TOXOPLASMOSIS AND ABORTION: SEROLOGICAL, EXPERIMENTAL ANIMAL STUDY AND AN ATTEMPT TO ARTIFICIAL CULTURE

DR. MUGHIS UDDIN AHMED

DEPARTMENT OF MICROBIOLOGY
B.M.S.I., J.P.M.C.
UNIVERSITY OF KARACHI, PAKISTAN
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Guide us in the right path
WHATEVER IS IN HEAVEN AND EARTH IS FOR ALLAH
TOXOPLASMOsis AND ABoRTION
SEROLOGICAL, EXPERIMENTAL
ANIMAL STUDY AND AN ATTEMPT
TO ARTIFICIAL CULTURE

By
MUGHIS UDDIN AHMED

Thesis
Submitted for the degree of Doctor of
Philosophy in Microbiology

Department of Microbiology
B.M.S.I., J.P.M.C.
University of Karachi
Karachi, Pakistan
CERTIFICATE

This thesis is submitted to the Faculty of Science, University of Karachi by Dr. Mughis Uddin Ahmed Department Microbiology Basic Medical Science Institute, Jinnah Postgraduate Medical Center and it satisfies the requirements for the degree of Doctor of Philosophy in Microbiology.

Internal Examiner
PROF. AMTUL HAFIZ

External Examiner
DR. BADAR IEHAN FAROOQI
DEDICATED TO

My Parents, Wife and Children

for their loving care

and

Professor Amtul Hafiz

for

her teaching & able guidance
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Thanks to Mr. Nisar Muhammad Usmani for typing my thesis well in time.
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<td>AB</td>
<td>Threatened, inevitable, incomplete and missed abortions.</td>
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<td>CFT</td>
<td>Complement Fixation Test.</td>
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<tr>
<td>DA</td>
<td>Direct Agglutination.</td>
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<tr>
<td>DT</td>
<td>Dye Test.</td>
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<tr>
<td>EIA</td>
<td>Enzyme immuno assay.</td>
</tr>
<tr>
<td>EIU</td>
<td>Enzyme immuno units.</td>
</tr>
<tr>
<td>ELIFA</td>
<td>Enzyme Linked Immuno Filtration Assay.</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay.</td>
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<td>G(g)</td>
<td>Gram</td>
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<tr>
<td>HIV</td>
<td>Human Immuno-deficiency Virus.</td>
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<td>hr</td>
<td>Hour</td>
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<tr>
<td>HP</td>
<td>High positive.</td>
</tr>
<tr>
<td>IFA</td>
<td>Immuno fluorescent assay.</td>
</tr>
<tr>
<td>IHA</td>
<td>Indirect Haemagglutination Assay.</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin.</td>
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<tr>
<td>ISAGA</td>
<td>Immunosorbent Agglutination Assay.</td>
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<td>LP</td>
<td>Low positive.</td>
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<td>M</td>
<td>Mughis</td>
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<td>mg</td>
<td>Milligram</td>
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LIST OF ABBREVIATIONS CONT'D.

min  Minute
ml   Milliliter.
mm   Millimeter.
nm   Nanometer
PO   Positive.
+ve  Positive.
P    Probability
PCR  Polymerase Chain Reaction.
PEF  Penetration Enhancing Factor.
rpm  Revolution per minute.
RIA  Radio Immuno Assay.
RT   Room temperature.
S.D.  Standard deviation.
μm   Micrometer.
UNP  Uncertain positive.
TA   Therapeutic abortion.
%    Percent
<    Less than.
>    More than.
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ABSTRACT
This study involved the analysis of serum samples and product of conception in 105 females. The serum analysis for IgM and IgG was done by enzyme immuno assay (EIA) in our population (Karachi). All human sera were also screened for human immuno deficiency virus (HIV) to rule out that acquisition of Toxoplasma gondii was not due to immuno suppression. The product of conception was used for making wet mount, stained preparation and mice inoculation (with saline control). The one group of mice was sacrificed on 3rd or 4th day and other on or about 3rd week. They were subjected to wet mounting, staining, EIA (IgM and IgG) and poured into artificial media. All IgM sero-positive cases were confirmed by IgM confirmatory kits.

Out of 105 samples tested for IgM antibodies 16 (15.2%) were sero-positive in both groups. In AB-group from 54 cases 14 (25.9%) were positive as compared to 2 (3.9%) out of 51 in TA group. IgM seropositivity was found to decrease with increase in age (inverse correlation) and highest number of cases were among threatened abortion.

In IgG type of antibodies out of total 105 there were 35 (33.3%) sero-positive. In AB group there were 27 (50.0%) sero-positive out of 54 screened and in TA group out of 51 cases tested 28 (15.7%) were sero-positive. IgG seropositivity was found to increase with increase in age (positive correlation). Highest number of cases
were also among threatened abortion.

HIV screening by EIA in both AB and TA groups revealed not a single sero-positive case. HIV results indicates that seropositivity of Toxoplasma was not due to immuno suppression.

Direct microscopy of the product of conception by wet mount and stained preparation (alkaline methylene blue) revealed parasite in two cases.

Serum study done in mice sacrificed on 3rd or 4th day showed no seropositivity for IgM and IgG type of antibodies, whereas mice sacrificed on 3rd week gave .2 (3.7%) seropositivity for IgM and IgG in AB group out of 54 cases.

Wet mount and stained preparation (alkaline methylene blue) revealed parasites from peritoneal wash of mice sacrificed on 3rd or 4th day.

In this study we have cultured *Toxoplasma gondii* in artificially prepared media for first time. The best growth was in TC-199 monophasic medium without antibiotic at 25°C and in the moist condition. The doubling time was 4 days for *Toxoplasma gondii* in this study. M-media was found very good for keeping toxoplasma for longer period at 4°C to 8°C.

Wet mount preparation shown us motility of toxoplasma, which was more in small (dwarf) form as compared to others. Three
forms of Toxoplasma gondii has been recognised in present study, large (Giant) form, medium (Intermediate) form and small (dwarf) form, keeping red cell as an index.

Artificial culture helped us in studying toxoplasma in detail by different stain and electron microscopy (Transmission & scanning) preparations.

Hallmark of this study was that for the first time we managed to culture toxoplasma in artificial media (monophasic TC-199 with salts & without antibiotics) and in M-medium they were kept alive for > 02 years at 4°C to 8°C.

Different forms of Toxoplasma and motility was also recognised for the first time.
The serum level of 105 patients was examined for the presence of anti-TA antibodies. Serum (Serum) was collected from all patients for analysis. The results showed that 87% of the patients had positive anti-TA antibodies, indicating a high prevalence of this antibody in the study population.

In a separate study, the serum level of 105 patients was examined for the presence of anti-M antibodies. Serum was collected from all patients for analysis. The results showed that 73% of the patients had positive anti-M antibodies, indicating a high prevalence of this antibody in the study population.

In another study, the serum level of 105 patients was examined for the presence of anti-G antibodies. Serum was collected from all patients for analysis. The results showed that 63% of the patients had positive anti-G antibodies, indicating a high prevalence of this antibody in the study population.

In addition, the serum level of 105 patients was examined for the presence of anti-54 antibodies. Serum was collected from all patients for analysis. The results showed that 54% of the patients had positive anti-54 antibodies, indicating a high prevalence of this antibody in the study population.

In a separate study, the serum level of 105 patients was examined for the presence of anti-TP antibodies. Serum was collected from all patients for analysis. The results showed that 43% of the patients had positive anti-TP antibodies, indicating a high prevalence of this antibody in the study population.
پاپازم) کو دوبارہ اس قبیلاً مساوی کے دو درد لازماً ترگوئی کی تو جلد مرگ میں قومت (سمند) پر کئی کسی اور TC-199 Monophasis میٹمیا پر نگیر اکیڈمی کو 25 سال متمم پر نم مثال میں میٹمیا پر ایک مسلسل تر کر کے تہجی حیرت کے 4 میٹمیا ترگوئی کو دوبارہ کیتا۔

میٹمیا سے 8 سمند میں نگیر پر زبردستی کے نقصان میں رکھا ہے۔

تمام مثال میں خیزدو معاہدے سے لازماً ترگوئی کی تعریفی دو مثال مساوی میں لازماً ترگوئی کی جن مثال وکل میں محمد میں نہیں

(سمند) میں مثال اور یہ کہ لازماً ترگوئی کے مثال مساوی کے نئے جم میں اس مثال میں گئی،

سے بیسی میں۔

تمام مثال میں خیزدو معاہدے سے لازماً ترگوئی کو کل کل زبردستی میں رکھا ہے۔

کئی زمرہ کے سے میں جملہ 19 سے گھو میں مثال مساوی کو 4 سے 8 سمند میں مثال کے نئے مثال اور یہ کہ لازماً ترگوئی کے مثال مساوی کے نئے مثال

سے کام کا جلد مرگ ہم ہو۔
INTRODUCTION
Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii* which affect a wide range of animals and birds (Jawetz et al., 1991). For the purpose of definition, toxoplasmosis is clinical disease caused by a coccidian protozoan parasite *Toxoplasma gondii*, whereas Toxoplasma infection refers to presence of either the tachyzoite form or the cyst in tissue irrespective of clinical disease (Macabe and Remington, 1984).

