolation of the parasite, microscopic demonstration of the organisms in tissue smears or sections, or demonstration of oocysts in the faeces of cats.

A. 6.1 Serological examination

Toxoplasmosis serology is fairly complex. The reviews on the "immunity to Toxoplasma infection" by Beattie (1963) and Remington and Krahenbuhl (1976) clearly indicate that antibodies are produced in toxoplasmosis. This has led to the development of a number of serological tests, a topic which has been reviewed by Fulton (1963), Jacobs (1976), and Lumsden (1979). The tests may be divided into broad groups: those utilizing the intact parasite as antigen and those using soluble fractions.
A. 6.1.1 **Methylene blue dye test.**

The methylene blue dye test of Sabin and Feldman (1948) is widely recognised as the reference test in most countries (Fleck, 1961, 1963; Denmark and Chessum, 1978), and has the advantage that the results can be compared with an agreed International Standard. The dye test is essentially a neutralizing test conducted *in vitro*. Despite many rigorous attempts at standardization (Beverley and Beattie, 1952; Waldeland, 1976b) which have improved the reproducibility and efficiency of the test, it still remains largely subjective, time consuming, expensive and potentially hazardous. It utilizes live virulent *Toxoplasma* parasites, which must constantly be maintained in the laboratory by animal passage. The test has been described by some authors as specific, sensitive and capable of giving quantitative and reproducible results (Wallace, 1969). However, the specificity of the dye test has been questioned by Awad (1954a and b) and Awad and Laison (1954) who recorded cross-reaction in animals infected with *Trichomonas*, *Sarcocystis*, and *Trypanosoma cruzi*. The value of the test has nevertheless been strongly emphasized...
by Jacobs and Lunde (1957).

In addition to specific antiserum, an accessory factor or "activator", derived from certain human sera is necessary. The accessory factor is heat-labile and loses activity rapidly except when frozen. Sabin and Feldman (1948) suggested that it was different from complement because it could not be replaced by $C_1$ and $C_2$ after heating the serum. Suzuki and Tsunematsu (1971) confirmed the importance of the 'activator' in the dye test reaction, and showed it to be properdin plus complement $C_1$, $C_2$, $C_3$, $C_4$ plus magnesium ions.

Recently, Hagiwara and Katsube (1981) have applied the dye test on meat extracts obtained from swine, and found that antibodies against *Toxoplasma* in the extracts could be detected by the test.

A. 6.1.2. Complement fixation test (CFT)

Complement fixation was first used by Nicolau and Ravelo (1937) as a diagnostic test for *Toxoplasma* antibodies. It was then adapted by Warren and Sabin (1942). They used an
extract of frozen and thawed infected rabbit brain as antigen and obtained specific results with serum from man and monkeys, but not with that of rabbits, dogs and cats. They observed that complement fixing antibodies appeared 1-4 weeks after infection, but tended to disappear about one month later. The same observations were made by Warren and Russ (1948). The studies of Warren and Sabin (1942) and Warren and Russ (1948) indicate that complement fixing antibodies differ from the dye test antibodies; since they appear later and disappear earlier (Sabin et al., 1952). Furthermore, sera with high titres of DT antibody may be devoid of complement fixing antibodies. The complement fixation is widely used; frequently in conjunction with other tests, and has the advantage that live parasites are not required.

Steen and Kass (1951) first described the preparation of an antigen from mouse peritoneal exudate for the
Toxoplasma complement fixation test. The antigen was water-soluble; not anti-complementary and fixed complement
specifically in the presence of human immune serum. Cooney et al. (1958) favoured the use of the antigen preparation of Steen and Kass in preference to that from chorionicallantoic membrane of chick embryos (Warren and Russ, 1948). Fulton and Sulton (1962) used a pure suspension of Toxoplasma freed from other cells as antigen for the complement fixation test with satisfactory results. Pettersen (1968) showed that alkaline extraction was a more efficient method for obtaining CFT antigen than the method of Warren and Sabin (1942).

A. 6.1.3. **Indirect fluorescent antibody test (IFAT)**

The use of immunofluorescence in the serodiagnosis of toxoplasmosis was developed by Goldman (1957 a, b). He observed that smears of Toxoplasma in mouse peritoneal exudate could be stained by fluorescein-labelled antibody prepared from the globulin fraction of the serum of infected humans or rabbits with high dye test titres. The results of the tests were compared with those obtained by the dye test and by complement fixation. In the first comparison there was a close correlation between the results, but not in the second.
The author points out some of the technical difficulties of the IFAT which include the use of a darkroom, temperature control, fluorescent equipment and staining as well as preparation of the labelled antibody. The test is equal to the methylene blue dye test in specificity and sensitivity and eliminates the need for living organisms and human accessory factor (Walton et al., 1966). Carver and Goldman (1959), investigating the possibility of staining *T. gondii* in tissues, found that formalin and other commonly used histological fixatives abolished the IFAT staining capacity. The IFAT detects antibodies elicited by surface antigens and which appear earlier during infection than antibodies to intracellular antigens measured by other serological methods. Kelen et al. (1962) first used the IFAT and for many years this technique has been used by many research workers with remarkable success (Fletcher, 1965; Jacobs, 1973).

In general, the IFAT agrees well with the dye test, and detects antibodies as early as the dye test, with comparable titres. False positive anti-toxoplasma IFA (based on detection of IgM) reactions may occur with sera from patients with antinuclear antibodies and rheumatoid factor (RF) (Araujo
et al., 1971). Remington (1969) adapted the IFAT to demonstrate the presence of IgM antibodies, but Camargo, et al. (1978) suggested that the IgM-IFAT was of limited use in the diagnosis of congenital toxoplasmosis in the newborn children.

A. 6.1.4. Indirect haemagglutination test (IHAT)

Jacobs and Lunde (1957) developed a haemagglutination test for the diagnosis of toxoplasmosis. The antigen was prepared by lysis of T. gondii harvests (in distilled water) from mouse peritoneal exudates 'cleaned' by the method of Fulton and Turk (1959), and used to sensitize tanned sheep cells. Karim and Ludlam (1975) found that the time course for the production of antibodies detected by the IHAT and the dye test differed, as the response detected by the dye test occurred earlier in the infection, and that of the IHAT later. Death and lysis of parasites is required for the liberation of soluble immunogenic materials, which would account for the late response measured by the IHAT, in which a soluble antigen is used. Kobayashi et al. (1971) performed a comparative study between the dye test and 3 different haemagglutination
techniques and found 94% agreement among the tests using human serum; and 83-92% agreement with cat serum, but only 83 to 88% agreement with dog serum. Several research workers have found IHAT a reliable and sensitive test in detection of antibodies against *Toxoplasma* (Behymer, *et al.*, 1973; Franti *et al.*, 1976; Handman, *et al.*, 1980; Makinde and Ezeh; (1981) have shown that the use of ultrasonicated and water-lysed soluble antigenic preparations of *T. gondii*, correlated closely in the IHAT, CFT and ELISA techniques. Recently, Hughes and Barfour (1981) have shown that antigen 6 which is heat-stable at 56°C for 1 hour, appears to be the major soluble reactive antigen in the IHAT.

The indirect haemagglutination test has some advantages such as: it can be done rapidly with one drop of serum or with blood collected on a filter paper disk, results can be determined in 2 hours, the antigen is commercially available and is stable for about 6 months, and confirmation of the current infection can be made if paired serum samples collected 2 weeks apart indicate a rise in titre
A. **6.1.5. Direct agglutination test (DAT)**

Fulton and Turk (1959) developed a direct agglutination test for toxoplasmosis in which pure suspensions of whole *T. gondii* organisms free from other cells were used. Further studies by Fulton (1965a, b) revealed that titres of direct agglutination test were similar to those found in the dye and complement fixation tests. The test appears to be specific as only the homologous organisms were capable of reducing the titres to *Toxoplasma* antigens in absorption experiments (Fulton, 1963, 1965a,b). Other absorption experiments were performed using antigens of *Leishmania donovani*, *Trypanosoma lewisi*, *T. cruzi*, *T. vivax*, *T. gambiense*, *Plasmodium berghei* or *Borrelia recurrentis* and no cross-reactions between them and *Toxoplasma* were observed (Lumsden, 1979).

Although the test is not widely used, it has a number of advantages over the other serological tests as it is easy to perform, a stable antigen of dead parasites is employed and a microtechnique system has been developed (Fulton, 1965a).
A. 6.1.6. **Precipitin test (PT)**

O'Connor (1957a, b) used a double agar gel diffusion technique (Ouchterlony method) to demonstrate anti-Toxoplasma precipitins in the aqueous humour of the eyes of patients suffering from uveitis. Antigen prepared from the peritoneal exudate of infected mice was used. Precipitin lines formed against *T. gondii* antigens, after incubation time of 2 days up to 3 weeks. Tonjum (1962), working on the extraction of soluble *T. gondii* antigens, found 2 lines of precipitation with human sera of high dye test titres (1:32,000). Chordi et al. (1964) found four precipitin lines.

A. 6.1.7 **Other serological test**

These include flocculation tests using acrylic particles (Siim and Lind, 1960), latex particles (Lunde and Jacobs, 1967b) coated with Toxoplasma antigen.

In general, the most useful and widely studied methods for the serodiagnosis of toxoplasmosis are the methylene blue dye test, indirect immunofluorescence, indirect haemagglutination, the complement fixation, and lately the enzyme-linked immunoasay, which will be described in detail later.
A. 6.2. **Isolation of the Toxoplasma organisms**

Diagnosis of toxoplasmosis by isolation of the parasite from infected tissues is briefly reviewed. Jacobs and Melton (1957) developed a method for the isolation of *Toxoplasma* by means of peptic digestion technique. Jacobs et al. (1960) used the peptic digestion method to isolate *Toxoplasma* from relatively large samples from cattle. Work (1967) used Jacobs and Melton's isolation technique to recover *Toxoplasma* from the flesh of sheep, swine, and cattle. Waldeland (1976a) detected *T. gondii* in the muscles of sheep of different ages by peptic digestion method (Jacobs and Melton, 1957) modified as recommended by Work (1967). Apart from the pepsin enzyme digesting the host tissues and the cyst wall of the parasite, it also tends to digest the bradyzoites. The trypsin digestion method has been found to be better than the former (Katsube et al., 1975; Jørgensen and Otwelo, 1978).

Isolation of *T. gondii* is carried out best by the inoculation of the mice. After isolation of the parasites from infected tissues, the mice are infected intra-peritoneally and examination after 6-10 weeks for the presence of cysts.
in the brain using both fresh squash preparations or permanently stained smears or sections as described by Beverley and Watson (1961). A simplified procedure for the isolation of oocysts of *T. gondii* from the faeces of cats has been reported (Wallace, 1971; Dubey et al., 1972).

