A SEROLOGICAL SURVEY OF THE PREVALENCE OF ANTIBODIES

TO <u>TOXOPLASMA</u> <u>GONDII</u> IN ANIMALS AND IN MAN IN KENYA

JOSPHAT OTWELO ABWAJO
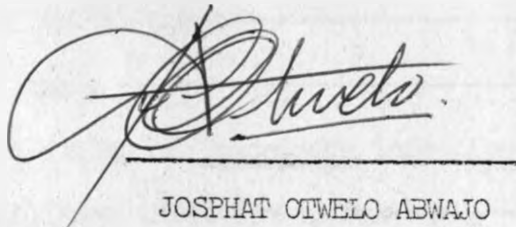B.V.M., Dipl. in Food Hyg. &
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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE

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FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF PUBLIC HEALTH,
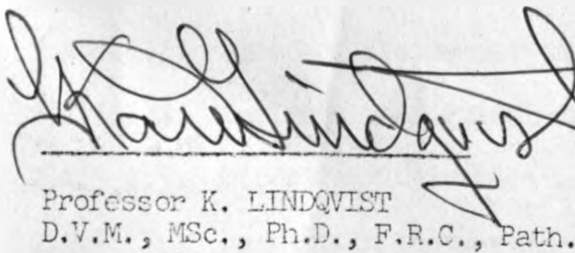PHARMACOLOGY AND TOXICOLOGY.

D E C L A R A T I O N

This thesis is my original work and has not been presented for a degree in any other University.
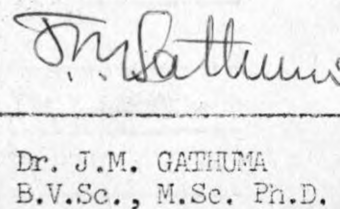
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This thesis has been submitted for examination with our approval as university supervisors

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S U M M A R Y

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 cooked 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 occured 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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D E D I C A T I O N S

Dedicated to my wife, Rebeccah and our three children

Sarah Ullah, Milkah and David.

1. 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 $\underline{T}$. $\underline{gondii}$ 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 toxo-

plasmosis 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-cyle, (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. ranae from

frogs, based solely on morphology.

A.   2.   <u>LIFE CYCLE</u>

In 1965, Hutchison demonstrated a resistant form of

<u>Toxoplasma</u> in cat faeces, and this formed a strong basis for

the establishment of the life cycle of <u>Toxoplasma</u>. Hutchison

<u>et al</u>., (1970, 1971) later demonstrated that <u>Toxoplasma</u> 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 <u>T. gondii</u>

are the domestic cat and a number of other members of the family

Felidae (Dubey, <u>et al</u>., 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 <u>T. gondii</u> in the intestine of the

felines are the resistant cocysts (Yilmaz and Hopkins, 1972;

Ruiz <u>et al</u>., 1973; Frenkel <u>et al</u>.,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 <u>et al</u>., 1972; Hartley and Munday, 1974; Frenkel <u>et al</u>.,

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., 1971 a; 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 Carne (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.

Chhabra 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, Berverley 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 Czchoslovakia.

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

prevalance of Toxoplasma infection in wild animals as

being 10.8%. of the 10.8% wild animals surveyed, 68%

bobcats, 28.0% coyotes, 27% grey foxes and 48% racoons

had antibodies against T. gondii. Toxoplasmosis

has been reported in Kenya in a colobus monkey by

Olubayo and Mwongela (1978); and tree hyrax (Olubayo

and Karstad, 1981). Roher et al. (1961) 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 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 a) 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..1976a; 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 1.5×10^5 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 conjuction 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

chorioallantoic 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

occured 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 ultra-

sonicated 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.   <u>Isolation of the Toxoplasma organisms</u>

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

<u>Toxoplasma</u> by means of peptic digestion technique. Jacobs

<u>et al</u>. (1960) used the peptic digestion method to isolate

<u>Toxoplasma</u> from relatively large samples from cattle.

Work (1967) used Jacobs and Melton's isolation technique to

recover <u>Toxoplasma</u> from the flesh of sheep, swine and cattle.

Waldeland (1976a) detected <u>T</u>. <u>gondii</u> 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 <u>et al</u>., 1975; Jørgensen and Otwelo, 1978).

Isolation of <u>T</u>. <u>gondii</u> is carried out best by the

innoculation 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, micro-

crystalline 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 B-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 photo-

metrically 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 (1978). Absorbance values of test samples at a

single serum dilution are converted into MONA using a

formula derived by Felgner (1978).

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 toxoplasmosis (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

toxoplasmosis. 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 5.8×10^6

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 ..... 44.......................... 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

g)   231/82 sheep ....   DT -ve

h)   Sheep II ....·.   DT -ve

i)   Sheep I **b** ....   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.Olsvik, tested in ELISA

and used as control sera:

a)   30/12/82 Goat ELISA Toxo- (+ve)·......   DT   +ve

b)   2/3/82 Goat immunized ....   DT   +ve

c)   Immunized goat C1 ....   DT   +ve

d)   "   "   02 .... DT   +ve

e)   "   ·· |/ 03 ....   DT   +ve

f)   "   "   04 ....   DT   +ve

g)   "   "   05 ....   DT   +ve

h)   "   "   06 ....   DT   +ve

i)   30/12/82 (585) Goat ELISA Toxo (-ve)...   DT   -ve

j)   Goat 02 ....   DT   -ve

k)   Goat 051 ....   DT   -ve

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

Identification (Animal Species and No.)	Prepartion for immunization	Antisera prepared
Goat No. 381 and No. 382	Human IgG	Goat (381/382)-anti-human IgG serum
Sheep No. 160 and No. 161	Goat IgG	Sheep (160/161)-anti-goat IgG serum
Goat No. 632 and No. 633	Sheep IgG	Goat(632/633)-anti-sheep IgG serum

3.3   Analysis of the antisera used in this study

Expriments 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. 8.