It is found to infect 5-95% of various human populations and is considered one of the most prevalent human diseases which may not always manifest clinically (Legnain et al., 1982 and Cohen et al., 1988). The number of human beings infected around the world are about 500 million (Jones, 1982). The clinical character of the disease varies with the organs attacked, which itself varies depending on whether the disease is congenital or acquired. The acquired form is usually seen in adults with lymphadenopathy and low grade fever as the most common manifestations. The other organs involved are lung, liver, heart, brain, eye and placentae of pregnant women. Immunity may be associated with chronic infection, which may recrudesce when a patient is immunosuppressed (Frenkel, 1984).

In pregnancy, the fetus is commonly invaded via an initial placentitis, which may result in abortion and if the fetus is carried to term it is commonly born dead or affected with congenital toxoplasmosis. Infection during pregnancy is almost always asymptomatic. Nevertheless
transplacental transmission following parasitemia in the mother may lead to fetal infection and congenital toxoplasmosis (Poulon et al., 1984).

The term abortion and miscarriage are synonymous and denote the expulsion of the conceptus before the end of the 28th week of pregnancy (Ahmed, 1996). Clinical observation suggests that more than 15% of pregnancies end in early abortion, but the loss of very early embryos may be greater than this. The most common time for clinically evident abortion to occur is between 8 and 13 weeks. The known causes are malformation of zygote, immunological rejection of foetus, general disease of mother (toxoplasmosis, rubella, cytomegalo-viral infection, syphilis, etc.), uterine abnormalities, hormonal insufficiency and other miscellaneous group (irradiation of the uterus, quinine, ergot, prostaglandins, accidental or operative trauma to uterus or the insertion of foreign bodies etc.). Clinical varieties of abortion are threatened, inevitable, complete, incomplete, septic, missed, recurrent, criminal and therapeutic abortions (Clayton, 1985).

Spontaneous abortion following toxoplasmosis in pregnancy occurs in < 10% of patients infected during the first trimester (Desmonts and Couvreur, 1974a). Most human infections are asymptomatic however, fulminant fatal infections may develop in patients with AIDS presumably by alteration of a chronic infection to an acute one (Jawetz et al., 1991, Jiming, 1995 and Ahmed et al., 1996).
Toxoplasma gondii has been frequently incriminated in causation of abortion, habitual or repeated miscarriages, premature births or still births and congenitally abnormal babies (Feldman, 1953a, Beattie, 1984, Mehta, 1987, Kagawala et al., 1989 and Ahmed, 1995). Toxoplasma gondii was isolated in 23 (32.8%) out of 70 cases in aborting women (Langer, 1963).

Toxoplasma was found as a cause of chronic infection of uterus (Remington et al., 1960 and 1961) and the organism may result in repeated chronic abortion (Remington, 1964). Organism has been incriminated in repeated congenital infection of fetuses (Gibson and Eyles, 1957 and Blantter, 1964). Another study carried out suggests that chronic Toxoplasma infection is an uncommon cause of habitual abortion, which contradict the above studies (Kimball et al., 1971). It has been oberved that primary infection with Toxoplasma gondii acquired during pregnancy, can result in congenital infection of offspring or in spontaneous abortion (Beckett and Flynn, 1953 and Desmonts et al., 1974b). Desmonts and courveur (1974a) published results of their study according to which 378 cases with increased antibody titers, 183 acquired during pregnancy 6.3 per 100 pregnancies with history of abortions, still births, severe cerebral and ocular defects in babies. Mild illness was seen in some cases. The parasite Toxoplasma gondii was
isolated from 25% of placentae of aborting female (Glasser and Delta, 1965). Immunity to Toxoplasma in females previously exposed to Toxoplasma infection has been reported (Obendor et al., 1990).

Human immuno deficiency virus (HIV) is the cause of the fatal condition called Acquired Immuno Deficiency Syndrome (AIDS). Cases of congenital toxoplasmosis have been observed in infants perinatally infected with human immuno deficiency virus (Mitchell et al., 1990, Locarnini, 1994, Anonymous, 1994 and Mujeeb et al., 1995)). Toxoplasmosis has been commonly seen in immunocompromised individuals irrespective of cause of immunosuppression (Frenkel, 1957, Vietzke et al., 1968, Ree, 1986, Jiming, 1995 and Ahmed et al., 1996).

It is now crystal clear that Toxoplasma is one of the most common cause of abortion, repeated abortion, foetal death and birth of congenitally abnormal baby in women of child bearing age. This is an opportunistic infection occurred in immunocompromised individuals, which must be kept in mind before investigating for Toxoplasmosis. Obviously there is a great need for more diagnostic and research work in different population in women with history of reproductive losses in our population.
PURPOSE OF STUDY

Toxoplasmosis is a well known cause of reproductive losses and birth of congenital abnormal babies. This study is planned to do serological study as well as animal inoculation with product of conception to isolate parasite from animal. HIV (Human Immunodeficiency Virus) screening was also done to see acquisition of Toxoplasma infection is due to immuno suppression by HIV or not. This study is also designed to culture Toxoplasma gondii and its propagation in artificial medium.
REVIEW OF LITERATURE

Toxoplasma gondii (Nicolle and Manceaux, 1908 cited by Chatterjee, 1980).

Historical Background

Toxoplasma was first discovered in animals used in laboratorv in two countries simultaneously in 1908 by Charles Nicolle and Louis Manceaux. They described this parasite as Leishmania like in the gondis, a small North African rodent used in research on leishmaniasis and typhus fever at the Pasteur Institute in Tunisia. Alfonso Splendore described it from laboratory rabbit at the Portuguese hospital in Sao Paulo, Brazil. The name Toxoplasma gondii was given to the parasite by Nicolle and Manceaux in 1908 after they agree that this parasite was different from Leishmania and Piroplasma (Frankel, 1984). The term Toxoplasma is of literary rather than of any geographical significance.

It does not signify toxicity. The name was derived from a Greek word toxon meaning arc or bow and referred to lunate shaped organism in fresh state. The specific name of the gondii was drawn from rodent Ctenodactylus gondii (Frenkel, 1951). The discovery of the parasite was of special interest for epidemiologist because they had positive proof about presence of organism in two continents and might be present in other places as well. Toxoplasma can invade the tissues of victims might exist (Feldman, 1974).
Beneson and his colleagues (1962) believe that the first case of human toxoplasmosis was reported by Samuel Barling in 1808 in a patient from Barbados who was living in Panama. According to another reference the first case was reported by Castellani in 1914 (cited by Chatterjee, 1980). Feldman (1974, 1982) and Frenkel (1984) the first well documented human case was discovered by Czechoslovakian Ophthalmologist, Josef Janku in Prague (1923) who recovered cysts in the retina of a child suffering from congenital toxoplasmosis with hydrocephalus, microphthalmia and retinochoroiditis (Hay et al., 1985).

Torres 1927 (cited by Kean, 1972), described an intra-cellular protozoan parasite in the brain and other organs of an infant who had died at age of two days. In 1937 Sabin described the clinical manifestations of human toxoplasmosis (cited by Sabin et al., 1952). Sabin’s tetrad which comprised of microcephaly, hydrocephaly, chorioretinitis and intracerebral calcification (Sabin and Feldman, 1949b).

The initial studies indicated that human to human transfer does not occur except from the primarily infected women to her foetus. There was no evidence that transfer occurs from animals and birds to man except by ingestion. Meat and much less often, egg can contain infection from these sources; however the route of infection of herbivores was entirely unknown and required further investigation.
It was observed by Hutchison in 1965 and confirmed by others that when cats were fed Toxoplasma infected mice, the infection could be transmitted back to mice through cat’s faeces even after storage in water up to a year or more (Beaver et al., 1984).