A. 6.3. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

At present, there are two widely accepted assay that employ labelled antibodies or antigens. They are immunofluorescence (Coons and Kaplon, 1950; Goldman, 1957a; Kelen, 1962; Fletcher, 1965), in which a fluorescent marker (fluorescein) is conjugated to the antibody or antigen, and the radio-immunoassay (RIA) (Yalow and Berson, 1959, 1960), in which isotopes are attached to the antibodies or antigens. In practice, immunofluorescence and radio-immunoassays present some practical problems.

Miles and Hales (1968) suggested the use of enzymes as labels of antibody or antigen in immunological assay systems provided a suitable immunoadsorbent could be made. Then Engvall and Perlmann (1971) described an enzyme-linked immunosorbent assay (ELISA) to estimate the protein concentration in fluids, particularly gammaglobulins, and in the same year, Van Weemen and Shuurs independently described an enzyme
immunoassay (EIA) for hormones. In essence, the four workers modified the radioimmunoassay (RIA) of Yalow and Berson (1959, 1960) by substituting an enzyme for the radiolabel of the antiserum.

For the past 12 years since its inception, ELISA has been shown to be applicable to a variety of diseases and conditions in endocrinology, microbiology, parasitology, immunopathology (Voller et al., 1976b, 1978, 1979). Reviews on enzyme immunoassays of Voller et al., (1976c, 1978, 1979); Shuurs and Van Weemen (1977); Voller (1981), Clark and Engvall (1981) classify the assay as follows: (i) Homogenous enzyme immunoassay and (ii) Heterogenous enzyme immunoassay. The homogenous enzyme immunoassay is a competitive assay for the antigen, and was used by Van Weemen and Shuurs (1971), Saunders and Wilder (1974), and Engvall and Perlmann (1971). In the heterogenous procedure of ELISA, one of the reactants is adsorbed onto a solid phase carrier i.e. the immunosorbent. The ELISA procedures most frequently used to detect antigens is the double antibody technique (Voller et al., 1978, 1979). The ELISA has been
used by several workers in the diagnosis of infectious diseases where the need is often for the measurement of antibody where the indirect method of ELISA is used (Engvall and Perlmann, 1972; Voller et al., 1976a). Various other modifications of ELISA have been used, such as an inhibition assay for antigen (Voller et al., 1978, 1979).

Various solid phases have been used in ELISA. These include particulate materials such as cellulose, agarose, polyacrylamide, cross-linked dextrans, silicone rubber, microcrystalline glass and plastic (van Weemen, 1974; Voller et al., 1978). Plastic carriers made of polyvinyl (Ruitenberg et al., 1974; Walls et al., 1977); polypropylene (Engvall and Perlmann, 1971), treated glass, polycarbonate or silicone rubber (Hamaguchi et al., 1976a, b) have all been tried. Voller et al., (1974) introduced the use of disposable microtitration plastic plates made of polystyrene as solid-phase carriers.

The ELISA utilizes conjugates which are made of immunoglobulins coupled to enzymes. Alkaline phosphatase and horseradish peroxidase (HRPO) are the two commonly employed enzymes in labelling antibody or antigen (Shuurs and van Weemen, 1977; WHO, 1976;
Nakane, 1979). Glucose oxidase and β-galactosidase are used to a more limited extent (Voller, 1981).

Substrates are usually chosen to give a colour product following enzymatic degradation. Ortho-phenylene-diamine is suitable for use with HRPO conjugates (Nakane, 1979), although it is light sensitive and reputed to have mutagenic properties (Voller et al., 1978). For alkaline phosphatase conjugates, para-nitrophenyl phosphate is an excellent substrate (Avrameas, 1969; Engvall and Perlmann, 1971; Bullock and Walls, 1977; Ruitenberg and Buys, 1977).

A. 6.3.1 Expressing the ELISA results

At present, there is no generally accepted way of expressing ELISA results (Voller et al., 1978; Voller and de Savigny, 1981). The end result of an ELISA test may be subjectively assessed by eye; or it can be measured photometrically. A few possible ways of recording photometrically derived results are listed below (Voller et al., 1978; de Savigny and Voller, 1980; Voller, 1981; Voller and de Savigny, 1981):

(i) As 'positive' or negative. All test samples giving an absorbance value above a threshold level are considered
as positive.

(ii) As "absorbance value". The absorbance values can be used as a direct measure of the sample's reactivity (Voller et al., 1976c).

(iii) As an end-point "titre". Samples are serially diluted and all dilutions are tested by ELISA. The 'titre' is the last dilution which yields an absorbance value above that of a group of known negative samples.

(iv) As a "ratio" (Positive/Negative or P/N ratio). The ratio of the absorbances of the test samples compared to a group of known reference negatives. The P/N ratios greater than 2 (or 3) are usually considered positive (de Savigny and Voller, 1980).

(v) As actual concentration of the substance being determined by reference to a standard curve prepared by plotting absorbance values of a series of reference substances containing different amounts of the substance.

(vi) As international unit per millilitre (iu/ml). Absorbance values of test samples at one serum dilution only converted to international units per millilitre and compared with the standard reference serum. (Lin et al., 1980).
(vii) As Multiple of Normal Activity (MONA) – proposed by Felgner (1973). Absorbance values of test samples at a single serum dilution are converted into MONA using a formula derived by Felgner (1973).

A. 6.3.2. Detection of antibodies to Toxoplasma gondii using ELISA

Engvall and Perlmann (1971) first showed that antibodies could be measured by the indirect ELISA using enzyme labelled antiglobulins as indicators. Voller et al. (1976a) introduced a microtitre ELISA for the diagnosis of toxoplasmosis. Several workers have tried to use ELISA to detect antibodies against T. gondii and found it specific, highly reproducible and sensitive (Walls et al., 1977; Milatovic and Braveny, 1980; Lin et al., 1980; Woodward, 1982). Other workers have tried to standardize ELISA technique using toxoplasmosis as a test model by which the parameters of the technique could be delineated (Bullock and Walls, 1977; Lin et al., 1980). The indirect immunofluorescence test for toxoplasmosis is well defined and standardized and thus made an excellent system for comparison with ELISA (Bullock and Walls, 1977).
The Toxoplasma ELISA data so far published compare favourably with current tests such as immunofluorescence, indirect haemagglutination, dye test, and complement fixation (Voller et al., 1976a, Bullock and Walls, 1977, Walls et al., 1977; Milatovic and Braveny, 1980; Lin et al., 1980). Most research workers have used the crude soluble Toxoplasma antigen first described by Steen and Kass (1951). Using this type of antigens in a micro-ELISA test, Voller et al. (1976a) and Milatovic et al. (1980) showed that the antibody titre obtained by ELISA correlated better with the IHAT than the DT. This seems to be based on the fact that ELISA and IHAT utilise soluble antigens (Karim and Ludlam, 1975) whereas the DT and IFAT are performed with intact parasite antigen (Karim and Ludlam, 1975). Walls et al. (1977) also showed that ELISA results were comparable to the IFAT. ELISA determination yields results which are presumably a summation of all the antibodies directed against several Toxoplasma antigens (Handman et al., 1980; Hughes and Barbour, 1981).
Several authors have recommended ELISA procedures for the demonstration of the IgG *T. gondii* antibodies in human toxoplamosis (Voller et al., 1976a; Walls et al., 1977; Lin et al., 1980; Woodward, 1982) as well as IgM (Camargo et al., 1978, Duermeyer et al., 1980; Naot and Remington, 1980; Naot et al., 1981a; Naot et al., 1983).

Camargo et al., (1978) investigated the diagnostic possibilities of the micro-ELISA, performed with enzyme-labelled anti-IgG and anti-IgM antibodies, in human toxoplamosis. They found excellent agreement between IgG-ELISA and IgG-IFA titres, IHAT titres in both recent and old infections. When they compared the IgM-ELISA and IHA test results in acute infections, large variations were frequently seen between the corresponding titres. The discrepancies existed because IgM-ELISA detects only IgM antibodies, and IHA test detects both IgG and IgM antibodies.

Following Camargo et al., (1978) observations on IgM-ELISA and IgG-ELISA, Naot and Remington, (1980); Duermeyer et al., (1980), and Naot et al., (1981a) described an ELISA
for the detection of IgM antibodies in acute toxoplasmosis in adults and congenital toxoplasmosis in children. They found that IgM-ELISA was significantly more specific and more sensitive than IgM-IFAT in detection of IgM antibodies to Toxoplasma. Serum containing antinuclear antibodies (ANA) (Araujo et al. 1971) or rheumatoid factor (RF) (Camargo et al. 1978) may cause false-positive results. An IgM-ELISA described by Naot and Remington, (1980); Duermeyer et al., (1980) and Naot et al.,(1981b) gave no false-positive results with sera containing RF and ANA. Recently, Naot et al. (1983), used IgM-ELISA and IgG-ELISA to show that IgM and IgG antibodies are produced in response to both protein and carbohydrate constituents of the invading parasite.

Berdal et al. (1983) seem to be the only workers who have described an ELISA sandwich procedure for detecting antibodies against Toxoplasma gondii in the goat. They also compared the ELISA with the standard DT and found a good correlation of 98% between their ELISA and the dye test.
3. MATERIALS AND METHODS

3.1 ANTIGEN

Toxoplasma gondii antigen was obtained from the Norwegian Defence Microbiological Laboratory (NDML) and the College of Veterinary Medicine (CVM), Oslo, Norway in a crude form through the courtesy of Dr. T. Omland and Mr. Olsvik.

The crude Toxoplasma gondii antigen

Briefly, the antigen had been prepared as follows:

Mice were injected intraperitoneally with live virulent tachyzoites of the RH strain of T. gondii. The parasites were harvested from the mice after 5-7 days post infection. The harvested peritoneal fluid containing live parasites was centrifuged at 1200xg for 10 minutes. The sediment was washed 3 times with phosphate buffered saline (PBS) by successive centrifugation. The washed parasites were killed by freeze-thawing three times. The crude antigen consisted of ruptured whole parasites at a concentration of $5.8 \times 10^6$ parasites per ml as quantitated by microscopy of a sample of the mouse peritoneal exudate. The antigen solution was stored in 2 ml aliquots at -20°C.
3.2 **SERUM SAMPLES**

Sera from human, goat and sheep were used for testing for antibodies against *T. gondii.* The *Toxoplasma* positive control sera from sheep and goat were produced by immunization of the animals with dead *T. gondii* in mouse peritoneal fluid.