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 Columbrook, 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

immunoscrption 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 1m 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 $+4^{\circ}$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 spectro-

photometer (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 $+4^{\circ}$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 antiserum by passing the serum through the sintered glass filter (No G3 - Pyrex, England). The immunsorbent 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 thiocynate

(KCNS), 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.
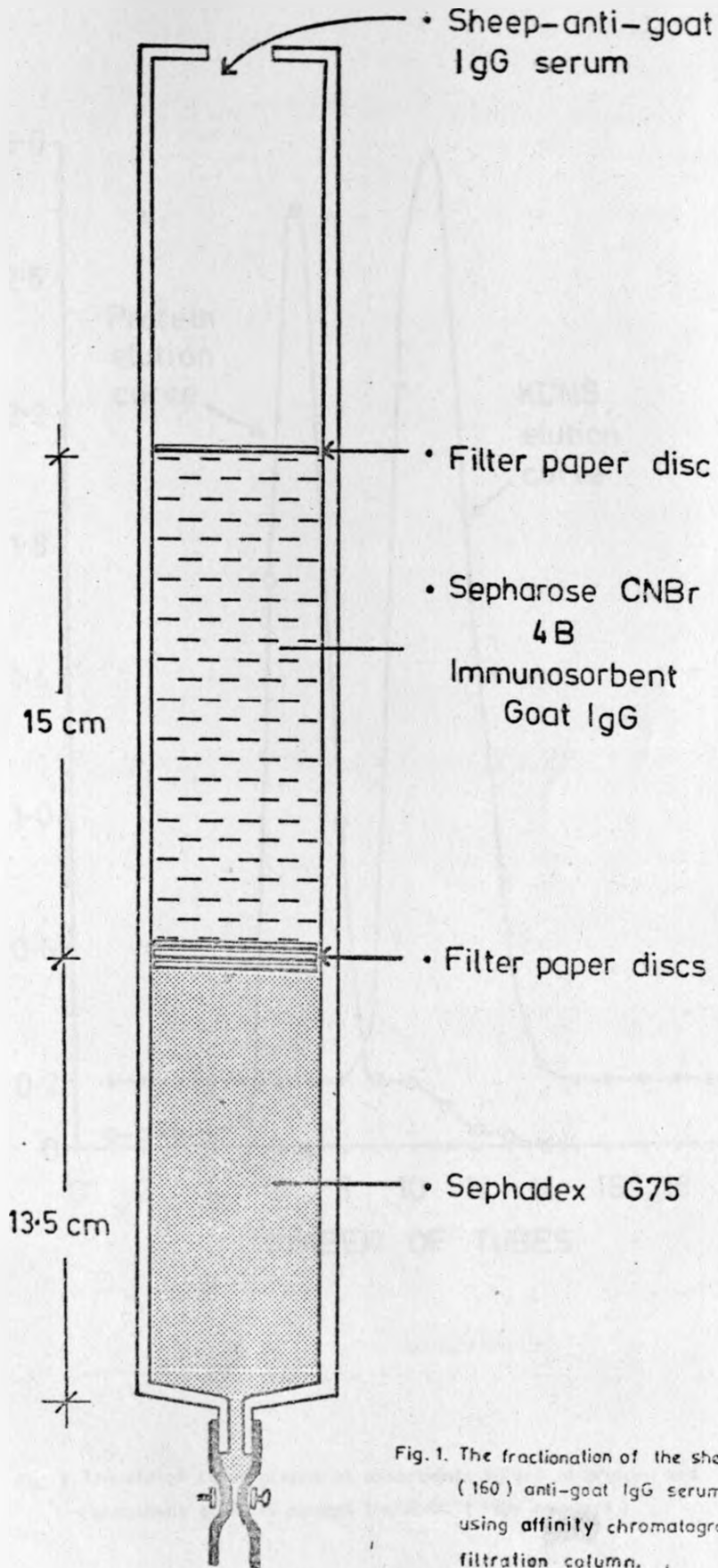
Fig. 1. The fractionation of the sheep (160) anti-goat IgG serum using **affinity** chromatography/gel filtration column.
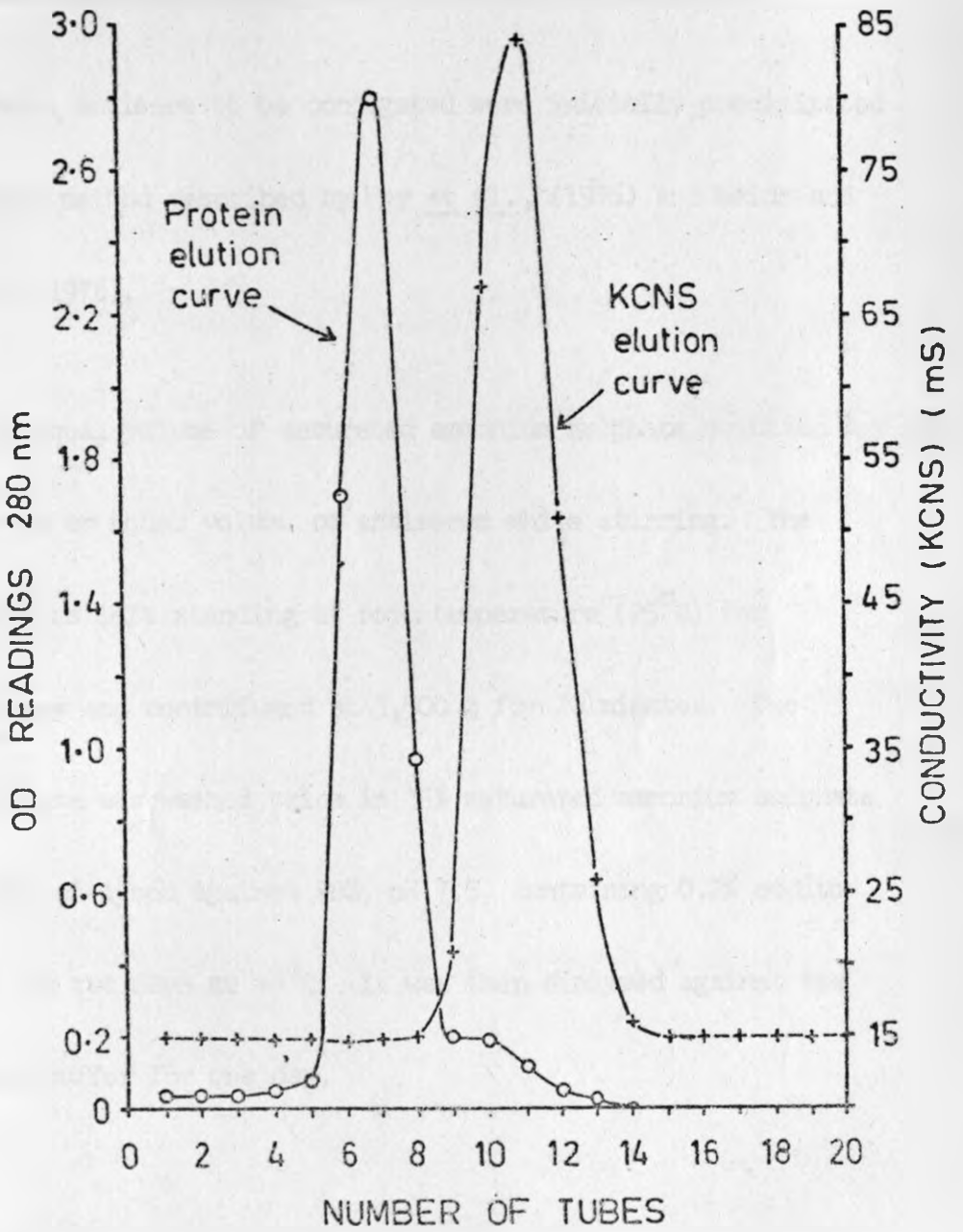
Fig. 2. The elution curve graphs of absorbance values of protein and conductivity of KCNS against fractions (tube numbers)

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 (1978).