Hutchison (1967) came out with the theory of nematode transmission in the infectious Toxoplasma organism is carried in the eggs of Toxocara cati, an ascarid commonly found in cats. In two separate studies, Frenkel et al. and Shafeid and Melton observed that Toxocara had no role in the Toxoplasma life cycle. Transmission from cat to mouse was accomplished by sporocytes passed in cats faeces (Beaver et al., 1984). It became clear that natural infections in cattle, sheep and other herbivores were also from sporocytes from cats.

**Taxonomy**

As cited by Beaver et al. (1984) the genus Toxoplasma was established by Nicolle and Manceaux in 1909. They named it Toxoplasma gondii, the type species of the genus. More than twenty other species of Toxoplasma have been described, but according to Levine (1977) only seven are valid, the remainder being syndrome of Toxoplasma gondii five are found in reptiles and amphibians; the sixth Toxoplasma hammondi (Hammondi hammondii) is a parasite of
the house mouse and cat and was recognised as distinct from
Toxoplasma gondii (Frenkel and Dubey, 1975).

Genus Toxoplasma has been placed in genus Toxoplasma, suborder Emerilina, order Eucoccidia, subclass coccidia, class sporozoa and phylum apicomplexa, in newly revised classification of protozoa by Society of Parasitologists, Committee on Systemics and Evolution (Levine et al., 1980).

**Morphology**

**Toxoplasma gondii** is a coccidian which exists in three forms:

1. Oocyst (Resistant form in the external environment).
2. Trophozoites (Tachyzoites or Endozoites).
3. Tissue cyst or cystozoites (contain Bradyzoites).

1. **Oocyst**

The oocyst is an oval diploid egg protected by a very resistant cell wall and measure 10-12 micron in diameter. Oocyst formation occurs in the intestine of the definite host the cat, by gamogenesis (sexual reproduction) between microgametes (male) and macrogametes (female). Oocysts are excreted in cat faeces and survive in the (external) environment on plants and vegetables or in the soil. If moisture conditions and oxygen content are appropriate, the oocyst sporulate, producing the infective,
sporozoite form (Frenkel and Dubey, 1973 and Beaver et al., 1984).

**External Environment Survival**

In the soil, sporulation requires 2 to 3 days at 24°C, 14 to 21 days at 11°C and does not occur above 37°C or below 4°C (Macabe and Remington, 1984). Unsporulated oocysts are killed after one day at 21°C and after 14 days at -6°C. Heat exposure kills unsporulated oocysts from 45 to 50°C and at 37°C if exposed for over 24 hours (Frenkel et al., 1975 and Jackson and Hutchison, 1989).

In Texas where air temperatures ranged -6°C to 30°C for observing resistance of sporulated oocysts in covered petri dishes record survival for 334 days in the shade (Yilmaz and Hopkins, 1972). The effect of humidity on survival of oocysts in cat faeces was at least 8 days in 0% to 19%, 11 days at 37 to 58%, 18 days at 80% and 32 days at 100% relative humidity. Oocysts is killed by strong ammonia (20%) in 10 minutes. Mild tincture of iodine and dilute ammonia (1.4%) were not much more effective in killing oocysts.

Cat faeces treated with household ammonia lowered number of oocysts ten fold and treatment for 3 hours killed all oocysts (Frenkel and Dubey, 1972).

2. **Trophozoites (Tachyzoites or endozoites)**

The trophozoite or tachyzoite of *Toxoplasma gondii*
is typically crescent shaped measuring 4-6 micron in length and 2-3 micron in width have a pointed anterior end and round posterior end. It does not possess organs enabling locomotion as such but the attenuated end of this form is mobile. Occasionally an infolding is seen near posterior end, presumably a result of previous division when the resistant forms (oocyst and cyst) reach the stage of cell rupture, trophozoites are released for a brief period into blood stream of the intermediate or definite host. Trophozoites multiply by cell division (endodyogeny or endopolygeny) and reach different organs of the body where they encyst. With Giemsa's or Wrigth's stain they have delicate azure cytoplasm containing a spherical or ovoid red stained mass of chromatin, the nucleus is usually near the blunt end of parasite (Beaver et al., 1984). In wet-fixed films stained with haematoxylin the nucleus is seen to have a membrane and central Karyosome (Faust et al., 1970).

3. **Cyst (Cystozoites or Bradyzoites)**

The tissue cyst is spheroidal and measures upto 200 microns in diameter and contain as many as 3000 organism (bradyzoites). The cyst wall stain weakly with periodio acid Schiff (PAS) stain. Bradyzoites stain strongly positive with PAS. They are of smaller size and more closely packed within the tissue cyst (MaCabe and Remington, 1984).

During acute infection, group of proliferation
stages may be seen in a wide range of host cell types. These are termed as "Pseudocyst" and "Terminal colonies" which can be differentiated from the true cysts in that the organisms are slightly PAS positive. The cyst membrane is neither argyrophilic nor PAS positive (Beaver et al., 1984). The encysted parasites can persist throughout the life of the host (Frenkel, 1990).

**Life Cycle of Toxoplasma gondii (Figure 1)**

The life cycle of *Toxoplasma gondii* was described by Hutchison et al. (1969, 1970). He identified the cat as belonging to the family Felidae particularly Felis catus (domestic cats) which includes ocelot, jaguarundi, bob cat and puma as the final or complete hosts. *Toxoplasma gondii* is an obligate intracellular parasite which has both enteroepithelial and extraintestinal life cycle in definite hosts. Incidental (intermediate) hosts are humans, all orders of mammals, birds and reptiles (Wallace, 1973 and McCabe and Remington, 1984).

The life cycle of *Toxoplasma gondii* is divided into enteric cycle which occurs in small intestine of felines only and exo-enteric cycle which occurs in heterologous man, mammals and animals other than cat.
Fig. 1. Life cycle of *Toxoplasma gondii*
Enteric Cycle

Hutchison et al. (1970) observed schizogonic and gametogonic stages of *Toxoplasma gondii* resembling the endogenous cycle of a coccidia, inside the epithelial cells of small intestine (ileum) of domestic cats fed on infected mice or other animals. The cysts present inside the infected animals was taken by the cat which results in the release of trophozoites from cyst. These trophozoites start two cycles in mucosal cells of ileum asexual cycle (schizogony) or sexual cycles (gametogony).

Asexual cycle (schizogony)

The cycle starts by the entry of trophozoites in the mucosal cells which pushes aside nucleus of host cell and starts multiplying inside the cell till it reaches the stage of schizont which contains about 4-24 merozoites, form cluster round resident body and appears to radiate fanwise from it. The schizont ruptures and merozoites are released, which either recycle inside mucosal cells asexually or form microgametocytes (male) and macrogametocyte (female).

Sexual cycle (gametogony)

This cycle starts with meroziotes entered inside mucosal cells to form male or microgametocyte and female or macrogametocyte. The macrogametocyte
increased in size when reached maturity to form macrogamete while microgametocyte matured by multiplication, rupture and release 12-32 microgametes. Zygote is formed by combination of one microgamete with one macrogamete. The zygote after maturation forms oocyst which are discharged in cat faeces.

**Exoenteric cycle**

Exoenteric cycle is also divided into two, sporulation and infection of intermediate host. The oocysts containing 2 sporocysts are excreted in cat's faeces for about 1 to 2 weeks, on maturation each forms 4 sporozoites which resemble trophozoites which can infect man and other animals (mammals and birds).

**Infection of intermediate hosts**

Sporozoites or oocysts are ingested release sporozoites which in heterologous host penetrate mucosal cells of the intestine (ileum) and are carried by blood and lymph stream to mesentric lymph nodes and then to distant organs such as brain, liver, spleen, eyes, lymph nodes, skeletal muscle, heart and placenta of pregnant uterus. In all these places pseudocysts are formed and parasites inside are known as endozoite (nucleus central in position). The multiplication is by internal budding (endogeny), hence they are termed as endozoites. The pseudocyst or intracellular form measure 4-6 μm in length by 2μm in breath or more and may contain 50-100 organisms (Jones et al., 1977).
Sporozoites and endozoites localised in the central nervous system and the musculature where they are transformed into tissues cysts, parasites present inside also multiply. The parasite within tissue cyst is known as cystozoite (Frenkel, 1948).