### 3.2.1 Control sera

#### 3.2.1.1. Human sera

Five dye test positive and three dye test negative human sera received from Oslo, Norway, through the courtesy of Dr. T. Omland and Mr. Olsvik were tested by ELISA. Their reference numbers are shown as follows:

- a) 0405/82 (IgM+ve) 300 iu/ml .......... DT +ve
- b) 018/82 (IgM+ve) 300 iu/ml .......... DT +ve
- c) 076/82 (IgM+ve) 300 iu/ml .......... DT +ve
- d) 16(20 iu/ml) .......... DT +ve
- e) 1024/82 (IgM+ve, ELISA-ve) .......... DT +ve
- f) 1064/82 .......... DT -ve
- g) 0422/82 .......... DT -ve
- h) 1045/82 .......... DT -ve
The Toxo-positive control sera and two negative control sera were received from the National Bacteriological Laboratory, Stockholm, Sweden, through the courtesy of Professor Gunnel Huldt. Their reference numbers are shown as follows:

a) No. 12 Toxo-positive control .......... DT +ve
b) Toxo-positive control ................. DT +ve
c) No. 14 Toxo-negative control ......... DT -ve
d) Toxo-negative control ................. DT -ve

3.2.1.2 Sheep sera

The following dye test positive and negative sheep sera were received from Oslo, Norway, through the courtesy of Dr. T. Omland and Mr. Olsvik, tested in ELISA and used as control sera:

a) 11/80 sheep ................................ DT +ve
b) Sheep 1600 iu/ml .......................... DT +ve
c) Naturally infected sheep (No. 256) .... DT +ve
d) Sheep I a .................................. DT +ve
e) Sheep III. (ELISA IgM +ve) ............. DT +ve
f) Sheep IV (ELISA IgM +ve, IgG +ve) .... DT +ve
3.2.1.3. Goat sera

The following dye test positive and negative goat sera were received from Oslo, Norway, through the courtesy of Dr. T. Omland and Mr.Ølsvik, tested in ELISA and used as control sera:

<table>
<thead>
<tr>
<th>Sera Description</th>
<th>DT Test Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>g) 231/82 sheep</td>
<td>DT -ve</td>
</tr>
<tr>
<td>h) Sheep II</td>
<td>DT -ve</td>
</tr>
<tr>
<td>i) Sheep I b</td>
<td>DT -ve</td>
</tr>
<tr>
<td>a) 30/12/82 Goat ELISA Toxo- (+ve)............</td>
<td>DT +ve</td>
</tr>
<tr>
<td>b) 2/3/82 Goat immunized</td>
<td>DT +ve</td>
</tr>
<tr>
<td>c) Immunized goat 01</td>
<td>DT +ve</td>
</tr>
<tr>
<td>d) Immunized goat 02</td>
<td>DT +ve</td>
</tr>
<tr>
<td>e) Immunized goat 03</td>
<td>DT +ve</td>
</tr>
<tr>
<td>f) Immunized goat 04</td>
<td>DT +ve</td>
</tr>
<tr>
<td>g) Immunized goat 05</td>
<td>DT +ve</td>
</tr>
<tr>
<td>h) Immunized goat 06</td>
<td>DT +ve</td>
</tr>
<tr>
<td>i) 30/12/82 (585) Goat ELISA Toxo (-ve)...</td>
<td>DT -ve</td>
</tr>
<tr>
<td>j) Goat 02</td>
<td>DT -ve</td>
</tr>
<tr>
<td>k) Goat 051</td>
<td>DT -ve</td>
</tr>
</tbody>
</table>
3.2.2 **Test serum samples**

One hundred and fifty eight sheep sera and one hundred and fifty five goat sera were collected randomly from slaughterhouses in Kenya. They were all tested by ELISA.

Two hundred and fourteen human serum samples were obtained from the blood bank, Nairobi, Kenya, and were tested by ELISA.

3.2.3 **Antisera to be used in the ELISA setup**

The antisera used are listed in Table 1. These antisera were prepared by colleagues in the Immunology Laboratory in the Department of Public Health, Pharmacology and Toxicology.
Table 1. Antisera used in ELISA

<table>
<thead>
<tr>
<th>Identification (Animal Species and No.)</th>
<th>Preparation for immunization</th>
<th>Antisera prepared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat No. 381 and No. 382</td>
<td>Human IgG</td>
<td>Goat (381/382)-anti-human IgG serum</td>
</tr>
<tr>
<td>Sheep No. 160 and No. 161</td>
<td>Goat IgG</td>
<td>Sheep (160/161)-anti-goat IgG serum</td>
</tr>
<tr>
<td>Goat No. 632 and No. 633</td>
<td>Sheep IgG</td>
<td>Goat (632/633)-anti-sheep IgG serum</td>
</tr>
</tbody>
</table>
3.3 Analysis of the antisera used in this study

Experiments were performed to ascertain the specificity of the antisera by immunodiffusion and immunoelectrophoretic techniques. The antisera had been raised by injections of 0.015M phosphate buffer, pH 8.0, eluate from DEAE - cellulose columns, which should contain purified IgG of a particular species. The antisera were thus expected to react with immunoglobulins - particularly IgG - of that species, and possibly with immunoglobulins - of some other species. The experiments were done to elucidate the specificity of these antisera for immunoglobulins of the homologous species and also their reactivity with serum components of heterologous species, in particular their cross-reactions with mouse serum components known to be present in Toxoplasma antigen used in the enzyme immunoassay.

3.3.1 Immunodiffusion and Immunoelectrophoresis.

3.3.1.1 Immunodiffusion.

The following antisera were tested:
i) Sheep (160/161)-anti-goat IgG serum

Sheep (160/161)-anti-goat IgG serum was tested in the immunodiffusion to check for its reactivity with other animal species (goat, bovine, human, mouse and rabbit) (Fig. 4).

ii) Goat (632/633)-anti-sheep IgG serum

The antibody activity and cross-reactivity of the antiserum was checked in immunodiffusion against other animal species (Fig. 9).

iii) Goat (381/382)-anti-human IgG serum

The unabsorbed goat (381/382)-anti-human IgG serum was tested for its cross-reactivity against serum of other animal species and also against crude *Toxoplasma* antigen (Fig 6).

The goat-anti-human IgG serum was then absorbed with insoluble immunosorbents prepared with mouse, rabbit, bovine, and sheep sera (Appendix 1) twice, before precipitation with sodium sulphate (18%), and seven times after precipitation, with mouse and rabbit serum immunosorbents only, and then tested for cross-reactions again as shown in Fig. 3.

The Ouchterlony double diffusion technique as described by
Crowle (1973) was used with slight modifications. Briefly, the method was as follows:

1% (W/V) of purified Oxoid agar (Oxoid Ltd., London, England) in PBS, pH 7.5 was used in the preparation of the gel; 0.1% sodium azide was added as a preservative (Appendix 7.1).

Melted agar in a volume of 3.5 ml was poured on each slide (size 75 mm x 25 mm). With a gel puncher (Gelman Instrument Co. Ann Arbor Michigan, U.S.A.), 6 wells of 4.0 mm diameter and 5 mm apart were cut in a hexagonal pattern with an additional central well. The central well was filled with 15.0 ul of different undiluted antisera once or twice, whereas the peripheral six wells received 15.0 ul of different antigens once or twice, depending on the potency of the antisera or antigens. The slides were initially kept in a dry atmosphere at room temperature (25°C) for 2 hours and later incubated in a humid chamber overnight at room temperature, then the gel was pressed as described by Weeke (1973). They were then left in 3% (W/V) trisodium citrate (Koch Light Laboratories Colunbrook, Buckshire, England) pH 8.5 for 24 hours in order to remove the non-precipitated proteins. After washing
and rinsing with distilled water, the slides were pressed, dried at room temperature and stained with either Coomassie blue or Ponceau "S" stain (Appendix 8) for 30 minutes and destained until the background was clear.

3.3.1.2. **Immunoelectrophoresis.**

Sheep (160)-anti-goat IgG serum showed some reactions in immunodiffusion experiments. It was decided to isolate specific antibodies from this antiserum, reacting only with goat IgG. These antibodies were purified by elution from Sepharose 4B CNBr to which purified IgG had been coupled (Fig. 1). The purified antibodies were then tested in immunoelectrophoresis to show whether the immunodiffusion reactions represented antibody activity to immunoglobulin classes, subclasses, and not to other serum proteins.

The immunoelectrophoresis microtechnique of Grabar and Williams (1953) following the method described by Scheidegger (1955), was used, with slight modification. About 15.0 ml of 1% (W/V) of litex agarose (Type HSA-Litex, Glostrup, Denmark) in sodium barbital - calcium lactate buffer, pH 8.6
(Appendix 6.1.1) was used. Two wells alongside one trough were made using a Gelman puncher in the agar (Fig. 5 a, b, c.), subsequently 10 ul of the antigen was filled into the wells. The slides were put into the Gelman electrophoretic chamber containing barbital calcium lactate buffer, Ph 8.0 as an electrolyte. Using a Gelman rectifier, a direct electric current 13mA, 40 volts) was passed through the agar at a flowing rate of 4 volts per centimeter for a period of 1 hour and 45 minutes. After the electrophoresis process, relevant antisera were placed in the troughs and diffusion allowed to take place for 48 hours. The pressing, drying and staining of the slides was performed as described for the double immunodiffusion.

3.4 Immunosorption of antisera

The antisera were all absorbed with several insoluble immunosorbents to which mouse, bovine, rabbit and sheep serum had been coupled. The immunosorbents used in this study are all shown in Appendix 1, which also shows the antisera and the percentages of protein coupled to the sepharose. The
immunosorption was performed in order to remove antibodies against immunoglobulins of sheep, mouse, rabbit, bovine, and goat from the antisera, thus rendering them species-specific for the IgG of the various species of animals. The absorbed antisera were later used in the preparation of the species-specific enzyme-conjugates used in this study.

3.4.1. Preparation of the insoluble immunosorbents

The preparation procedure for insoluble immunosorbents was carried out as recommended by the manufacturers (Pharmacia Fine Chemicals - Uppsala, Sweden) of cyanogen bromide activated Sepharose 4B CNBr-activated Sepharose.

Briefly the method was as follows:

A required amount of CNBr-activated Sepharose was weighed out. The Sepharose was then washed with 1 M HCl solution in the ratio of 200 ml. per gramme of dry Sepharose. This was followed by rinsing several times with distilled water. The protein to be coupled was dissolved in buffer (0.1M sodium bicarbonate buffer, pH 8.3 containing 0.5M sodium chloride) (Appendix 2.1). The protein solution was mixed with the suspension of the gel on the rotary mixer (Voss of Maldon, Essex, England) for 2 hours at room
temperature (25°C) and then for 18 hours at +4°C. The uncoupled protein was then washed off with the coupling buffer and the remaining active groups were blocked by 1M diethanolamine, pH 9.0 for 2 hours at room temperature. The blocking reagent was washed off using 1000 ml 0.1M acetate buffer, pH 4.0 containing 0.5M sodium chloride and finally rinsed with 1000 ml of coupling buffer and 1000 ml saline.