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 $+4^{\circ}$C. It was then dialysed against the

initial buffer for one day.

The precipitate and the dialysed gammaglobulin fraction

(25.0ml) was further purified on a diethylamino ethyl-

cellulose (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 (NaIO$_4$) 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 $-20^{\circ}C$.

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 $+4^{o}$C. The latter two parasitic

antigens were crude extracts, concentrated by lyophylization

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 occured 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
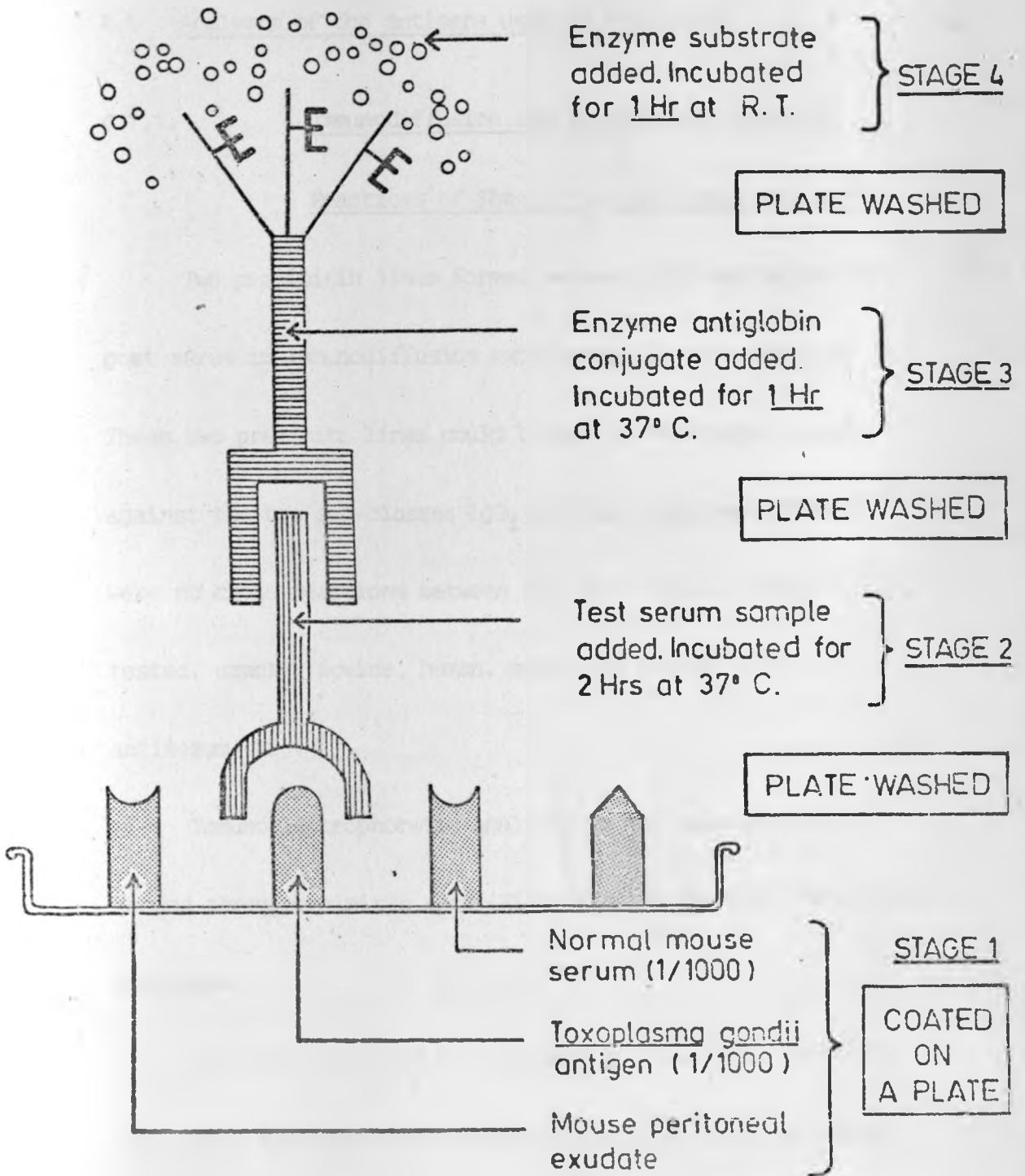
Fig 3   An indirect Toxoplasma gondii ELISA used in detecting anti-Toxoplasma antibodies in humans, sheep and goats.

4. RESULTS

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 precipitn lines could be due to reactions formed

against the two sub-classes IgG_1, and IgG_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 immuno-

globulin 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 Immunodiffussion 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

$-20^{\circ}C$ 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

44% between the DT and ELISA.