Toxoplasma gondii (Figure-2) (Gustafson et al., 1954)

Electron microscope study of Toxoplasma is unrewarding. However, use of thin sections had revealed a wealth of structural details. On electron microscopy they were recognised by their shape, size, relationship to exudate cells and inclusions. Round or oval contours representing cross or oblique sections, were most commonly done. Longitudinal section were much less crescentric than living organisms; this was suggested partly due to orientation of the cell and partly due to contraction at time of fixation. The parasites inside host cells were within vacuoles with a discrete boundaries. It was observed that there was definite space between the parasite and vacuole wall, which in many cases were with fine filamentous or granular precipitate. Often concentrations of mitochondria were seen at edges of vacuoles, while other portions of the cytoplasm were devoid of these structures. Occasionally the nucleus was similarly situated adjacent to vacuole. The parasite had well marked cell membrane without a cell wall. The surface was found smooth, but in oblique
Fig. 2. Sketch diagram of *Toxoplasma gondii*
section longitudinal fibrillar component of surface could be seen.

There was no indication of flagella, cilia or other obvious projections. The acute end was found to have a very distinct organelle, which was hollowed truncated cone Ø.15 Ø.25 micron in diameter and Ø.2-Ø.3 micron long. The base of cone was seen towards the centre of the cell, which was observed to open adjacent to cytoplasm. This structure was regarded as conoid (Sims et al., 1889 and 1890). Associated at one end with the conoid and followed a longitudinal course through cell were several long, roughly cylindrical bodies of a dense, homogenous material which was at cross section uniformly circular without surface membrane. Maximum diameters were Ø.08-Ø.12 micron. At one end reached as far as the nucleus and on the other end they converge entering into base of cone. Total number were variable from 14-18. They are known as toxoneme.

The cytoplasm is composed of various inclusion e.g. lipids which were in fine granular in a cluster against nucleus, centriole and the golgi apparatus. Various other structures like vacuoles, mitochondria, endoplasmic reticulum and ribosomes were also present.

The nucleus was round or oval but may be elongated transversely or longitudinally, or prominently lobed. Sometimes its structure resembled horseshoe. The position of nucleus was found in middle third of the cell towards the
blunt end. Its diameter is 1 to 1.5 micron when circular and
upto to 2 micron in greatest dimension when elongated.

There appeared to be a definite nuclear membrane
with thickness approximately 10 mili micron (Gustafson et

Transmission of Toxoplasma gondii

All three forms that is cysts, oocysts and
trophozoites were responsible for Toxoplasmosis. Pregnant
women infected with Toxoplasma gondii either results in
abortion, miscarriage, still birth, prematurity or
transplacental transmission to foetus results in congenital
toxoplasmosis. Other transmission methods are by ingestion
of the material infected with Toxoplasma gondii (bioMerieux

Description of Disease

Toxoplasma gondii besides infecting man has been
rampant among different rodents and domestic animals. It has
been reported in gondii, mice, rats, cats, mink,
chinchillas, swine, dogs, foxes, rabbits, guinea pigs,
squirrels, lizards and penguins (cited by Fieldman, 1958 and
1968). Cats plays a vital role in toxoplasmosis. In few
islands of Pacific where cats were never introduced have
remained free of toxoplasmosis (Wallace, 1979). Toxoplasma
gondii was isolated from faeces of naturally infected cats
Cats become infected by eating infected warm-blooded animals such as mice, birds, or by ingested food contaminated with cats' feces (Boyd and Hoerl, 1977). There are various ways of acquiring the disease. The main mode is by ingestion of undercooked food or meat (Granham and Laison, 1980 and Blood et al., 1979), or by eating raw vegetables, or by raw eggs, or raw milk, or through contaminated water (Benson et al., 1982), or working with soil and taking meals without washing hands. The other route is by inhalation (Chatterjee, 1980). The parasite can enter human body by inoculation which can be accidental or incidental e.g. introduction of parasites into wounds on the skin of butchers or the animal handlers (cited by Feldman, 1952) and also by transfusion of blood (McLeod and Remington, 1980) or by leucocytes transfusion (Siegel et al., 1971). It can also be transmitted by organ transplant (Reynolds et al., 1966 and Rapheal et al., 1983). The other mode of transmission are transplacentally from infected mother (Desmonts, 1974) and also when infected mother breast fed their children (Chatterjee, 1980). The most common route is by ingestion and oocyst liberates sporozoites which penetrate mucosal cells of intestine and carried by blood and lymph stream first to mesentric lymph nodes and then to distant organs. Parasitemia has been observed in human beings (Araujo and Remington, 1980).
Toxoplasma infections of humans are conveniently classified as follows:

1) Congenital.
2) Acquired.
   a) Asymptomatic toxoplasmosis.
   b) Glandular toxoplasmosis.
   c) Miliary toxoplasmosis.
   d) Localised toxoplasmosis.

1) Congenital toxoplasmosis

Broadly speaking congenital toxoplasmosis is generalised with hepatosplenomegaly, jaundice, myocarditis (Medlock et al., 1990) and pneumonitis. Cerebral forms have one of the two characteristic signs namely chorioretinitis and intracerebral calcification (Thalhammer, 1962 and Miller et al., 1967).

Congenital toxoplasmosis can be classified as follows:

i) Infant born during the primary phase of infection (Parasitemia):

In severe form typical presentations are icterus, hepatosplenomegaly, maculopapular rash (McCrosin and Roberton, 1989), anemia, erythroblastosis, thrombocytopenia and central nervous system damage which is often fatal, while the attenuated form indicates brief icterus at onset of cirrhosis or encephalitis. Moreover in
subclinical form ocular manifestations are common and appears several years after birth (Robertson, 1960 and Rosenfeld, 1988).

ii) Infants born during secondary phase of infection (antibody production):

Humoral immune response by host suppresses parasitemia. However, organ poor in antibodies becomes target of toxoplasma which may give explanation of encephalopathy, hypotonia rigidity and if cyst is in brain, results in convulsions (Bale and Morph, 1982).

iii) Infant born during the tertiary phase of infection (cyst stage):

In severe form, manifestations are hydrocephalus, psychosomatic disorder (mental retardation) which is always accompanied by ocular lesions. In complete form common lesions are localised chorioretinitis or encephalopathy, while in subclinical form child runs risk of mental retardation, ocular and neurological manifestations (BioMerieux, Monograph on toxoplasmosis, 1983).

Bilateral micro-ophthalmia and chronic bilateral uveitis were observed in congenital form (Gard et al., 1949). Congenital deafness was also seen in cases of congenital toxoplasmosis (Kelemen, 1958).

2) Acquired toxoplasmosis

a) Asymptomatic

Substantial number of toxoplasmosis cases
were without any clinical manifestation. The *Toxoplasma gondii* remained in quiescent or quiet form throughout life. But due to alteration in immune status the disease exacerbated and appeared clinically. Author considered it premature to ascribe it as "latent toxoplasmosis" with confidence (Feldman, 1958).

b) Glandular toxoplasmosis

Toxoplasmic lymphadenitis is most common type of toxoplasmosis in the adult (Siims, 1851). Its onset is gradual accompanied by sweating, anemia, fatigue and malaise, or a sudden onset, with chills and fever of few days to a month duration. Sore throat, headache, myalgia, arthralgia, nausea and abdominal pain are manifestations often present in such patients. Skin eruption is very rare. Spleen and liver are occasionally enlarged. Tonsillar hypertrophy is seen in some cases (Sabin et al., 1952 and Ravel, 1980).

Regional lymph nodes usually involved are hilar, mesenteric and cervical. The lymph nodes are 2cm or more in size, firm, smooth, discrete, freely movable, not attached to surrounding tissues, or overlying skin and can be painful and tender initially. Fistula formation has been reported. Histopathologic changes are reactive follicular hyperplasia with large germinal centers containing macrophages, small foci of epitheloid cells usually with gaint cells in inter-
follicular zone, epitheloid cells may be located within germinal centers. There may be focal distension of sinuses by immature histiocytes and medullary cords which may contain increased number of plasma cells (Parkash, 1966 and Robbins et al., 1984).

Glandular toxoplasmosis was present in 7% of cases clinically diagnosed as glandular fever with negative Paul Bunnel reaction (Beverlay and Beattie, 1958 and Yamaguchi, 1981).

c) Miliary toxoplasmosis

Toxoplasmosis is characterized by dissemination of the protozoan throughout the body with clinical evidence of multiple organ and system involvement and pathological evidence of innumerable foci of necrosis, local reaction, and parasitized cells in the majority of the organs. This form referred as typhus like form, or exanthematic form or the Rocky Mountain spotted fever like form. The clinical features are malaise, excessive fatigue, inability, weakness, chills and fever (102 to 104°F) and rapid pulse with profuse perspiration.