The uncoupled protein was estimated by using a spectrophotometer (Beckman, U.S.A., Model 25) at 280 nm wavelength and the percentage of the coupled protein was determined.

The protein Sepharose conjugate was stored at +4°C as a 50% suspension in saline to which sodium azide was added as a preservative to a final concentration of 0.1%.

3.4.2 Procedure for immunosorption of the anti-sera using the insoluble immunosorbents.

The absorption procedures with various insoluble immunosorbents were generally the same for all the antisera (Appendix 1). The general procedure for all the absorptions was as follows:- An equal volume of the antiserum was mixed with
an equal volume of the immunosorbent and rotated at room temperature on an end-to-end rotator for 2 hours, and then rotated in the cold (+4°C) for 18 hours.

The immunosorbent was then separated from the antisem by passing the serum through the sintered glass filter (No G3 - Pyrex, England). The immunosorbent was regenerated by washing it with 600 ml of 0.2M glycine/HCL buffer, pH 2.5, followed by 1000 ml of PBS, 1000 ml of coupling buffer, and finally suspended in saline with 0.1% sodium azide for storage. The immunsorbents were re-used several times.

A modified version of the purification of antibodies from sheep(160)-anti-goat IgG serum was performed. Briefly, it was performed as follows:- The coupled Sepharose 4B CNBr was packed in a small column of 15 cm high and 1.5 cm diameter. It was layered on top of a sephadex - G75 (Pharmacia, Fine Chemical, Uppsala, Sweden), separated by filter paper discs (Fig. 1), and left to pack in PBS for 2 hours at room temperature. The sephadex-G75 column was 13.4 cm high, constituting a total height of 28.5 cm of the affinity chromatography/gel filtration
column. The column was washed with 3.0ml of 3.5M potassium thiocyanate (KCN), which was used as the chaotropic buffer eluent in all the elution processes. The column was washed with 500 ml of PBS, and 50.0 ml of sheep-anti-goat IgG serum pumped through it using a peristaltic pump (Variperpex, LKB, Bromma Sweden). The column was then washed with 500ml PBS to remove all the unabsorbed contaminants from the column. The sheep-anti-goat antibodies were eluted with 3.0ml of 3.5M KCNS at pH 7.5. The protein content in each elution process was strictly monitored at 280nm wavelength till all the proteins had been eluted (absorbance values reading less than 0.10). The concentration of KCNS in each fraction was also determined using a conductivity meter (Type CDM 2f No. 274816, Denmark). In order to avoid pooling fractions containing high concentrations of toxic KCNS, elution curve graphs of the absorbance values (280nm wavelength) of the protein conductivity against fractions ("tubes") were drawn as shown in Fig. 2. Only those immunoglobulin fractions without or with very little KCNS concentration were pooled, concentrated by Amicon ultrafiltration using Amicon filter (Model 75PSI, 402, Amicon, Lexington U.S.A.) with a cut off point of 50,000 daltons, and later used for the preparation of the species-specific conjugates.
Fig. 1. The fractionation of the sheep (160) anti-goat IgG serum using affinity chromatography/gel filtration column.
Fig. 2. The elution curve graphs of absorbance values of protein and conductivity of KCNS against fractions (tube numbers)
3.5 **Purification of the immunoglobulins for conjugation.**

Whole antisera to be conjugated were initially precipitated using the method described by Fey *et al.*, (1976) and Heide and Schwick (1973).

An equal volume of saturated ammonium sulphate solution was added slowly to an equal volume of antiserum while stirring. The mixture was left standing at room temperature (25°C) for 10 minutes and centrifuged at 3,500 g for 20 minutes. The precipitate was washed twice in 35% saturated ammonium sulphate and then dialysed against PBS, pH 7.5, containing 0.2% sodium azide for two days at +4°C. It was then dialysed against the initial buffer for one day.

The precipitate and the dialysed gammaglobulin fraction (25.0 ml) was further purified on a diethylamino ethylcellulose (DEAE) - anion - exchange cellulose chromatography (Bio Rad. Lab. Richmond, California), pre-equilibrated with 0.02M phosphate buffer, pH 8.0. The same buffer was used
as an eluent of the immunoglobulins from the chromatographic column, and the fractions collected using a 700 Ultrarac Fraction collector and recorder (LKB, Bromma, Sweden).

3.6 Conjugation of horseradish peroxidase and glucose oxidase to purified IgG.

Most of the enzyme-labelled immunoglobulins (conjugates) used in this study were prepared in our laboratory according to the method of Wilson and Nakane (1978) with few modifications.

The following conjugates were prepared: Goat (381/382)-anti-human IgG glucose oxidase - Ab (17/7/83), Goat (632/633)-anti-sheep IgG horseradish peroxidase (HRPO)-Ab (6/3/82), sheep (160/161)-anti-goat IgG (HRPO)-Ab (16/10/82), and sheep (160/161)-anti-goat glucose oxidase - Ab (16/10/82).

3.6.1 Horseradish peroxidase conjugates.

A brief description of the preparation of the sheep (160/161)-anti-goat IgG HRPO-Ab (16/10/82) conjugate will serve as an illustration of the preparations of all the peroxidase conjugates.

The enzyme quality used was horseradish peroxidase
type VI (Sigma Chemical Co., St. Louis, Mo.) (HRPO)

For the oxidation of the enzyme, 0.10 ml of freshly prepared 0.15M sodium metaperiodate (NaIO₄) solution was mixed with 7 mg of HRPO dissolved in 0.9 ml of distilled water. The mixture whose colour changed from brown to green, was then kept in the dark at room temperature for 30 minutes.

It was then dialysed for 45 minutes against cold (+4°C) 1mM acetic acid/sodium acetate buffer, pH 4.4 (Appendix 9.1).

In order to inactivate the remaining excess sodium metaperiodate, 0.5 ml polyethyleneglycol (PEG) was added to the dialysing buffer. The oxidized HRPO enzyme was then dialysed and coupled with 12.5 ml of protein solution containing 20 mg of purified sheep-anti-goat IgG (Coupling ratio: Enzyme/Protein: W/W = 1/3), and the pH of the mixture carefully adjusted to 9.0 using 0.5 ml of 0.2M sodium carbonate buffer, pH 9.0 (Appendix 9.2). The mixture of the enzyme and protein was left to couple for 2 hours at room temperature in the dark, with slow stirring.

The pH was re-adjusted back to 7.4-7.8 using 0.1 ml. 1N HCl and then the mixture was left overnight at +4°C without stirring. After overnight incubation, 20 mg of L-
lysine was added, mixed on a magnetic stirrer at room
temperature for 2 hours to block the uncoupled sites of the
enzyme. The conjugate was purified by precipitation using 50%
saturated ammonium sulphate. Normal sheep serum was added
to the purified conjugate as a stabilizer to a final
concentration of 10%. An equal volume of glycerol was added
to the volume of the conjugate and stored at -20°C.

3.6.2. Glucose oxidase conjugates

All the steps described for the preparation of the
horseradish peroxidase conjugates were followed when
preparing the glucose oxidase conjugates (Refer to 3.6.1.).

3.7 Enzyme-linked immunosorbent assay (ELISA) for
the detection of Toxoplasma gondii antibodies.

The ELISA was performed as described by Voller et al.,
1976a); Walls et al., (1977); Lin et al., (1980) and
Berdal et al., (1983) for the detection of Toxoplasma
antibodies.

Briefly, the test was carried out as follows:- The
Toxoplasma gondii antigen was adsorbed to the wells of microtitre polystyrene plastic plates namely, NUNC A/S Immunoplates I and II (A/S NUNC Roskilde, Denmark), and Falcon plates (Div. Becton, Dickinson, and Co. Oxnard, California, U.S.A.). Various dilutions of the antigen were tested on the two types of plates. Several coating buffers, namely, carbonate buffer, pH 9.6, PBS with or without 2% PEG, PBS (1/100) + 2% PEG, and 0.015M phosphate buffer, pH 7.5 + 0.4M NaCl + 2% PEG were also tested (Appendix 3.1). As a control for cross-reactivity between mouse and other animal species, each plate was also coated with normal mouse serum (1/1000) in alternating columns of wells.

The diluted antigen and mouse serum were added in a volume of 100 ul per well and incubated overnight at +4°C in a humid chamber and at -20°C after incubation until use. The antigen and mouse serum coated plates were then washed with PBS + 0.05% Tween 20, pH 7.5 (Appendix 3.2) 6 times each wash, at an interval of 2 minutes.

The test serum samples were added in one dilution of
1/50 in different serum diluents namely: PBS + 0.05% Tween 20, 0.05M phosphate buffer, pH 7.5 + KCl/EDTA/0.5% Tween 80 with or without 2% PEG, with or without 2% mouse serum, with or without 2% bovine serum, with or without 2% sheep serum. For dilution of goat serum samples, PBS + 0.5% Tween 20 + 10% normal sheep serum + 10% normal mouse serum was used, while PBS + 0.5% Tween 20 + 2% normal goat serum + 2% normal mouse serum was used for the dilution of sheep serum samples (Appendix 5.1). Positive and negative reference sera were always included on each plate for the calibration of unknown test samples. Controls for the non-specific adsorptions of the reference sera, conjugates, and substrates were included in all the tests.

The plates with the test serum samples, and reference sera were incubated for 2 hours at 37°C in a humid atmosphere. The plates were then washed as mentioned, and 100 ul of a specific conjugate added per well in a conjugate diluent namely: 0.5M phosphate buffer, pH 7.5 + KCl + EDTA + 0.5% Tween 80 + 5% normal serum of the species of animal in
which the antiserum had been produced (Appendix 5.2).

The plates with the conjugate were then incubated for 1 hour at 37°C in a humid chamber. The plates were washed as previously described, and 100 ul of a specific substrate (Appendix 4) made in a specific substrate buffer (Appendix 3.3) was added.

All the enzymes reactions were incubated for 1 hour at room temperature in the dark, and then all their absorbances read using a mini-reader (Dynatech Mini-reader MR 590, Dynatech, England).

When the substrate (Appendix 4.2) for horseradish peroxidase enzyme was added, the colour developed to a deep yellow one. Depending on its intensity, the absorbance was read at 410 nm or the reaction was stopped by adding 50 ul of 2N sulphuric acid and the absorbances read at 490 nm, which is close to the wavelength for maximum absorbance of the acidified end-product of the enzyme reaction.