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 158 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.
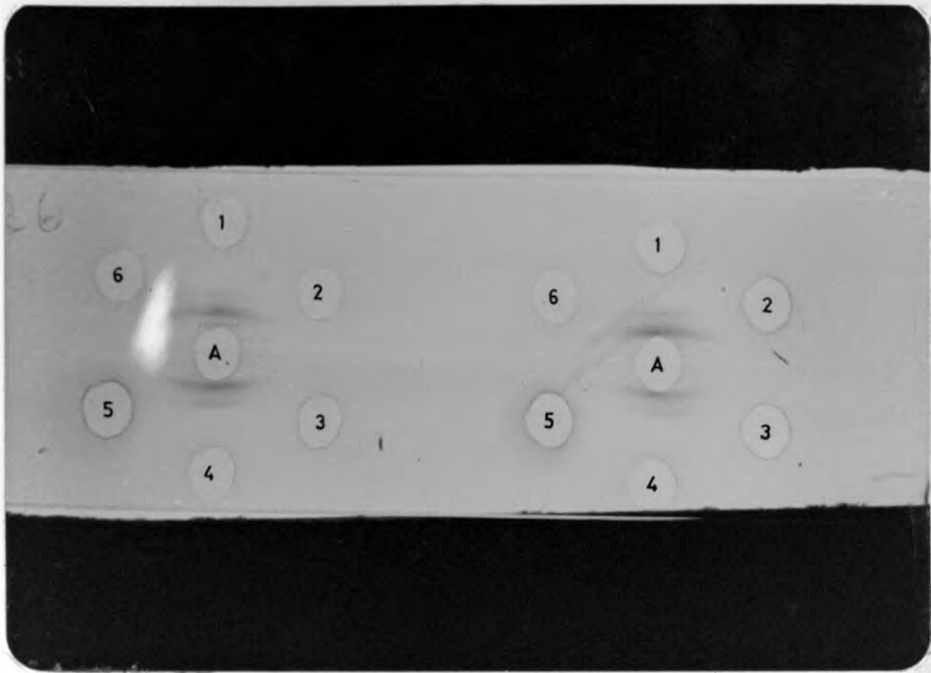
Fig. 4 :   Immunudiffusion of Sheep-anti-goat IgG serum
                  against other animal species.

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   Immunoelectrophoretic 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
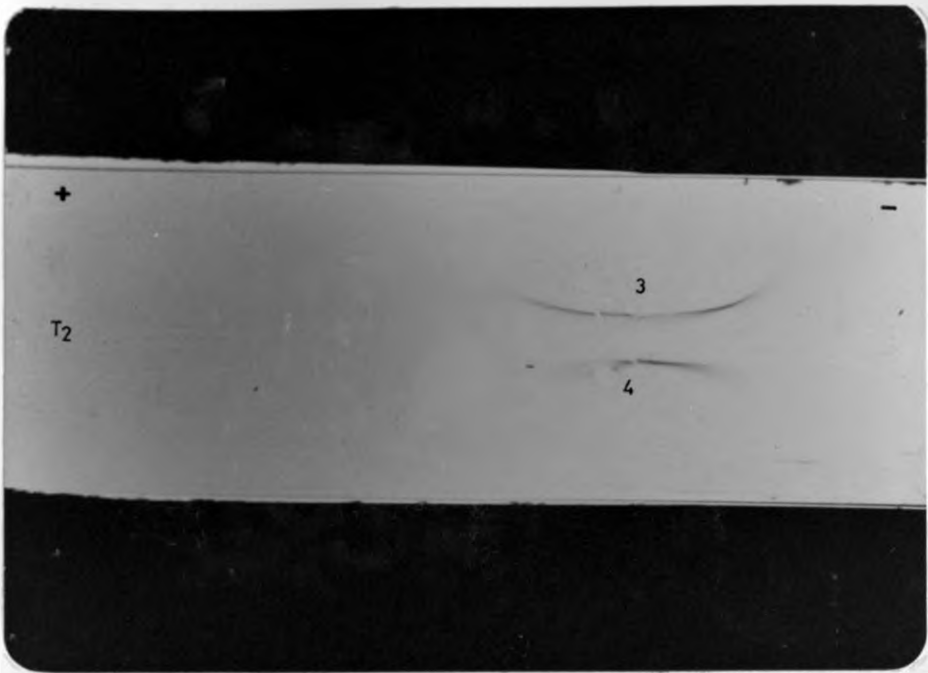
Fig. 5b :   Immunoelectrophoretic analysis of sheep

(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
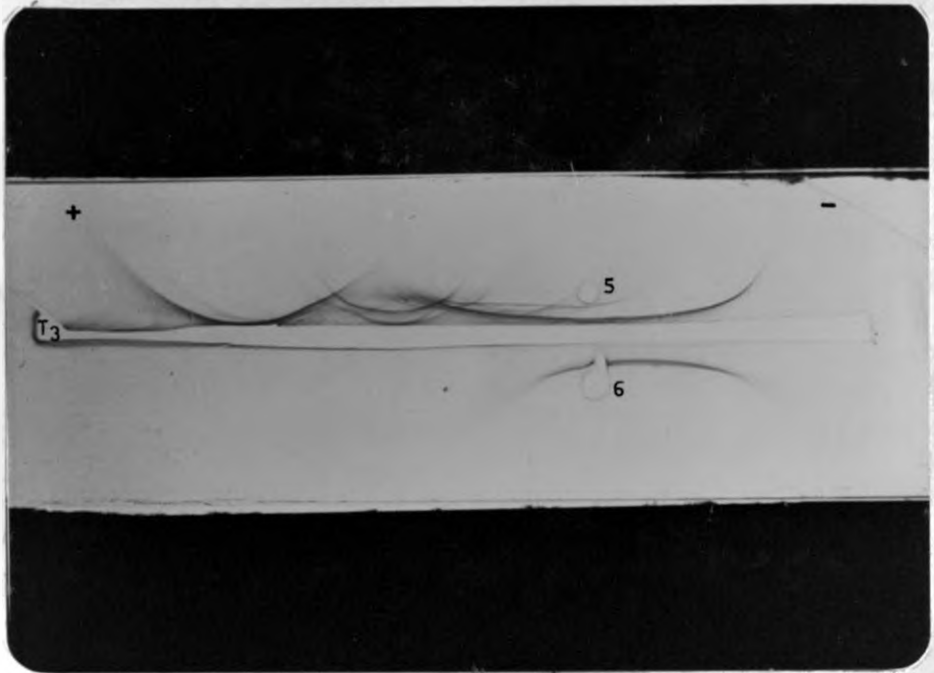
Fig 5c:　　　Immunoelectrophoretic analysis of rabbit-
　　　　　　　anti-goat serum against neat goat serum

　　　　　　　and goat IgG.

　　　　　　　Used as control and showing IgG class in neat

　　　　　　　goat serum.