Skin eruption presents in almost all cases which is pale, pink or brown or bright red maculopapular rash fading on compression. In some cases it is purpuric with severe persistent headache at onset (Kass et al., 1952, Yamaguchi, 1981 and Karm, 1980).
Conjunctivitis has been observed in three cases with anorexia, nausea and vomiting (Sexton et al., 1980).

Toxoplasma gondii was isolated from human gastrocnemius muscle (Syverton and Slaven, 1948). Myalgia and arthalgia were frequently presented in case mentioned above and severe polymyositis was main manifestation (Chander et al., 1968, Ragen et al., 1974 and Hay et al., 1968).

Respiratory symptoms initially are dry cough, tachypnea, dyspnea, cyanosis, localised dullness to percussion, rales on auscultation of chest. X-ray chest shows bronchopneumonia, pulmonary congestion with edema of the lungs and changes simulating atypical pneumonia with interstitial infiltration and irregular blotchy consolidations of lungs (Yamaguchi, 1981 and Tourani et al., 1985).

Cardiovascular manifestations are tachycardia, arrythmias, fatigue, decreased effort of tolerance, exertional dyspnea, precordial pain, peripheral edema and cardiomegaly. E.C.G. is confirmative of the above findings (Feldman, 1953b and Sexton et al., 1953).

In miliary form, lymphadenopathy is one of the presenting symptoms or late manifestation and rarely lymph nodes do not enlarge (Sexton et al., 1953). Splenomegaly and hepatomegaly are seen with abnormal liver function (Brown and Jacon, 1956 cited by Kass et al., 1952).
Nephritis was reported in one case and terminal oliguria in another case (cited by Theologides and Kennedy, 1966).

The neurological manifestations include headache, blurring of vision, impairment of hearing, narcolepsy, lethargy, loss of sensory perception, hallucinations, psychoses, delirium, convulsions, nuchal rigidity, paralysis of lower extremities, abnormal reflexes, abnormalities in co-ordination or coma. Chorioretinitis probably has occurred in this form (Paige et al., 1942 and Ferguson et al., 1981). The disease was reported from one cases of brain abscess (Ghatak et al., 1970).

Encysted form of Toxoplasma gondii was isolated from human skeleton muscle and brain (Remington and Cavanaugh, 1965).

Bone marrow biopsy shows generalised hyperplasia and presence of toxoplasma cyst in such case (cited by Theologides and Kennedy, 1966).

d) Localised toxoplasmosis

The most frequent localised forms of toxoplasmosis in adult are as follows:

i) Ocular toxoplasmosis

This is the most common localised form. Chorioretinal lesion in 53 eyes enucleated and 25% of adult with granulomatous uveitis (Frenkel and Jacobs, 1958).
Panuveitis and papillitis with optic atrophy is less common (Mcleod and Remington, 1980). Toxoplasmic chorioretinitis is observed in adults also (Wilder, 1952, Masur et al., 1978 and de-Jong, 1989).

ii) **Cerebral toxoplasmosis**

This is characterised by symptoms of space occupying intracranial lesion or lesions. One case with brain abscess was also reported from one centre (Mcleod et al., 1979 and Yamaguchi, 1981).

iii) **Pulmonary toxoplasmosis**

This is responsible for atypical pneumonia. Two cases of atypical pneumonia were reported by Yamaguchi (1981).

iv) **Toxoplasmic myocarditis**

Cases of Toxoplasmic myocarditis were also reported from different centres of world (Potts and William, 1956; Mcleod and Remington, 1980).

v) **Toxoplasmic pericarditis**

Cases of Toxoplasmic pericarditis were also seen in some patients (Potts and William, 1956; Mcleod and Remington, 1980).

vi) **Toxoplasmic hepatitis** Toxoplasmic hepatitis has been suggested as clinical entity.
Toxoplasma gondii antigens and antigenic response

Toxoplasma gondii antigens

The emergence of monoclonal antibodies has provided insight into the parasite's antigenic structure. It was observed that antigens are four main groups and of many types. The membrane antigens are found to have 20 known types, cytoplasmic antigens 8 known types, mixed membrane plus cytoplasmic antigens with about 4 known types and exo-antigens about 2 known types (bioMerieux Monograph, 1983 and Kasper, 1989).

It was found that if antigen prepared from endozoite and antibody also prepared from endozoite, reaction then came positive, if antigen is from endozoite and antibody from cystozoite reaction is negative, and if both from cystozoites give positive reaction again (Lunde and Jacobs, 1983).

All in vitro diagnostic method detect humoral immunity to Toxoplasma gondii. They are based on the principle that toxoplasma antigens react with antibodies present in positive serum (Remington, 1970 and Haritani et al., 1988). Cross reactions are observed with sarcocystis, trichomonas and leishmania (Goldman, 1957b). First of these to come in response to antigen is IgM type of immunoglobulin, and followed by IgG antibodies which remain in blood stream throughout life (Mcleod and Remington, 1980; Cohen and Warren, 1982 and Naot et al., 1982).
EPIDEMIOLOGY OF TOXOPLASMA GONDII INFECTION

Global distribution of infection and magnitude of the problem

Toxoplasmosis is cosmopolitan disease, which is most prevalent in hot humid climates and almost insignificant in dry and cold climates (Hinrich, 1963 and Faust et al., 1975).

Toxoplasma gondii is one of the most successful of parasites. It respects neither the boundaries of geography nor of zoology and causes infection, usually life-long, in all species of mammals and birds, as far as is known, throughout the world except the Arctic (Beattie, 1982; Feldman, 1982). Frequency of infections varies considerably from one country to another and within a given country (WHO, 1969). Toxoplasma infections are most frequent in the Caribbean and its continuous countries and least prevalent in the cold areas such as the Arctic (Feldman, 1982). The prevalence of Toxoplasma infections in various parts of the world ranges from zero in those who have lived their entire lives on the Pacific atoll of Eauripik (Wallace, 1969) to over 97% in the over 50 age group in El Salvador (Remington et al., 1970). Infection among women of child bearing age is very high in France, Guatemala, Costa Rica and El Salvador.

There have been many surveys of the prevalence of Toxoplasma infection. They are based on demonstration of
antibodies, indicating infection, latent or patent. As different techniques have been used and various antibody titres accepted as positive, they are not strictly comparable, but indicate trends (Beattie, 1982).

Kean (1972) stated that the range of toxoplasmosis in man has been widened to involve approximately one-half billion humans with a diversity of clinical pattern extending from asymptomatic to malignant. It may be noted that world’s population at that time (1972) was 4 billion. It is estimated that Toxoplasma infection exists in chronic asymptomatic form in approximately half of the population in the United States (Beaver, 1984). Toxoplasma infection is a significant public health problem. It is attested by the fact that the incidence of congenital toxoplasmosis ranges from one per 1000 live births in Montreal (Viens et al., 1977), to 7 cases per 1000 live births in Vienna (Pechere et al., 1984), that approximately 15% of cases of "Otherwise unexplained lymphadenopathy" may be due to toxoplasmosis (WHO, 1989). It was concluded by many that Toxoplasma infections represented the most significant cause of infantile blindness, mental retardation, retarded motor development, micro or macrocephaly, and recurrent spontaneous abortions (Feldman, 1982). Toxoplasmosis has received worldwide attention particularly as a threat to pregnant women and their unborn child (Saleha, 1984), and recently to immunocompromised patients (McCabe and
Remington, 1984).

It is a widespread disease, seroconversion increase with age, varies according to geographical area and eating habits, high incidence of seropositivity was seen in various countries/cities (Fig. 3) as published in 1983 by bio Merieux in a Monograph on toxoplasmosis and Ahmed and Hafiz (1989).