For the alkaline phosphatase reaction, the colour developed to greenish yellow after the addition of the
alkaline phosphatase substrate (Appendix 4.3). The reaction was stopped by adding 50 ml of 4N sodium hydroxide and the absorbance read at 410 nm.

A bluish colour developed after the addition of the glucose oxidase substrate (Appendix 4.1). The reaction was not stopped.

A summary of the *Toxoplasma gondii* ELISA which was used in detecting anti-*Toxoplasma* antibodies is shown in Fig. 3.

3.8 Analysis of ELISA absorbance values:

Various methods for mathematical transformation of absorbance values have been evaluated (de Savigny and Voller, 1980) and no single method was found to satisfy all the standard requirements.

The Positive : Negative (P/N) Ratio method (de Savigny and Voller, 1980, Voller and de Savigny, 1981) was used in analysing the ELISA absorbance results in this study. The absorbance values (read at either OD 410 nm or 490 nm wavelengths) were mathematically converted into P/N ratios. The P/N ratios greater than 2 (or 3) are usually considered positives (de Savigny and Voller, 1980).
Example of P/N Ratio Calculation.

The average absorbance value of seven positive human serum samples was 0.33 (read at OD 410 nm wavelength) (Fig. 10). The average absorbance value of six negative human serum samples was 0.06 (Fig. 10).

Therefore, the P/N ratio was calculated as follows:

\[
\frac{0.33 \text{ (positive)}}{0.06 \text{ (negative)}} = 5.50
\]

Having established the positive threshold value, the prevalence of anti-Toxoplasma antibody in 214 human, 155 goat and 158 sheep serum samples assayed at a single serum dilution of 1/50 was analysed using this P/N ratio method. After transforming the absorbance values mathematically into P/N ratios, samples that gave the P/N ratios above 2.0 were considered to be positive.

3.9 Comparison of the dye test and ELISA

A comparative study was performed using reference sera with known dye test titres (see 3.2.1). A total
of 13 human, 11 goat, and 9 sheep reference sera were tested in ELISA using a single serum dilution of 1/50. The above named control sera were absorbed with normal mouse serum added to a final concentration of 2%, 10% and 2%, respectively, prior to testing in ELISA.

3.10 Absorption experiments.

To test for the specificity of ELISA values for Toxoplasma antibodies, absorption experiments were performed. Nine human serum specimens with known dye test titres (8 positives and 1 negative) were pre-incubated with T. gondii antigen, normal mouse serum, C. bovis, E. granulosus antigens, or no antigen, for 2 days at +4°C. The latter two parasitic antigens were crude extracts, concentrated by lyophilization in our laboratory.

The mixtures were then centrifuged, and the supernate assayed by ELISA at a single serum dilution of 1/50 to determine whether absorption of the antibody in the sample had occurred by the particular antigen.
Enzyme substrate added. Incubated for 1 Hr at R. T.

STAGE 4

PLATE WASHED

Enzyme antiglobin conjugate added. Incubated for 1 Hr at 37°C.

STAGE 3

PLATE WASHED

Test serum sample added. Incubated for 2 Hrs at 37°C.

STAGE 2

PLATE WASHED

Normal mouse serum (1/1000)

Toxoplasma gondii antigen (1/1000)

Mouse peritoneal exudate

STAGE 1

COATED ON A PLATE

Fig 3 An indirect Toxoplasma gondii ELISA used in detecting anti-Toxoplasma antibodies in humans, sheep and goats.
4.1 Analysis of the antisera used in this study

4.1.1 Immunodiffusion and Immunoelectrophoretic Reactions of Sheep (160)-anti-goat-IgG Serum

Two precipitin lines formed between this antiserum and goat serum in immunodiffusion experiments as shown in Fig. 4. These two precipitin lines could be due to reactions formed against the two sub-classes IgG\textsubscript{1} and IgG\textsubscript{2} of sheep. There were no cross-reactions between the other animal species tested, namely, bovine, human, mouse and rabbit, and this antiserum (Fig. 4).

Immunoelectrophoretic analysis of the same antiserum showed three precipitin arcs (Fig. 5a) with the non-fractionated antiserum.

Since it could not be concluded with absolute certainty that these reactions represented antibody activity to immunoglobulin classes or sub-classes, and not to other serum proteins, it was decided to isolate specific antibodies reacting only with goat IgG. These antibodies were purified by elution from
sepharose 4B to which purified goat IgG had been coupled (Fig. 1).

Only a single precipitin arc formed in the slide containing
purified antibodies from the sheep (160)-anti-goat IgG serum
in the trough (Fig. 5b). This antibody preparation was used
for the preparation of enzyme-conjugates specific for
goat immunoglobulins. Rabbit-anti-goat serum was used as a
control in Fig. 5c, and indicates that the slow-migrating
immunoglobulin belongs to the IgG class.

4.1.2 Immunodiffusion reactions of other antisera

a) Goat (381/382)-anti-human IgG serum.

The Toxoplasma antigen consisting of infected mouse
peritoneal exudate reacted strongly with unabsorbed goat
(381/382)-anti-human IgG serum, as illustrated in Fig. 6.

The precipitin line showed complete identity between some
components in the mouse serum (Well:6) and the crude antigen
(Well:5). There was no reaction of identity between rabbit
serum and mouse serum components using this antiserum. There
was partial identity between a component in the human
(Well:2) and in mouse serum (Well:3).

The cross-reactions between this unabsorbed antiserum
and the bovine, mouse and rabbit sera are shown in Fig. 7.

Fig. 8 shows the results of immunodiffusion experiments of the absorbed antiserum. Due to inadequate absorption, a weak precipitin line still formed between the mouse serum and the antiserum after absorption with insoluble immunosorbents to which mouse, bovine, rabbit and sheep serum had been coupled (Fig. 8). Therefore, normal mouse serum and normal rabbit serum, to a 2% final concentration of each, were added to the absorbed goat (381/382)-anti-human IgG conjugate to abolish completely the cross-reactivity with mouse serum.

b) Goat (632/633)-anti-sheep IgG serum

This antiserum gave a strong reaction with IgG of sheep serum, but showed no cross-reactions with serum from other animal species as shown in Fig. 9.

4.2 Conjugates

The enzyme-labelled immunoglobulins (conjugates) used in this study were prepared from the immunoglobulin fraction of the absorbed serum or from purified antibodies (Table 1, page 48).
Preliminary tests and titrations of the conjugates in ELISA showed that they had been rendered species-specific for IgG of the various species of animals. When stored at 

-20°C in an equal volume of glycerol, and normal serum used as a stabilizer, to a final concentration of 5-10%, they were found to keep well for some months without appreciable loss of antibody activity.

4.3 Comparison of the dye test and ELISA

The results of a comparative study of DT and ELISA are shown in Fig. 10. Out of 13 human sera tested, 6 (46%) were recorded as positive in both DT and ELISA assays, while 6 (46%) were negative in both assays. One sample (8%) was positive in DT and negative in ELISA.

ELISA identified as positive all 6 DT positive sera, except one, and all the 6 DT negative sera as negative.

Therefore, there was a good correlation of 92% between the DT and ELISA in human sera. The DT negative sera gave an average absorbance value of 0.05, whereas most of the DT positive sera gave absorbance values that were above 0.10 (Fig. 10).
Shown in Fig. 11 are the results of a comparative study of the DT and ELISA in 11 goat sera, derived from goats immunized with *Toxoplasma* antigen plus mouse peritoneal fluid. Normal mouse serum was added to the diluted goat serum samples to a final concentration of 10%. Out of the 11 goat sera, 8 (73%) were recorded as positive in both the DT and ELISA assays, while 3 (27%) were negative in both assays. ELISA detected all the DT positive control sera as positive and all the DT negative control sera as negative. Therefore, a 100% agreement between the DT and ELISA was observed.

Fig. 12 shows the results of a comparative study of the DT and ELISA in 9 sheep sera. Normal mouse serum was added to the diluted sheep serum samples to a final concentration of 2%. Five sheep serum samples were found to be negative in ELISA, while being positive in the dye test. Only one sheep serum sample was recorded as positive in both DT and ELISA assays, while 3 were negative in both DT and ELISA. Therefore out of 9 sheep serum samples, derived from sheep immunized with mouse peritoneal fluid and *Toxoplasma* antigen, four samples gave a poor correlation of
4.4 Results of ELISA of random serum samples

4.4.1 Prevalence of Toxoplasma gondii antibodies in 214 human sera.

The P/N ratio for the standard negative serum was 2.0. Out of 214 human sera tested, 91 (43%) gave P/N ratios above 2.0, and therefore were considered to be positive, while 123 (57%) gave P/N ratios less than 2.0 and were considered to be negative for Toxoplasma antibody activity.

4.4.2 Prevalence of Toxoplasma gondii antibodies in 155 goat sera.

Three known dye test negative goat serum samples, gave an average absorbance value of 0.02 in ELISA, which was used in the calculation of the P/N ratios. Out of 155 goat sera tested, 33 (21%) gave P/N ratios above 2.0 and therefore were recorded as positive, while 122 (79%) gave P/N ratios less than 2.0 and were recorded as negative for Toxoplasma antibody activity.

4.4.3 Prevalence of T. gondii antibodies in 153 sheep sera

Three negative sheep sera with known dye test titres gave
an absorbance value of zero in ELISA (Fig. 12). This value could not be used in the calculation of the P/N ratios. Therefore, the average absorbance value for the negative reference samples was considered to be 0.02 (as for the goats) and was used to calculate the P/N ratios. The results of the prevalence of anti-Toxoplasma antibodies in 158 sheep serum samples was found to be 56%.

4.5 Absorption experiment.

The specificity of ELISA for Toxoplasma antibodies was confirmed by absorption experiments.

It is clear from the results shown in Fig. 13 that the Toxoplasma antigen was the only preparation capable of significantly reducing the ELISA values of the positive specimens, thus showing the immunological specificity of the present ELISA for Toxoplasma antibodies in human sera.
Fig. 4: Immunodiffusion of Sheep-anti-goat IgG serum against other animal species.

Well: A  Sheep (160)-anti-goat IgG serum
Well: 1  Goat serum
Well: 2  Bovine serum
Well: 3  Human serum
Well: 4  Goat serum
Well: 5  Mouse serum
Well: 6  Rabbit serum
Fig. 5a  Immuno-electrophoretic analysis of sheep (160)-anti-goat IgG serum against neat goat serum and goat IgG.