Trough　:　T_3　　= Rabbit-anti-goat serum

Well　　:　5　　= Neat goat serum

Well　　:　6　　= Goat IgG
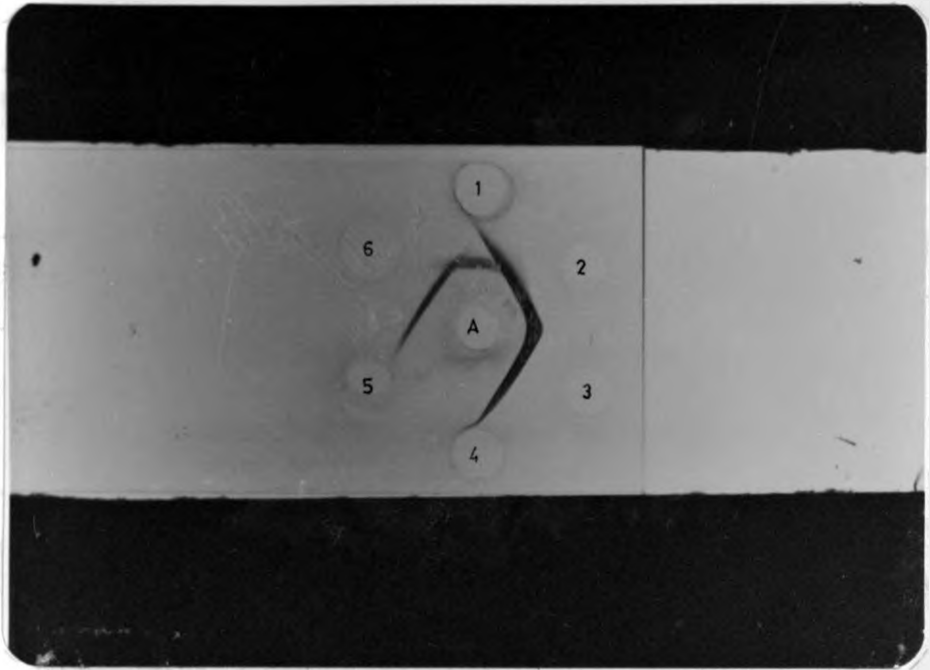
Fig. 7 :   Immunodiffusion of unabsorbed goat (381/382)-

anti-human IgG serum against other animal

species.

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

Well : 1   Mouse serum

Well : 2   Human serum

Well : 3   Human serum

Well : 4   Bovine serum

Well : 5   Sheep serum

Well : 6   Rabbit serum
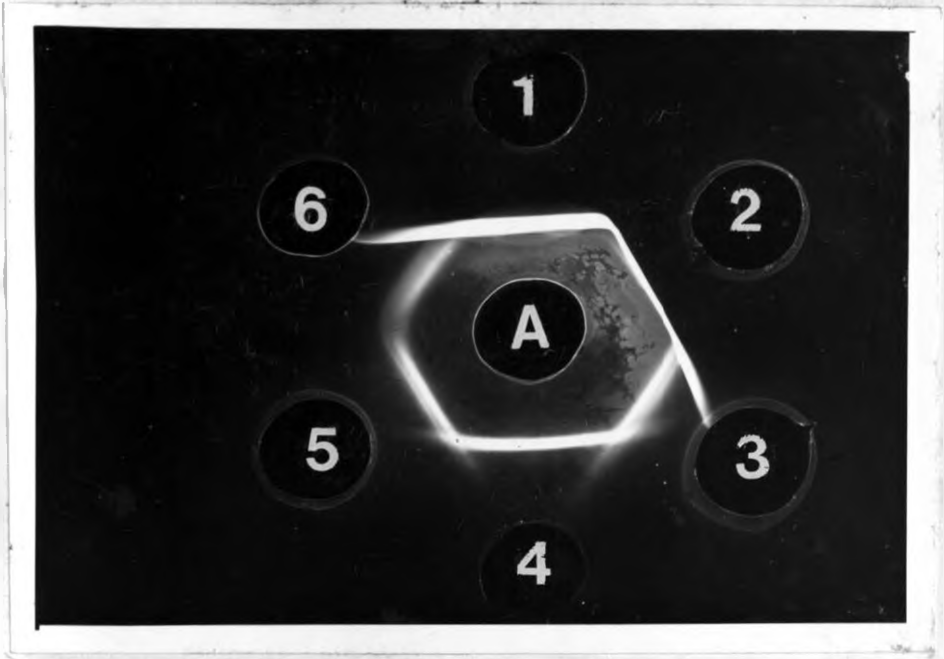
Fig. 6 :   Immunodiffusion of unabsorbed goat (381/382)-

               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
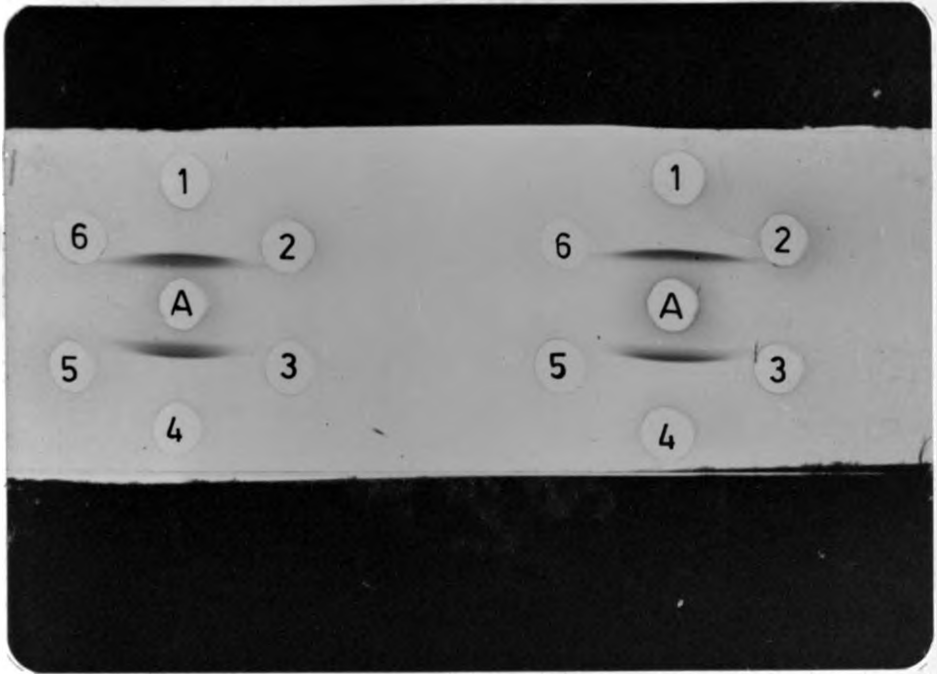
Fig. 9 :   Immunodiffusion of goat (632/633)-anti-