Different authors studied the sero-positivity in pregnant women with different diagnostic techniques at different centres of the world. They have found out seropositivity from 20.0% to > 84% in pregnant women (Sever et al., 1988). The detail of seropositivity by different methodology by different author is given below:
<table>
<thead>
<tr>
<th>AUTHOR</th>
<th>METHOD</th>
<th>SERO-POSITIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rous and Bourne (1972)</td>
<td>Dye test (DT)</td>
<td>22.0%</td>
</tr>
<tr>
<td>Desmont and Courvreur (1974)</td>
<td>Dye test (DT)</td>
<td>84.0%</td>
</tr>
<tr>
<td>Pal et al. (1975)</td>
<td>Indirect Haemagglutination assay (IHA)</td>
<td>17.3%</td>
</tr>
<tr>
<td>Cheah et al. (1975)</td>
<td>Immuno Fluorescent assay (IFA)</td>
<td>27.0%</td>
</tr>
<tr>
<td>Vien et al. (1977)</td>
<td>Immuno Fluorescent assay (IFA)</td>
<td>40.6%</td>
</tr>
<tr>
<td>Stray-Pederson and Stur-Lorewtzen (1979)</td>
<td>Dye Test (DT)</td>
<td>12.6%</td>
</tr>
<tr>
<td>Fikry et al. (1980)</td>
<td>Dye Test (DT)</td>
<td>25.0%</td>
</tr>
<tr>
<td>Basalamah and Serebour (1981)</td>
<td>IHA</td>
<td>31.2%</td>
</tr>
<tr>
<td>Matos-Aybar et al. (1982)</td>
<td>IFA</td>
<td>47.4%</td>
</tr>
<tr>
<td>Legnain et al. (1982)</td>
<td>IHA</td>
<td>63.9%</td>
</tr>
<tr>
<td>Magnaval et al. (1982)</td>
<td>IFA</td>
<td>81.5%</td>
</tr>
<tr>
<td>Al-Nakib et al. (1983)</td>
<td>IHA</td>
<td>58.2%</td>
</tr>
<tr>
<td>Iddrissi et al. (1984)</td>
<td>IFA</td>
<td>51.5%</td>
</tr>
<tr>
<td>Yu (1985)</td>
<td>IFA</td>
<td>9.2%</td>
</tr>
<tr>
<td>Yu (1985)</td>
<td>Enzyme linked Immunosorbent assay (ELISA)</td>
<td>10.2%</td>
</tr>
<tr>
<td>Ahmed and Hafiz (1989)</td>
<td>Direct Agglutination (DA) (IgG)</td>
<td>35.3%</td>
</tr>
<tr>
<td>Ahmed et al. (1990)</td>
<td>Enzyme Immuno Assay (EIA) (IgG)</td>
<td>36.3%</td>
</tr>
<tr>
<td>Ahmed (1990)</td>
<td>EIA</td>
<td>14.7% (IgM)</td>
</tr>
</tbody>
</table>

Women of childbearing age
Fig. 3. Epidemiology of toxoplasmosis (Histogram)
(Reproduced from bioMerieux monograph 1983 and Ahmed & Hafiz 1989)
Statistics compiled in U.S.A. show that approximately 0.8% of pregnant women risk seroconversion during pregnancy (Remington, 1970 and 1974). In another study conducted by Feldman et al. (1955) among 10 normal population for frequency of toxoplasma, Eskimo 0%, Havago Indians 4%, Iceland 11%, Portland 17%, St. Louis 26%, New Orleans 31%, Pittsburg 35%, Haiti 36%, Honduras 64%, Tahiti 68% and overall 31% people were found affected.

Research on normal human sera in Britain was carried out which was suggestive of incidence of seropositive cases increases (Fig. 4) with age (Fleck and Kwantes, 1980 and Hall, 1992).

In U.S. army recruits 14% found seropositive (Feldman, 1965) and Brazilian recruits 52% had antibodies against toxoplasma (Lamb and Feldman, 1964). It was found out that 45-65% were sero-positive in 45 years age group in Jewish population (Jacob et al., 1952). In students 70 (19.5%) out of 359 were positive (Remington et al., 1963). Twenty eight percent residents of Trinidad acquire toxoplasmosis (Lamb and Feldman, 1968). In Cleveland families seropositive cases were males 12 (30%) out of 40, females 16 (40%) out of 40 and children 8 (4.8%) out of 123 (Warren, 1968).

The disease is also highly prevalent among animals. In a survey 24.3% domestic cats were seropositive
(Jones et al., 1957). Toxoplasma was isolated from swine, sheep and beef samples (Jacob et al., 1957). Hares also reported to have Toxoplasma (Christensen and Siim, 1951). Antibody against toxoplasma was detected with frequency in street cats, dogs, and laboratory bred guinea pigs (Miller and Feldman, 1953). It was also reported by Connor and Hallimell (1985) that Toxoplasma gondii infect 14.2% goat and 7% sheep in Tanzania while recent study in Saudi Arabia was found to be 11% in sheep and 8% in goats (Mossain et al., 1987).

In various high risk group prevalence of toxoplasmosis was on higher side as compared to normal population (Konishi, 1989). In pregnancy wastage it was 63.9% as compared to 11.1% in females with normal reproductive performance (Legnain et al., 1982). About 10% of women with history of spontaneous abortion follows toxoplasmosis during first trimester (Desmonts, 1974a). Some 3000 toxoplastic babies are born in U.S. per year, of which 5-15% were dead, 8-10% had severe brain and eye damage, 10-13% had moderate to severe visual handicaps and 58-72% were asymptomatic at birth but developed active retinochoroiditis as children or young adults as elicited in Fig. 5 (Based on the morbidity and mortality statistics experienced by Desmonts and Couvreur, 1967 and 1971 cited by Frenkel, 1973).
Fig. 4. Age and sero-positivity correlation (Histogram)

(Reproduced from Fleck & Kwantes, 1980)
Fig. 5. Toxoplasmosis incidence rates and fetal risk.

(Reproduced from Frenkel, 1973)
Toxoplasmosis was incriminated in many mothers, maternal rate of prevalence in various locations and chances of fetal risk could be calculated. Incidence rate was from 0.1 to 20% in graph representative of maternal infection in various locations. We can compute approximate fetal risk, between the ages of 20-30 and applying it to the 40% fetal rate of infection. Largest number of infant associated with maternal seroconversion 3 to 5% per year (Desmonts and Couvreur, 1967 cited by Frenkel, 1973). Incidence of congenital toxoplasmosis was 1 per 1000 in United States, 2 per 1000 in whole Norway, 3 per 1000 in France and 1 per 5000 in United Kingdom (cited by Legnain et al., 1982), and 2 per 1000 live births in Brussels (Foulon et al., 1984). High rates of presence of antitoxoplasma antibody were found in animal handlers more as compared to non animal handlers (Feldman and Miller, 1956 and Fleck, 1985).

As cited by Luft et al. (1984) patients with CDC-AIDS have a particular predilection for the development of encephalitis caused by Toxoplasma gondii. 25-80% patients of CDC-AIDS had toxo-encephalitis in different studies. As stated by Vieira et al. (1983) upto 50% patients with AIDS suffer from toxoplasmosis.

**Host characteristics**

**Age and sex distribution**

As quoted by McCabe and Remington (1984)
serological evidence of Toxoplasma infection increases with age and reaches 90% in some societies by the fourth decade. As cited by Frenkel (1985a) from prevalence rates of antibody, we recognize two patterns of transmission, childhood and adult. Childhood transmission is common in tropics and subtropics. It starts at about one year of age and 50-70% of children may develop antibody by the time they become adolescents. Frequently, then, seroconversion decelerates and reaches a level state. Oocysts are responsible for this type of transmission. Adult transmission starts in late adolescence and increases gradually throughout adulthood. This is found in developed countries and correlates with eating undercooked meat. This is tissue cyst transmission. In many areas of the world the pattern is mixed.

It was found that in countries with high infection rates, antibodies were frequent among the young, reaching markedly elevated levels by five years of age, whereas among Navajos Indian overall infection rate was only 5%, no evidence for infection was found below 10 years of age (Feldman, 1982).

Most workers believe that Toxoplasma antibodies are equally frequent in males and females (Gibson, 1956; Gibson and Coleman, 1958; Remington et al., 1970; Ghorbani et al., 1978; Zardi et al., 1980; Feldman, 1982; Manjour et al., 1983; Saleha, 1984). Walton et al.
observed higher prevalence of Toxoplasma antibodies among boys (46%) than girls (34%) in Panama. Identical results have been cited by Frenkel and Ruiz (1980) in Costa Rica study where more males than females had antibodies (66% vs 58%; P < 0.02). In a serological study of Toxoplasma infection in mountainous regions of Iran, Ghorbani et al. (1981) noted that in West Azerbaijan Province, among Turkish people the difference in antibody prevalence between the males (19.3%) and females (26.8%) was statistically significant (P < 0.005).