Showing three precipitin arcs in non-fractionated sheep-anti-goat IgG serum

Trough: $T_1$ = Sheep (160)-anti-goat IgG serum

Well : 1 = Neat goat serum

Well : 2 = Goat IgG
Fig. 5b: Immunoelectrophoretic analysis of sheep (160)-anti-goat IgG serum against neat goat serum and goat IgG.

Showing the IgG class in the purified antibodies of sheep (160)-anti-goat IgG serum.

Trough: \( T_2 \) = purified antibodies of sheep (160)-anti-goat IgG serum.

Well: 3 = Neat goat serum

Well: 4 = Goat IgG
Fig 5c: Immunoelectrophoretic analysis of rabbit-anti-goat serum against neat goat serum and goat IgG.

Used as control and showing IgG class in neat goat serum.

Trough : T3 = Rabbit-anti-goat serum

Well : 5 = Neat goat serum

Well : 6 = Goat IgG
Fig. 7: Immunodiffusion of unabsorbed goat (381/382)-anti-human IgG serum against other animal species.

Well 1: Goat (381 & 382) anti-human IgG serum (unabsorbed)

Well 2: Mouse serum

Well 3: Human serum

Well 4: Human serum

Well 5: Bovine serum

Well 6: Sheep serum

Well 7: Rabbit serum
Fig. 6: Immunodiffusion of unabsorbed goat (381/382)-human IgG serum against other animal species and crude *Toxoplasma* antigen.

Well : A  Goat (381/382)-anti-human IgG serum (unabsorbed)
Well : 1  Human serum
Well : 2  Human serum
Well : 3  Mouse serum
Well : 4  Rabbit serum
Well : 5  Mouse serum
Well : 6  *Toxoplasma* antigen/Mouse peritoneal exudate
Fig. 8: Immunodiffusion of absorbed goat (381/382)-anti-human IgG against other animal species.

Well : A Absorbed and purified goat (381/382)-anti-human IgG.

Well : 1 Mouse serum
Well : 2 Human serum
Well : 3 Rabbit serum
Well : 4 Mouse serum
Well : 5 Bovine serum
Well : 6 Sheep serum
Fig. 9: Immunodiffusion of goat (632/633)-anti-sheep serum against other animal species.

Well : A  Goat (632 & 633) anti-sheep serum
Well : 1  Sheep serum
Well : 2  Bovine serum
Well : 3  Human serum
Well : 4  Sheep serum
Well : 5  Mouse serum
Well : 6  Rabbit serum
Fig. 10. Comparison of ELISA with dye test in 13 human serum samples.
Dye tested goat serum samples in Oslo

- ○ Dye test negative
- □ Dye test positive

Fig. 11. Comparison of ELISA with dye test in 11 goat serum samples.
Fig. 12. Comparison of ELISA with dye test in 9 sheep serum samples.
Fig 13. Demonstration of the immunological specificity of ELISA reactions for detection of *Toxoplasma* antibodies by absorptions experiments in 3 human serum specimens.
5. DISCUSSION

ELISA has been used by several workers to detect antibodies against *T. gondii* in human serum specimens (Voller *et al.*, 1976a; Walls *et al.*, 1977; Lin *et al.* 1980; Woodward, 1982 and Naot *et al.* 1983). Berdal *et al.* (1983) are the only workers who have used ELISA for detecting antibodies against *T. gondii* in goats. From the available literature, it appears that nobody has used the ELISA procedure for the detection of antibodies against *T. gondii* in sheep. In this study, an indirect ELISA was used for the detection of antibodies against *T. gondii* in humans, goats and sheep.

Dr. T. Omland and Mr. Ø Olsvik, working in Norway, had found human serum sample No. 1024/82 DT positive and ELISA negative, and that its antibody activity belonged to the IgM class. They also found that sample No. 018/82 was DT positive due to IgM, but they did not test it in ELISA (see 3.2.1.1). The data on the comparison of the DT and ELISA in Fig. 10 shows that the same sample No. 1024/82 was found to be ELISA negative in this study. The reason for this sample being ELISA negative
when it was DT positive could be explained by the possibility that the anti-Toxoplasma antibody activity was too low in this particular sample, and therefore could not be detected by this type of ELISA (Fig. 10). In absorption experiments, this sample also gave similar results as sample No. 018/82 (IgM DT +ve), and the negative control (Fig. 13).

Discrepancies between the DT titres and ELISA absorbance values were first noted by Voller et al. (1976a). Their sandwich ELISA procedure did not distinguish between sera with medium DT titres (256-612) and those with high titres (over 1024).

Pettersen (1981) used rabbits immunized with live parasites to study the immune response to Toxoplasma infection. In the early stages of the disease, there was a rapid antibody rise measured by dye test, including both IgM and IgG antibodies. The ELISA absorbance values rose slowly, probably because a sandwich ELISA detects IgG antibodies better than IgM antibodies (Pettersen, 1981). This discrepancy between the DT and ELISA was important within the first 5 weeks of infection and then reached a plateau.
Camargo et al. (1978) showed that surface antigens are important in the DT, while cytoplasmic antigens may be of greater importance in ELISA. However, the ELISA determination is thought to yield results which are presumably a summation of the antibodies directed against several antigens known to induce antibody responses in humans (Handman et al. 1980, Hughes and Barfour, 1981).

It should also be remembered that all the conjugates employed in the present study were produced by immunization with the IgG fraction of human, goat and sheep serum (containing both light and heavy chains of the immunoglobulin) and hence these conjugates were expected to detect anti-Toxoplasma antibodies of all immunoglobulin classes reacting in ELISA.

Although the distinction between the various classes of antibodies elicited in Toxoplasma infection is clinically and epidemiologically important, no attempt was made to determine the classes of antibodies in human, goat and sheep serum samples in this study due to a limited period of time.

Apart from the minor discrepancy mentioned for human
serum No. 1024/82 the results of this study indicate that the
ELISA results correlated quite well with DT results in humans
and goats, but not in sheep. A good correlation of 92%
between the DT and ELISA was observed in humans (Fig. 10). This
is in accordance with the results of Voller et al. (1976a),
Lin et al. (1980), Mondesire et al. (1981) and Woodward
(1982) with regard to DT and micro-ELISA values in man.

The prevalence of anti-Toxoplasma antibodies in 214
human serum samples assayed was found to be 43%.
These results compare favourably with those of other workers
with regard to the prevalence of Toxoplasma infection in man in
Africa. Mas Bakal et al. (1968) found a prevalence of 56% DT
positive among 106 human sera in Kenya. Ludlam (1965) detected
Toxoplasma antibodies in 83.3% of adult Nigerians. Fulton
et al. (1966) found that 50% of Bagarda adults and 33% of
Masai infants gave positive dye tests, while Omland et al.
(1977) found an overall prevalence of Toxoplasma antibodies
of about 60% in the native population of Liberia.
When a total of 9 sheep reference serum samples and 11 goat reference serum samples with known dye test titres were absorbed with 2% and 10% normal mouse serum, respectively, and tested in ELISA (Figs. 11, 12), a 44% correlation between the DT and ELISA was observed with sheep sera while there was a 100% agreement between the DT and ELISA in goats. The figure in goats is of the same order of magnitude of 98% as reported by Berdal et al., (1983) in Norwegian goats.

The prevalence of Toxoplasma antibodies in 155 goat serum samples was found to be 21% in ELISA.

Toxoplasma infections have also been diagnosed in goats in other parts of the world (Reiman et al., 1975, Durfee et al., 1976, Chhabra et al., 1981). Since different standards and methods have been used during the previous surveys of Toxoplasma antibodies in goats, it is difficult to compare the results obtained in the present survey with those of other workers. The prevalence rate of antibodies recorded in goats and sheep in this study is the first one to be reported from this country.

In other parts of the world, recent surveys in sheep
and goats have utilised the IHAT and DT for the detection of
Toxoplasma antibodies with satisfactory results (Work, 1967;
Waldeland, 1976c; Chhabra et al., 1981).

Using ELISA for the detection of Toxoplasma antibodies,
a higher antibody prevalence rate (56%) was recorded in sheep
than in goats (21%) in the present epidemiological survey.
This is of the same order of magnitude as reported by Jacobs
et al. (1963) who detected the parasite in about 67% of 34
sheep with DT titres 1/16; by Work (1967) who recovered
the parasite from 7 of 9 sheep with the titres 1/50, and
Waldeland (1976a) who recovered the parasite from about 58%
of 116 DT positive sheep. In an investigation on the prevalence
of Toxoplasma antibodies in sheep in Norway, serological
evidence of the infection was found in about 46% of the mature
ewes, and in about 26% of the lambs (Waldeland, 1976c).

Since most of the sheep slaughtered in our slaughter-houses
are mature ones, the data presented in this study lead to the
conclusion that 56% of adult sheep examined may have been infected
with T. gondii. It should be noted that there was a poor
agreement between the DT and ELISA in sheep and therefore this
comparison of the prevalence rates may not be entirely valid. The high prevalence of *Toxoplasma* antibodies in sheep might be due to the fact that they are strongly exposed to infection by the ingestion of oocysts of *Toxoplasma gondii* from contaminated pastures, as they graze the herbage close to the ground. The goats browse on the shrub and other vegetation and rarely graze close to the ground. The ingestion of sporocysts is probably the principal mode of natural transmission in herbivorous animals (Jacobs 1973, Hartley and Munday, 1974).

The observation by Work (1967) and Waldeland (1976a) that herbivorous animals frequently harbour *Toxoplasma* cysts in their tissues, which could serve as an important source of human infection, is further strengthened by this study. The public health significance of higher prevalence rates of 21% and 56% among goats and sheep, respectively, might prove to be significant in view of the high prevalence rate of 43% which was found in the human population in this study.
The cause(s) of the negative ELISA-IgG results when the DT results are positive in sheep is unclear. The efficiency of the serological tests in bovine, sheep, goat and pig sera depends in some tests on the inactivation of a heat-stable non-specific anti-Toxoplasma factor. A temperature of 60°C for 30 minutes is required to remove this non-specific effect (Jacobs, 1976). Chhabra et al. (1981) inactivated the goat test sera at 56°C for 30 minutes, and found a high prevalence rate of 34% using IHA test. They also removed the heterophile antibodies by absorption with normal cells. Lin et al. (1980) found that pre-treatment of the test specimens was not required in humans. Therefore, lack of pre-treatment of the goat and sheep sera before testing could have contributed to the apparent low prevalence rate in goats, and even a lower prevalence rate in sheep.

Kenyan goats and sheep tend to be heavily infected with various parasites which may result in false positive reactions in serological tests. Absorption experiments with some of the common parasites showed that there were no major cross-reactions between them and *T. gondii* (Fig. 13). The same conclusions had been reached by Ruitenbergen and Buys (1977) when they tested
sera from patients with toxoplasmosis, malaria, schistosomiasis and hydatidosis. Further studies utilizing several other parasites are needed to resolve this question.