                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
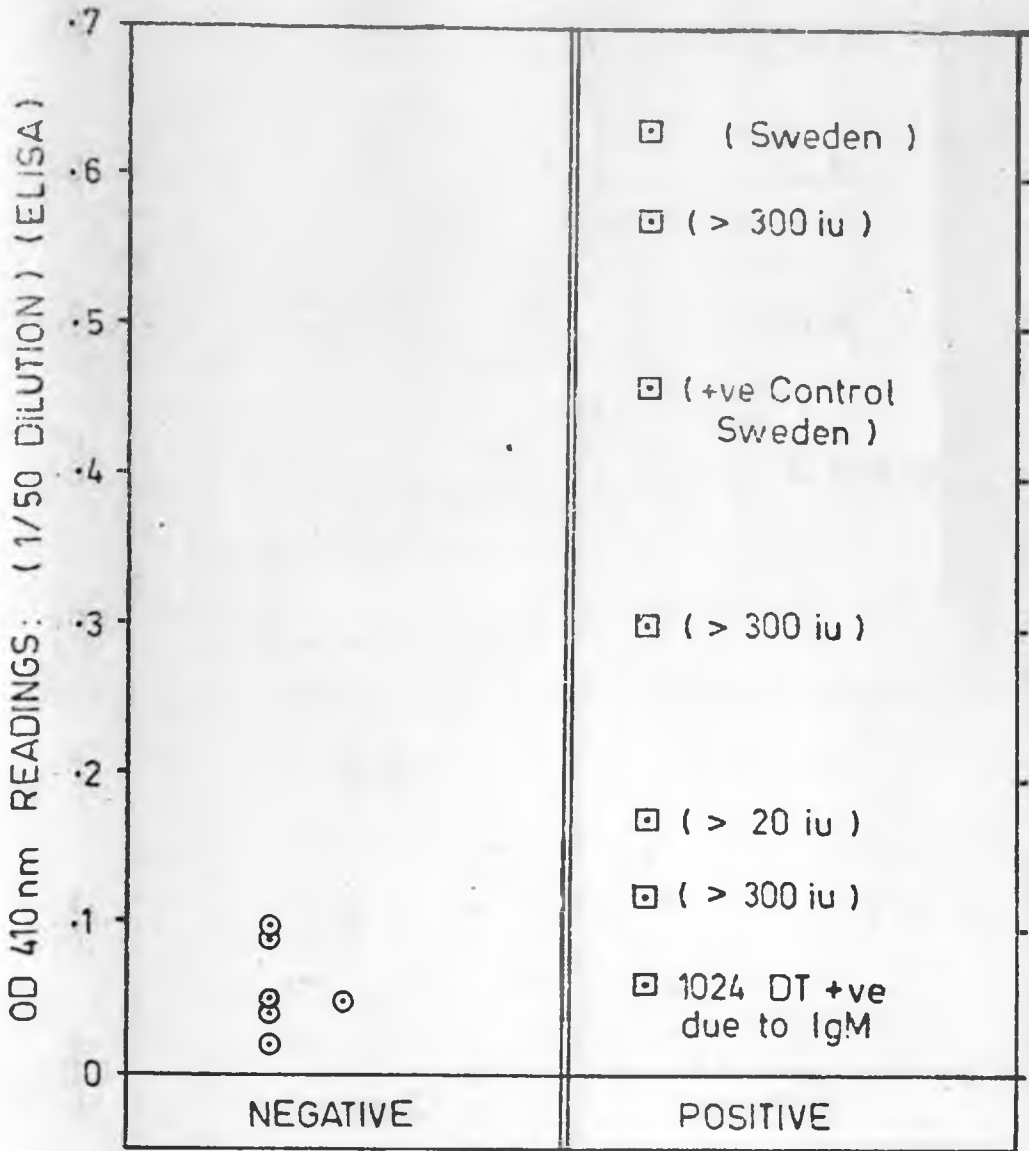
Well : 6   Rabbit serum