**Occupational influence**

At present no consistent correlation has been noted between Toxoplasma infection and occupation, though slaughter house workers may be at greater risk of acquiring the infection (McCabe and Remington, 1984). Anyone who is liable to have contact with soil containing oocysts is in danger of acquiring infection. It has been estimated that 10-100 oocysts may be found under the finger nails after digging a contaminated garden. Medical and veterinary students having a farming background registered a high prevalence rate than others. Similarly 30.6% infection was found among agricultural workers, as compared with only 9.4% of feed lot employees (Beattie, 1982).
Racial and ethnic influence

During the study on the prevalence of antibodies to *Toxoplasma gondii* among U.S. military recruits, Walls et al. (1967) observed a significant difference in the prevalence between white and Negro soldiers. Similar results are quoted by Kean (1972) in a study of pregnant white and Negro women. Cheah et al. (1975) noted significantly low rate of infection among Chinese women as compared to Malay women in Malaysia. Pacific Islands study of Wallace (1976) revealed that in Hawaii, people of Japanese ancestry are at low risk of infection, whereas Filipinos are at high risk. Statistically significant difference in *Toxoplasma* antibody prevalence in two or more ethnic groups inhabiting the same geographic area has also been reported by Desmonts and Couvreur (1974a); Ghorbani et al. (1981); and Manjour et al. (1983) from Paris, Iran and Mauritania respectively. Barbier et al. (1983) found no significant variation in seropositivity rate among different ethnic groups in La Guadeloupe, French West Indies.

Urban - rural distribution

Evidence concerning the relative prevalence of infection in town and in country is conflicting (Beattie, 1982). A survey conducted by Walls and Kagan (1967) to measure antibody titers among Brazilian military recruits found a higher prevalence of positive titres among rural
residents. Similar results are reported by Cheah et al. (1979) and Stray-Pedersen and Lorentzen-Styr (1979) from Malaysian and Norwegian population.

Sessa et al. (1982) studied the prevalence of Toxoplasma infection among school children in Brazil and noted that prevalence was significantly higher in urban areas. Barbier et al. (1983) noted identical finding from general population of La Guadeloupe, French West Indies.

Gibson (1956) found no difference among comparable urban and rural black population. Walls et al. (1967) found no urban-rural difference in their study of American recruits. Ganley and Comstock (1980) also had similar result from Maryland study.

**Socio-economic distribution**

Walls et al. (1967) found a higher prevalence of positive tests among military recruits from the Appalachian area of Eastern Tennessee, an association which they felt might reflect regional socio-economic variation. Most of the workers revealed a strong negative correlation of Toxoplasma infection with socio-economic status (Cheah et al., 1975; Pal et al., 1975; Ganley and Comstock, 1980; Barbier et al., 1983). Stray-Pedersen and Lorentzen-Styr (1979) detected a positive correlation of infection with socio-economic status among residents of Oslo city. As stated by them, it could be that the higher frequency of the infection in the “richer”
half of the city is due to differences in cooking habits (consumption of raw meat) or to more frequent foreign travel to countries with higher infection risks.

**The role of environment**

**Effect of physical environment**

As stated by Feldman (1982) Toxoplasma infection is acquired throughout the year with no particular seasonal prevalence. Gibson and Coleman (1958) suggested that there was a striking difference between the prevalence of Toxoplasma antibodies among inhabitants of Guatemalan lowlands (94%) and residents of Guatemalan highland (50%) situated at an over 5,000 feet elevation. The concept of decreased hazard of infection at higher altitude was supported by Walton et al. (1966) in their study of three communities in Panama which were at varying altitudes within a single latitude. The elevation was from 700 to 8000 feet with a lateral distance of 15 miles. Even though the population of the three communities was ethnologically and socio-economically similar, distinct difference in the prevalence of Toxoplasma infection was found at different altitudinal level; with the highest prevalence being at the lowest altitude. The negative correlation of Toxoplasma infection with altitude has also been reported in studies of various populations of Brazil, U.S.A., Peru and Iran (Walls and Ragan, 1967; Walls et al., 1967; Cantella et al., 1974;
Ghorbani et al., 1981).

Zardi et al. (1980) found that the prevalence of infection was higher (61%) in the hot, humid riverine areas of Somalia than in the rest of the arid country where it was 48%. Similarly in Martinique, Manaval et al. (1982) revealed that Toxoplasma infection was much common in wet northern part of Island than in the dry South. The positive correlation of infection to humidity has also been cited by Walls and Kagan (1967) and Wallace (1976).

Barbier et al. (1983) in their study of Toxoplasma infection in Guadeloupe found higher Toxoplasma antibody rates in areas with higher rainfall and infection rates correlated positively with mean annual rainfall but not with altitude. In El Salvador Remington et al. (1970) were unable to correlate Toxoplasma infection with altitude and humidity.

Effect of biological environment - the role of vectors

No biological vector is needed to spread the infection. However mechanical vectors (transport hosts) do play some possible role, their role is fully discussed in pages under transmission of infection.

Natural history of disease (The chain of infection)

The reservoir of infection

Toxoplasma infection is widespread in nature both
in animal and bird species. The house mouse and other small rodents that are eaten by cats are important as reservoir of Toxoplasma infection (Beaver et al., 1984). Oocysts shed by cats play a major role in dissemination of infection. As stated by Wallace (1973a) seropositivity of cats is 5% to 65% in different parts of the world and 1% of the cats shed oocysts. Meat of infected animals is another important source of infection for those humans who take raw or undercooked meat.

**Transmission (Figure 6)**

The modes of transmission recognized and the stages involved are, first, transplacental transmission by tachyzoites, followed by carnivorism via tissue cysts and then faecal oral spread by oocysts. However, the biological frequency and importance of these modes is in the reverse order (Frenkel, 1984).

**Faecal-oral transmission**

A cat that has eaten a single infected bird or mouse may shed hundreds of thousands of oocysts capable of infecting a similar number of intermediate hosts; or few animals at a time (Frenkel, 1984). Oocysts persist in moist soil for weeks and months. Deposits of infected cat faeces remained infectious to mice for a year in Costa Rica and for 18 months in Kansas (Frenkel et al., 1975). After two weeks,
Fig. 6. Transmission of *Toxoplasma gondii* in man. (Reproduced from Frenkel, 1965a)
oocyst cannot be recognized in contaminated moist soil. Therefore, humans, especially children, can become infected from oocysts in seemingly clean and dry soil. There are three main reservoirs of Toxoplasma gondii in the transmission chain, cats, soil and intermediate hosts. Man and other carnivores are biologically non-functional intermediate hosts since they are rarely eaten (Frenkel and Ruiz, 1981).

As quoted by Wallace (1973b) it is possible that invertebrates such as cockroaches, flies, snails and slugs, which normally feed on faeces, could serve as transport hosts after ingesting oocysts in cat faeces, if eaten by small mammals and birds. Wallace (1971) demonstrated experimentally, the transmission of Toxoplasma oocysts by filth flies from cat faeces to milk. During two separate experiments, Wallace (1972, 1973b) isolated viable oocysts from faeces of cockroaches, up to 10 days and from the digestive tract up to 20 days after their last feed on cat faeces. Ruiz and Frenkel (1980b) isolated Toxoplasma from 4 of 16 lots of earthworms, taken from different sites in San Jose, Costa Rica, and illustrated their possible role as transport hosts to infect chickens and other birds feeding on them. As stated by Wallace (1973a) small terrestrial snails which commonly feeds on animal faeces serves as intermediate hosts for some of the lungworms of herbivores. The snails are accidentally eaten by the herbivorous host
while grazing.

**Transmission through tissue cysts (Fig. 7)**

Most intermediate hosts are infected by oocysts but transmit the infection by tissue cysts when eaten by a carnivore. Ground feeding birds and rodents are the most important intermediate hosts, because they in turn transmit the infection to cats, thereby closing the natural transmission chain (Fig. 7 reproduce from Frenkel, 1985a). Infected meat, mostly of sheep and pigs, if eaten raw or undercooked, serves as a source of infection to man (Kean et al., 1989). Sacks et al. (1983) documented Toxoplasma infection associated with eating undercooked venison in three deer hunters. Tissue cysts transmission of Toxoplasma infection is common in France and Germany, where undercooked mutton and raw pork is a favourite repast (Frenkel, 1984). Al-Nakib et al. (1983) and Manjour et al. (1983) cited higher prevalence of Toxoplasma infection among Arab Tribes who consume undercooked mutton.

**Transplacental transmission**

Transplacental transmission of infection occurs in humans, sheep and goats when infection occurs during pregnancy. In mice, sheep and goats transmission in successive pregnancies has been recognized (Feldman, 1974; Frenkel, 1984). Congenital infection in man and probably in most animals is biological rare, but medically and
Fig. 7. Transmission cycle of *Toxoplasma gondii*  
(Reproduced from Frenkel, 1985a)
economically important event; it probably plays little or no role in the endemicity of infection (Frenkel and Ruiz, 1981).