The naturally occurring antibodies reacting with mouse serum and peritoneal fluid components, present in sheep and goat sera was a significant observation made in this study. This led to a change in the procedure which necessitated the absorption of all the test and reference sera of goats and sheep with normal mouse serum, before testing in ELISA to avoid false positive reactions.

Since only crude Toxoplasma antigen was used in the coating of the microtitre polystyrene plastic plates, and for immunizing the goats and sheep, sera from these animals contained antibodies to mouse peritoneal fluid components, which necessitated absorption of these sera before testing them for the presence of Toxoplasma antibodies.

Lin, et al. (1980) performed absorption experiments on human sera, which revealed that only the Toxoplasma antigen, but not normal mouse plasma or Amoeba or Trichinella antigens,
were able to significantly reduce the ELISA values in the specimens. The same observation was made in this study on human samples (Fig. 13). Our data on the ELISA system indicate that ELISA procedures were reliable and specific for the demonstration of *T. gondii* antibodies of IgG class in human toxoplasmosis.

The results of the absorption experiments (Fig. 13), preliminary tests and titrations, indicate that there are no naturally occurring antibodies that react with the mouse serum components, present in human serum. However, Foucard et al. (1975) showed that approximately 70% normal human blood donors have antibodies reacting with immunoglobulins of cow, sheep, goat, guinea pig and a small extent with horse, swine, and rabbits.

In this study, it was shown in immunodiffusion experiments (Fig. 6, 7) that the mouse, rabbit and bovine serum components reacted with anti-human IgG serum. It was also shown that the mouse serum components in the crude *Toxoplasma* antigen reacted with anti-human IgG serum, and that some components in the mouse and human sera were partially
 identical (Fig. 6). Lartea and Falk (1980) also found
that some components in the mouse and human sera were
partially identical.

Absorption studies of the goat (381/382)-anti-human
IgG serum, using mouse, rabbit, sheep and bovine insoluble
immunosorbents, produced a species-specific antiserum
that reacted only with human immunoglobulins in ELISA and
immunodiffusion tests (Fig. 8), and was used for the preparation
of anti-human IgG enzyme conjugates.

When goat and sheep test and reference sera were absorbed
with 10% and 2% normal mouse serum, respectively, the ELISA
absorbance values were greatly reduced, presumably now
representing antibody activity to Toxoplasma antigens only.
It was also noticed that sheep gave a strong immune response
to mouse peritoneal fluid when the crude Toxoplasma antigen
was used for immunization, while the response to Toxoplasma
antigenic components was poor.

One problem associated with toxoplasmosis serology in
general and ELISA specifically, is the lack of appropriate
standards and a common unit for expressing results. Lin et
al. (1980) and Woodward (1982) recommended the reporting of
ELISA results in international units based upon the World Health Organization's International Standard which would eliminate the potential skewing of ELISA standard curves. This is critical in a screening programme in which it is of primary importance to differentiate positive from negative specimens. Whereas their recommendations are acceptable and relevant to the work done in this study, the P/N ratio was used in reporting the ELISA results for the following reasons:

(1) It automatically compares the test specimen with the "normal" population P/N or positive reference serum in a quantitative manner. (2) The results are easily understood. (3) It requires tests at a single-serum dilution only. (4) The method is used only when small numbers (\( \leq 10 \)) of normal reference sera are available. The use of P/N ratio is in accordance with the recommendations of de Savigny and Voller (1980).

The ELISA procedure described in this study has several advantages over other commonly used assays for detecting and measuring antibodies to \( T. gondii \). The DT, IFAT, IHAT and most of the micro-ELISA tests require serial dilutions of the test samples for establishing the level of antibodies,
whereas this ELISA yields approximate quantitative values on the basis of a determination using a single dilution of 1/50 of the test sample. However, Bullock and Walls (1977) found that titration curves indicated that the use of a single dilution is not adequate for quantitative tests.

Several workers have used for human toxoplasmosis a test system by which ELISA could be evaluated (Bullock and Walls, 1977; Lin et al. 1980; Mondesire et al. 1981).

In this study several experiments were performed to establish the optimal conditions for ELISA. The common 0.06M carbonate buffer, pH 9.6, recommended by most investigators as a good coating buffer in ELISA, was found to be inadequate. A coating buffer consisting of 0.015M phosphate buffer + 0.4M NaCl + 2% PEG, pH 7.5 was found to be more efficient for coating Toxoplasma antigens in this study. For coating serum proteins, PBS diluted 1/100 and containing 2% PEG, pH 7.5 was an efficient coating buffer.

Ortho-phenylene diamine dihydrochloride was one of the enzyme substrates used in ELISA tests. Although it is
reputed to be toxic by inhalation, in contact with skin and if swallowed (Aldrich Chemical Co. Ltd, Dorset, England), it is a useful substrate because the enzyme reaction can be stopped instantly with $2\text{N H}_2\text{SO}_4$.

Lin et al (1980) found that the Toxoplasma antigen on a solid phase could even be kept at room temperature ($23^\circ\text{C}$) for at least 10 months without loss of activity. Microtitre plastic plates coated with T. gondii antigen at either $+4^\circ\text{C}$ or room temperature ($25^\circ\text{C}$) overnight were found to keep well for over 3 months at $-20^\circ\text{C}$ without loss of activity in this study.

Chessum and Denmark (1978) suggested that ELISA may produce erratic results due to the non-uniform adsorbent quality of the microtitre plastic plates. The same observations were made during our study on toxoplasmosis using ELISA. There were minimal variations between individual wells, but considerable variations between plates from different manufacturers.

However, Bullock and Walls (1977), in their evaluation of some of the factors affecting ELISA, found that there was no difference between the types of microtitre plates they used.
The only main variable in the type of ELISA used in this study was the serum diluent which differed for all the three species of animals, namely human, goat and sheep (Appendix 5). Otherwise, it seems evident that a single ELISA procedure could be used to detect *Toxoplasma* antibodies in all the animals tested.

Non-specific binding of serum proteins to the plastic plates is an important factor in all such tests. Ruitenberg et al. (1976) and Bullock and Walls (1977) investigated this problem and agreed that washing with Tween-20 in saline or water is as effective as incubation with BSA and virtually eliminates background reactions. Bullock and Walls (1977) found no advantage in using BSA and Tween-20. Although Lin et al. (1980) and Woodward (1982) have used PBS containing 0.05% Tween-20 + 5% BSA as a serum diluent, they did not discuss problems related to non-specific adsorption.

We have found a very high background when PBS containing 0.05% Tween-20 (Appendix 5.1.1) was used as serum diluent for goat and sheep sera. Our observations agree with those of other investigators that PBS containing 0.05% Tween-20 is a suitable

Efforts were made to reduce the background by examining the effect of various concentrations of Tween-20 and normal serum in the diluents. It was found that an increase in the percentage of Tween-20 (upto 0.5%) and 2-10% normal serum reduced the background significantly, but did not eliminate it completely.

Due to difficulties encountered in eliminating the background in ELISA used for the detection of Toxoplasma antibodies in human sera, a different serum diluent (Appendix 5.1.2) that gave an acceptable background was formulated.

It was also observed in this study that frozen and thawed serum samples adsorbed highly to the non-coated wells, producing a very high background.

It was noted that the conjugates used at their optimal concentrations did not bind non-specifically when diluted in the conjugate diluent described in Appendix 5.2.
Although the numbers of human, goat and sheep sera examined so far were small, it is concluded that, *Toxoplasma* infections occur among humans, goats and sheep in Kenya. These findings, in conjunction with those of Mas Bäkal et al., (1968); Olubayo and Mwongela (1978); and Olubayo and Karstad (1981) confirm that *Toxoplasma* infections exist in humans, domestic animals and wildlife in Kenya.

It is therefore likely that sheep and goats may play an important role in the epidemiology of *Toxoplasma* infections by maintaining a widespread distribution of the parasite. Nevertheless, these findings on the prevalence of *Toxoplasma* antibodies only indicate that toxoplasmosis may be widely distributed in Kenya and it is suggested that further investigations should be carried out on other common meat-producing animals.

The possibility of cross-reactions due to infections with parasites possessing antigen(s) in common with *T. gondii* was not extensively examined, and therefore it is suggested
that such investigations should be carried out.

Further studies should be performed on the sera of various species of animals, to determine whether the naturally occurring antibodies reacting with mouse serum components are present in their sera. In this study, it was concluded that these antibodies are present in goat and sheep sera, but not in human sera.

In view of the data presented here and by others (Bullock and Walls, 1977; Lin et al. 1980; Woodward, 1982; Berdal et al. 1983), ELISA may be considered an adequate method for population screening for anti-Toxoplasma antibodies. It was also observed that standardization of ELISA procedures is possible, but slight modifications would be required to suit a particular type of assay.
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### APPENDIX I: Immunosorbents used and the antisera absorbed in this study

<table>
<thead>
<tr>
<th>Antigen coupled to CNBr-Sepharose</th>
<th>Amount of total protein determined by spectrophotometry</th>
<th>Percentage coupled to Sepharose</th>
<th>Antisera absorbed/purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat purified IgG</td>
<td>163.0 mg protein</td>
<td>74%</td>
<td>Sheep anti-goat serum (purified)</td>
</tr>
<tr>
<td>Mouse-serum protein ppt. 50% (NH₄)₂SO₄</td>
<td>6.83 mg protein</td>
<td>99%</td>
<td>Goat-anti-human IgG serum</td>
</tr>
<tr>
<td>Whole bovine serum</td>
<td>ND</td>
<td>ND</td>
<td>Goat-anti-human IgG serum</td>
</tr>
<tr>
<td>Whole rabbit serum</td>
<td>ND</td>
<td>ND</td>
<td>Goat-anti-human IgG serum</td>
</tr>
<tr>
<td>Whole sheep serum</td>
<td>ND</td>
<td>ND</td>
<td>Goat-anti-human IgG sheep</td>
</tr>
</tbody>
</table>

ND = Not done
Appendix 2: Buffers and solutions used in the preparation of
of the insoluble immunosorbents

2.1 0.1M Sodium bicarbonate (NaH Co.), pH 8.3 with 0.5 M
sodium chloride (protein coupling buffer)

Sodium bicarbonate ......................8.40 gm
Sodium chloride .......................29.22 gm

8.4 gm of sodium bicarbonate was dissolved into 500 ml of distilled
water, the pH was adjusted to 8.3 with 1M sodium hydroxide
solution. Under magnetic stirring, 29.22 gm of sodium chloride
was added and stirring continued until the salt had dissolve and
the pH checked again. The buffer was then transferred into the
volumetric flask and the volume made upto 1000ml.