Fig. 10 Comparison of ELISA with dye test in 13 human serum samples.
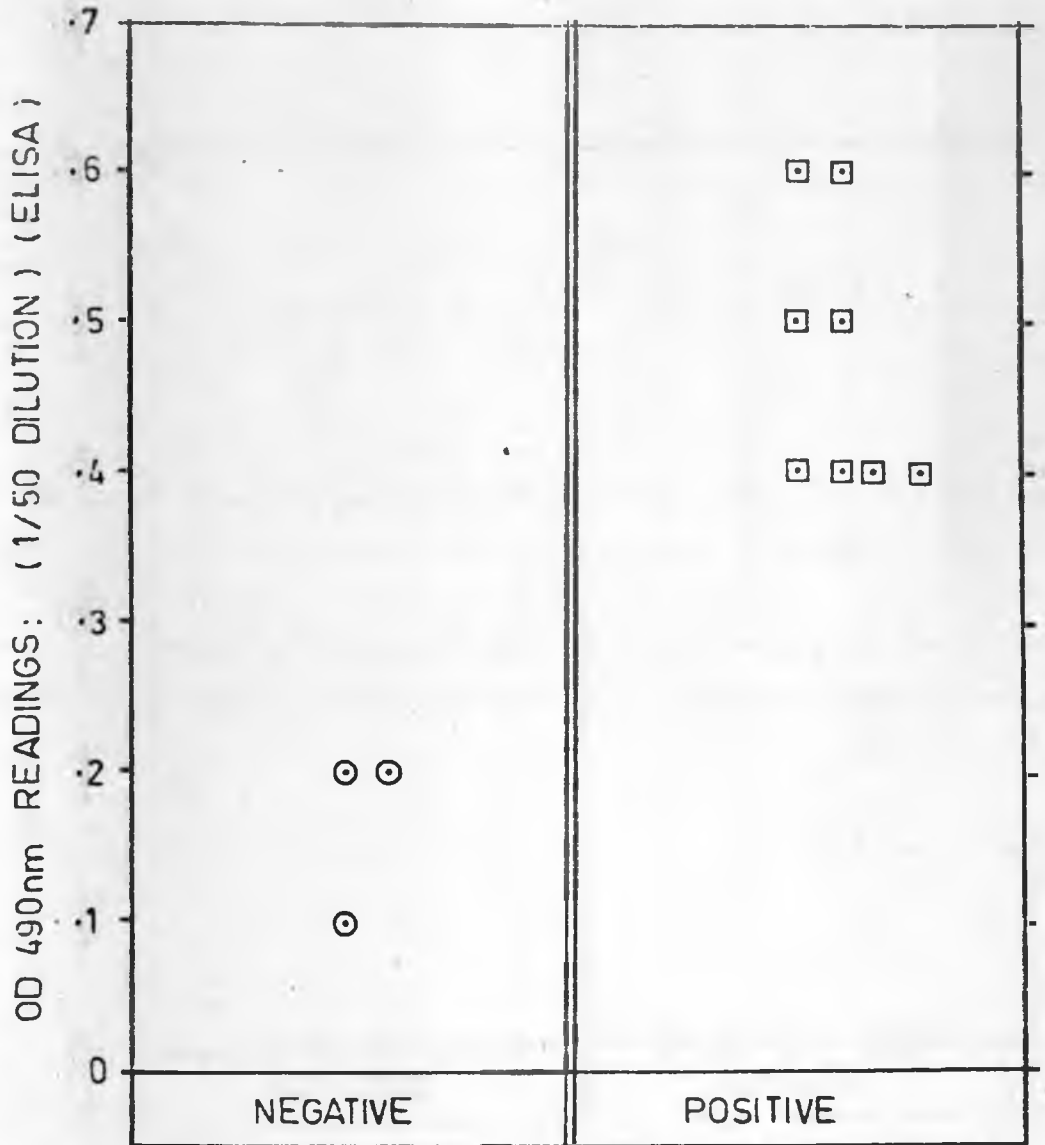
Fig. 11. Comparison of ELISA with dye test in 11 goat serum samples.
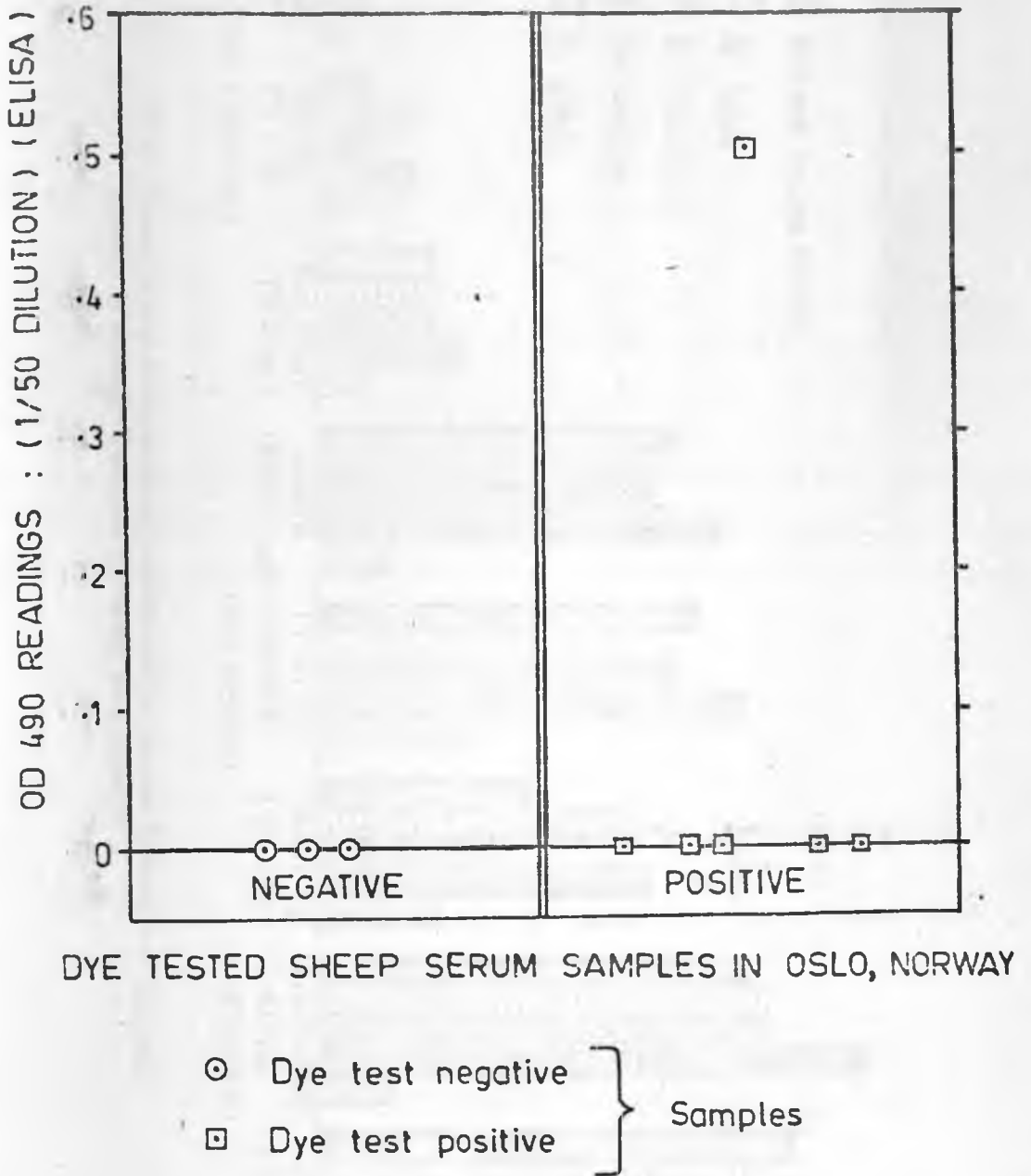
Fig. 12. Comparison of ELISA with dye test in 9 sheep serum samples.
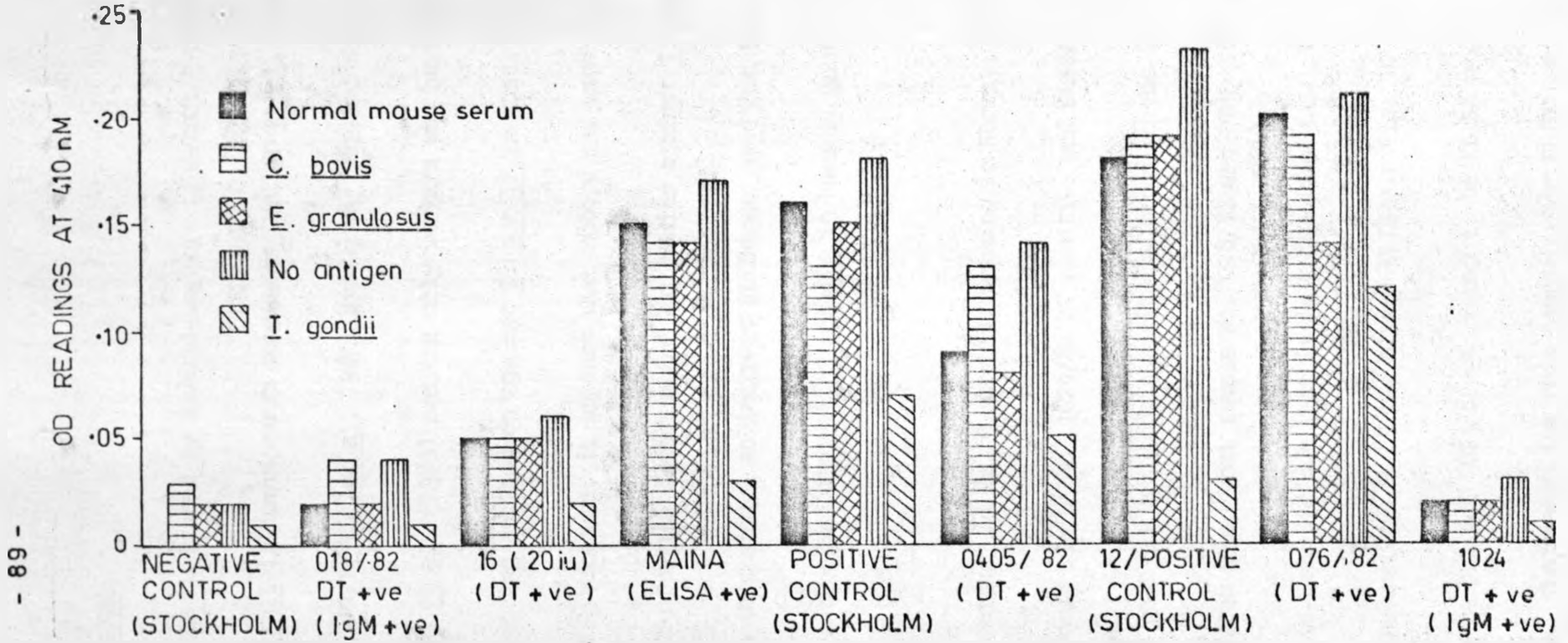
Fig 13. Demonstration of the immunological specificity of ELISA reactions for detection of Toxoplasma antibodies by absorptions experiments in 9 human serum specimens

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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 Baganda 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 Toxoplama 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 brouse 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 signifcance of higher prevalence rates of 21% and 56% among goats and sheep, respectively, might prove to be significant in view of the high prevalance 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 Ruitenberg 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 occuring 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 occuring 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). Letarte 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-phenylendiamine 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 2N H_2SO_4.

Lin et al (1980) found that the Toxoplasma antigen

on a solid phase could even be kept at room temperature

($23^{o}C$) for at least 10 months without loss of activity.

Microtitre plastic plates coated with T. gondii antigen

at either $+4^{o}C$ or room temperature ($25^{o}C$) overnight were

found to keep well for over 3 months at $-20^{o}C$ 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

investigated.

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

washing solution for ELISA (Mondesire et al. 1981; Woodward,

1982; Berdal et al. 1983).

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 encoutered 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

thawn 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.

C O N C L U S I O N

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 conjuction with those of Mas Bakal 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 occuring

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.

R E F E R E N C E S

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A P P E N D I X

Appendix I: Immunosorbents used and the antisera absorbed in this study

Antigen coupled to CNBr - Sepharose	Amount of total protein determined by spectro-photometry	Percentage coupled to Sepharose	Antisera absorbed/ purified
Goat purifed IgG	163.0 mg protein	74%	Sheep anti-goat serum (purified)
Mouse-serum protein ppt. 50% $(NH_4)_2SO_4$	6.83 mg protein	99%	Goat-anti-human IgG serum
Whole bovine serum	ND	ND	Goat-anti-human IgG serum
Whole rabbit serum	ND	ND	Goat-anti-human IgG serum
Whole sheep serum	ND	ND	Goat-anti-human IgG sheep

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$_3$), 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 <u>0.1M acetate buffer, pH 4.0 containing 0.5M sodium chloride</u>

    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: <u>Buffers used in ELISA</u>

3.1.   <u>Coating buffers</u>

3.1.1. <u>0.06 M carbonate buffer, pH 9.6</u>

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

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 (mw = 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 upto 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 (Mg $Cl_2.6H_2O$)

Adjust pH to 9.6 with 1 N HCl.

Appendix 4: Enzyme substrates

The substrates were made up feeshly 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

β - 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 - phenylendiamine 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 H_2O_2 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) cf 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   <u>PBS + 0.5% Tween 20 + 10% normal sheep serum + 10%</u>

<u>normal mouse serum.</u>

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.   <u>PBS + 0.5% Tween 20 + 2% normal goat serum + 2% normal</u>

<u>mouse serum:</u>

96.0 ml PBS + 0.5% Tween 20

+ 2.0 ml normal goat serum

+ 2.0 ml normal mouse serum.

5.2 <u>Conjugate diluents</u>

1000.0 ml 0.05 M phosphate buffer, pH 7.5

+ 75.0 gm KCL

+ 1.0 gm Na$_2$ - 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.

<u>Appendix 6.</u> <u>List of buffers and reagents used in immuno-</u>
<u>electrophoresis and immunodiffusion</u>

6.1   <u>Barbital calcium lactate buffer for immunoelectro-</u>
<u>phoresis</u>

6.1.1.   (<u>For electrophoresis chamber</u>)

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) (Na_2HPO4)....... MW 141.96

Sodium dihydrogen orthophosphate (Na_2H_2PO4) .... MW 156.01

0.15 M Na_2HPO4 $=$ 21.3 gm/litre

$=$ 63.9 gm/3 litres

Dissolve 63.9 gm Na_2HPO4 in 3 litres of distilled water. Dissolve

23.4 gm Na_2H_2PO4. $2H_2O$ in 1 litre of distilled water. Adjust

the pH of 0.015 M Na_2HPO4 solution to pH 7.5, by adding

0.15 M Na_2H_2PO4. $2H_2O$ solution.

Appendix 7.   Agar and agarose used in the immunodiffusion and

immunoelectrinhoresis

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

Purified Oxoid Agar ....:....... 2gm

PBS ....50 ml

Distilled water ....150 ml

Sodium azide (Na N_3) .... 0.02 gm

0.1% (W/V) sodium azide was added as a preservative to prevent

microbial to 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 coomasie 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/1
    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 (NaHCO$_3$) .... M Wt 84.01

8.4 gm sodium bicarbonate

+ 500 mls. distilled water

Adjust the pH to 9.0 using solid sodium carbonate.