2.2 1mM hydrochloric acid solution for using the freeze dried
cyanogen bromide activated Sepharose 4B.

1mM hydrochloric acid solution was prepared by dilution of 36%
concentrated acid sp. gr. 1.18, i.e. 0.086 ml conc.HCl solution
per litre (V/V) of distilled water.

2.3 TM diethanolamine pure, pH 9.0 (blocking agent).

Diethanolamine pure ................... 105.14 gm
dissolved into 1 litre distilled water.
2.4 0.1M acetate buffer, pH 4.0 containing 0.5M sodium chloride

Acetic acetate ............................ 5.77 gm
Sodium acetate ............................ 8.2 gm
Sodium chloride ............................29.22 gm

Dissolve 5.77 gm acetic acid into 1 litre of distilled water 0.1M. Adjust pH of 0.1 M acetic acid with 0.1 M sodium acetate (8.20 gm/l) to pH 4.0.
Now add 29.22 gm/l of sodium chloride (0.5M).

Appendix 3: Buffers used in ELISA

3.1. Coating buffers

3.1.1. 0.06 M carbonate buffer, pH 9.6

Was freshly made from 1M stock solutions. 5 ml 1M sodium carbonate + 10 ml 1M sodium bicarbonate + 235 ml distilled water gave a pH close to pH 9.6.

3.1.2. Phosphate buffered saline (PBS) diluted 1/100 + 2% polyethylene glycol (PEG), pH 7.5

10.0 ml PBS diluted in 1000 ml distilled water plus 20 gm PEG;
0.1% sodium azide added as a preservative.
3.1.3. **0.015 phosphate buffer, pH 7.5 + 0.4 M sodium chloride + 2% PEG.**

1M sodium chloride was made (58.44 g/l dist. water). Then 0.4 M sodium chloride was made. 1 volume of 0.15 M phosphate buffered saline, pH 7.5 was mixed with 9 volumes of 0.4 M sodium chloride. 2% polyethylene glycol was added and preserved with 0.02% sodium azide.

3.2. **ELISA Washing Buffer Solution**

0.05% Tween 20, pH 7.5 PBS

4.5 ml Tween 20 was added into 900 ml of PBS and mixed thoroughly. No preservative was added.

3.3. **Enzyme substrate buffers**

3.3.1. **Substrate buffer for horseradish peroxidase (HRPO) and glucose oxidase.**

0.05 M citrate/ammonium acetate buffer, pH 5.0 +

0.1% benzoic acid.

800 ml distilled water

+ 10.5 gm citric acid (\( m_w = 210.14 \))

+ 3.0 ml glacial acetic acid (17.5M)

+ 1.0 gm benzoic acid.

pH was adjusted to 5.0 with concentrated ammonia solution, and then filled up to 1000 ml with distilled water.
3.3.3. Diethanolamine buffer, pH 9.6 for alkaline Phosphatase substrate

1000 ml distilled water
+ 10% (V/V) Diethanolamine pure
+ 200 mg Magnesium chloride (MgCl₂.6H₂O)

Adjust pH to 9.6 with 1 N HCl.

Appendix 4: Enzyme substrates

The substrates were made up freshly each time, used immediately and kept in the dark when made.

4.1. Glucose oxidase substrate

For ONE microtitre plate:

10.0 ml 0.05 M citrate ammonium acetate buffer, pH 5.0

(Appendix 3.3.1.)

+ 1.0 ml 20 or 18% glucose sol.
+ 0.1 ml HRPO solution (1.0 mg/ml)
+ 0.1 ml 2, 2- azinodi - (3-Ethyl benzthiazoline sulphonic) (ABTS) solution (25 mg/ml).

**Final Concentration**

| B - D - Glucose solution in 11.2 ml substrate | = 17.85 mg/ml |
| HRPO | 8.93 ug/ml |
| ABTS | 0.225 mg/ml |
4.2 Horseradish peroxidase substrate

(Ortho-phenylenediamine dihydrochloride) (OPD)

For ONE microtitre plate (96 wells):

11.0 ml 0.05 M citrate ammonium acetate buffer, pH 5.0
+ 0.1 ml STOCK OPD (100 mg/ml)
+ 0.1 ml 1% hydrogen peroxide
   (1/30 H₂O₂ in distilled water).

4.3 Alkaline phosphate substrate (1 mg/ml)

Sigma 104 phosphate substrate tablets. (p-nitrophenyl
phosphate Disodium)

10.0 ml diethanolamine buffer, pH 9.6 (Appendix 3.3.2).
+ 10.0 mg (2 tablets) substrate

(1 tablet contains 5.0 mg substrate plus filler).

Appendix 5. Diluents

5.1. Serum diluents

5.1.1. PBS + 0.05% Tween 20, pH 7.5

100 ml PBS
+ 0.5 ml Tween 20
5.1.2. 1000.0 ml 0.5 M phosphate buffer, pH 7.5

+ 75.0 gm KCL

+ 1.0 gm Na$_2$ - EDTA

+ 2.5 gm Benzoic acid

Varying amounts (percentages) of sera (2%, 5%, 10%), Tween 80 (0.5%, 0.1%, 0.05%), and PEG (2%, 4%) were added to the serum diluents in order to determine a suitable diluent.

5.1.3 PBS + 0.5% Tween 20 + 10% normal sheep serum + 10%

normal mouse serum.

80.0 ml PBS + 0.5% Tween 20

+ 10 ml normal sheep serum

+ 10 ml normal mouse serum.

The 10% normal mouse serum was added to the diluent to remove the natural anti-mouse activity in the goat samples. 10% normal sheep serum was added to reduce the non-specific binding in the non-coated wells.

5.1.4. PBS + 0.5% Tween 20 + 2% normal goat serum + 2% normal mouse serum:

96.0 ml PBS + 0.5% Tween 20

+ 2.0 ml normal goat serum

+ 2.0 ml normal mouse serum.
5.2 **Conjugate diluents**

1000.0 ml 0.05 M phosphate buffer, pH 7.5

+ 75.0 gm KCL

+ 1.0 gm Na₂ - EDTA

+ 2.5 gm Benzoic acid

+ 0.5 ml Tween 80 (0.05%) 

+ 5% of serum of the species of animal in which the antiserum was produced. No PEG was added to the conjugate diluent.

**Appendix 6. List of buffers and reagents used in immuno-electrophoresis and immunodiffusion**

6.1 **Barbital calcium lactate buffer for immunoelectrophoresis**

6.1.1. *(For electrophoresis chamber)*

Sodium barbital (sodium 5,5-diethylbarbiturate) .................. 105.0 gm

Barbital (5,5 diethylbarbituric acid) ... 16.6 gm.

Calcium lactate ......................... 15.2 gm

This was made into 10.0 l of distilled water. pH was adjusted to 8.4 or 8.6.
6.1.2. For preparation of agarose

The above buffer was diluted with distilled water in the ratio of 2:1 (2 parts buffer and 1 part water V/V).

6.2 Phosphate buffered saline (PBS) for the preparation of agar gel for immunodiffusion and other uses

1 volume of phosphate buffer 0.15 M, pH 7.5, plus 9 volumes of saline. (0.9% sodium chloride in distilled water).

6.2.1. Preparation of 0.15 M phosphate buffer, pH 7.5

Di-sodium hydrogen phosphate (anhydrous) \((\text{Na}_2\text{HPO}_4)\) ........ MW 141.96

Sodium dihydrogen orthophosphate \((\text{Na}_2\text{H}_2\text{PO}_4)\) ................ MW 156.01

0.15 M \(\text{Na}_2\text{HPO}_4\)

\[= \frac{21.3 \text{ gm/litre}}{} = \frac{63.9 \text{ gm/3 litres}}{}\]

Dissolve 63.9 gm \(\text{Na}_2\text{HPO}_4\) in 3 litres of distilled water. Dissolve 23.4 gm \(\text{Na}_2\text{H}_2\text{PO}_4\). \(2\text{H}_2\text{O}\) in 1 litre of distilled water. Adjust the pH of 0.015 M \(\text{Na}_2\text{HPO}_4\) solution to pH 7.5, by adding 0.15 M \(\text{Na}_2\text{H}_2\text{PO}_4\). \(2\text{H}_2\text{O}\) solution.
Appendix 7. Agar and agarose used in the immunodiffusion and immunoelectrophoresis

7.1. 1% Agar in phosphate buffered saline, pH 7.5, for immunodiffusion

Purified Oxoid Agar ......................... 2 gm
PBS ............................................. 50 ml
Distilled water .............................. 150 ml
Sodium azide (Na\(_3\)) ........................ 0.02 gm

0.1% (W/V) sodium azide was added as a preservative to prevent microbial growth on the agar during immunodiffusion.

7.2 1% agarose in the barbital lactate buffer for immunoelectrophoresis

Litex agarose (Type HSA) .................. 2 gm
Barbital lactate buffer ................... 50 ml
Distilled water ............................ 150 ml

Appendix 8. Protein staining and destaining solution

8.1 Coomasie Brilliant Blue 250 R ...... 10 gm
Ethanol ........................................ 900 ml
Glacial acetic acid ......................... 200 ml
Distilled water ............................ 900 ml
8.2 **Destaining solution - procedure for Coomassie blue solution.**

- Ethanol ..................................... 900 ml
- Glacial acetic acid ...................... 200 ml
- Distilled water ............................. 900 ml

8.3 **Ponceau "S" solution.**

- Ponceau S .................................. 2 gm
- 1 M acetic acid ............................ 1000 ml
- 0.1 M Sodium acetate .................... 1000 ml

8.4 **Destaining solution for Ponceau 'S' staining procedure.**

- 3% (V/V) glacial acetic acid in distilled water.

Appendix 9. **Other buffers.**

9.1 **1m M acetic acid/sodium acetate buffer, pH 4.4 (used in the conjugation procedures).**

- Acetic acid .................................. 17.4 M
- 1 M acetic acid ............................. 57.47 ml/l
- 1m M acetic acid ............................. 0.058 ml
- .............................................. 0.06 ml.
0.06 ml acetic acid added to 1000 ml distilled water, pH adjusted to 4.4 using 1 N NaOH, forming sodium acetate.

9.2 0.2 M carbonate buffer, pH 9.0 (used in the conjugation procedures).

Sodium bicarbonate (NaHCO₃) .......... M Wt 84.01

8.4 gm sodium bicarbonate

+ 500 mls. distilled water

Adjust the pH to 9.0 using solid sodium carbonate.