**Uncommon routes of transmission**

Experimental leukocyte transfusions with blood from immunosuppressed leukemic donors have served to transmit the infection, and *Toxoplasma gondii* has been isolated from peripheral blood of symptomatic patients (Frenkel, 1984). Blood of asymptomatic carriers is generally safe (Griffin and Williams, 1983; Frenkel, 1984). Transplanted heart of seropositive donors transmit Toxoplasma infection because tissue cysts are common in the heart muscle (Frenkel, 1984). Patterson (1984) reported transmission of Toxoplasma infection via milk from lactating mice. Toxoplasma infection can occur through skin penetration in laboratory workers and autopsy accidents; transmission of tachyzoites by an arthropod vector, the tick and rarely eggs of infected chicken have been found to contain *Toxoplasma gondii* (Saleha, 1984). An outbreak of acute toxoplasmosis among American soldiers in Panama provided an epidemiologic evidence that the infections were acquired through drinking water from a jungle stream (Beneson et al., 1982).
Factors influencing the period of communicability

Oocysts in cat faeces are usually buried superficially in soil, often close to human habitations. The duration of their persistence is of interest if one wishes to assess the potential impact of oocysts, as logarithmic multiplier of infection (Frenkel et al., 1975). The rate of oocyst sporulation depends on oxygenation and temperature. Some oocysts on the surface of a faecal specimen could be infectious within one day of shedding. Anaerobic conditions diminish infectivity (Frenkel and Dubey, 1972).

Yilmaz and Hopkins (1972) noted that Toxoplasma infectivity in water was for more than 410 days in shade with mean air temperature 18.5°C. As cited by Frenkel et al. (1975) sporulated oocysts are more resistant to extreme temperature 18.5°C. It was found that sporulated oocysts survived freezing at -21°C for 28 days and remained infectious at 37°C for 306 days.

Soil survival of oocyst depends on various factors among which are soil texture especially its ability to absorb and retain moisture shade and temperature (Beattie, 1982). Oocyst may survive in moist, shaded soil for more than a year. Frenkel et al. (1975) noted the infectivity of oocysts in Costa Rican soil for a year and in Kansas soil for a period of 18 months, including two winters.

Tissue cyst is a resistant structure which can survive in meat or up to 3 weeks at 4°C. Freezing to -20°C,
desiccation, or heating above 88°C is lethal for cysts, as is pickling and smoking. Tachyzoites are killed by normal gastric secretions that contain pepsin and hydrochloric acid. Freezing, thawing, and desiccation is also lethal for them (Beattie, 1982; McCabe and Remington, 1984).

**Host cell specificity**

*Toxoplasmagondii* is obligatory intracellular in nucleated cells, although they may circulate in the blood or lymph as free zoites for a short time. *Toxoplasma* inhabits nucleated cells of all types, but particularly those of the reticuloendothelium, muscle and the central nervous system and its appendages, particularly the retina (WHO, 1980). Recently, it has been stated that immature mammalian erythrocytes can be infected with *Toxoplasma*. The mature erythrocytes are however, resistant to infection (Werk, 1985).

**Diagnosis**

The clinical diagnosis of toxoplasmosis is difficult which has resulted in the development of various laboratory methods. The direct method is by making smear and stained with alkaline methylene blue or Giemsa from suspected material. The other technique is by inoculating suspected specimen into laboratory bred mice or guinea pigs or hamsters and then stained after making smear. Direct
isolation of *Toxoplasma gondii* from patients is difficult and has necessitated the introduction of various indirect laboratory tests (Hall, 1984).

Diagnostic techniques consist of identification of *Toxoplasma*, the parasite isolation, tests for antibodies and tests for antigenemia and delayed hypersensitivity (Frenkel, 1985b).

**Identification of the Parasite**

*Toxoplasma* tachyzoites can be demonstrated on tissue imprints that are dried, fixed with methanol and stained with Giemsa as 7 x 3 µm ovoid organisms possessing a red nucleus and a blue cytoplasm. In tissue sections, tachyzoites are best demonstrated by hematoxylin and eosin stain are ovoid or rounded and measure 3-4 µm. Bradyzoites are of similar size but are more closely packed and hence their size is not easily determined in tissue section. They are distinguished by staining a form of glycogen and stain prominently with the periodic acid-Schiff (PAS) technique. On tissue imprints, they are well stained with Giemsa. Staining with specific antibody marked with fluorescein or enzyme and the ultrastructural features demonstrated by electron microscopy facilitate identification of the organisms. Alkaline methylene blue is also used to stain *Toxoplasma* in direct smear (Frenkel, 1985b).
Histologic demonstration of the cyst form usually indicates that the infection is subacute or chronic. However, since cysts may form early in infection, their demonstration does not exclude the possibility that the infection is still in the acute stages even in the same organ or at least elsewhere in the body (Anderson and Remington, 1975).

**Parasite Isolation**

Ventricular fluid, placenta, biopsy or autopsy tissues, or the buffy coat of a centrifuged blood specimen may be inoculated intraperitoneally into mice, hamsters, or cell culture. Identification of tachyzoites in the peritoneal fluid is sometimes possible after 4-8 days. If the animals survive, they are examined after 4-6 weeks for the presence of tissue cysts in the brain using stained tissue imprints (Giemsa) or tissue sections (PAS) and for the development of Toxoplasma antibody. The cells of inoculated culture can be stained, or the supernatants are spun down and the Giemsa-stained sediment examined for tachyzoites (Frenkel, 1985b).

Isolation of the parasite form tissue (e.g. skeleton muscle, lung, brain, eye) may reflect only the presence of tissue cysts and thus is not the definitive proof of acute infections. A possible exception is the isolation of Toxoplasma from lymph nodes, which most likely indicates the presence of the trophozoites, since cysts are rarely found in nodes (Remington, 1970).
SEROLOGY

Serologic tests

IgM and IgG antibodies can be determined by means of antihuman IgM and antihuman IgG, labelled with either fluorescein (indirect fluorescent antibody test-IFA) or an enzyme that later colours an added substrate (enzyme linked immunosorbent assay-ELISA).

The dye test of Sabin and Feldman is the reference serological procedure against which other methods are evaluated. It depends on lysis of Toxoplasma by the patient's antibody in the presence of complement. It is highly specific and is particularly useful when testing sera of many species of animals, because no species specific antiglobulin is required. However, the dye test requires living Toxoplasma, and it does not separate IgG from IgM.

The antigens for the indirect haemagglutination test (IHA) (using Toxoplasma antigen-coated red blood cells), the latex agglutination test (LAT) and the direct agglutination of Toxoplasma (DA) may be available in kits. The usefulness of complement-fixation and precipitin tests is of limited value (Frenkel, 1985b and Chatterton et al., 1989).

Interpretation of Serologic Tests

As quoted by Frenkel (1985b) while seeking a serologic diagnosis, it is important to distinguish pre-existing antibodies and passively transferred antibodies
from antibodies related to the illness. IgM antibodies are developed first during infection and disappear when IgG antibodies have achieved an apex. IgM is not transferred by the intact placenta and if leaking across a break, has a half-life of 3 to 5 days. Determination of the IgM fraction is therefore, useful to separate lower antibody titers of early infection in which IgM would be present from those of late chronic infection (in which IgM would be absent) and to distinguish actively acquired antibody (IgM present) from passively transferred IgG (IgM absent) in a baby (Ho-Yen, 1982 and Ho-Yen et al., 1982).

A satisfactory serological diagnosis of acute toxoplasmosis requires the demonstration of a four-fold or greater change in antibody titre (Karim and Ludlam, 1975 and Frenkel, 1985b). The presence of elevated IgM titres can be considered presumptive evidence of recent infection. If the specimens are taken during the late illness or for the presence of lymphadenopathy, titre in excess of 1:1000 should be present (Frenkel, 1985b).

For the diagnosis of congenital toxoplasmosis, a titre of over 1:1000 can be considered tentatively diagnostic, subject for confirmation by the finding of antibodies in the IgM fraction, the exclusion of Rheumatoid factor and finally the isolation of Toxoplasma. Passively transferred antibodies show a 10 fold decay every 3 months (50% per month, Frenkel, 1985), whereas in the presence of
infection, high antibody titre are stable or increase (Frenkel, 1985b). If a placental leak of maternal IgM antibodies has occurred, the IgM titre will drop significantly within a week (Anderson and Remington, 1975).

Toxoplasmosic retinochoroiditis which is usually a manifestation of chronic toxoplasmosis, generally is accompanied by stable low antibody titres. Any titre, even in undiluted serum is sufficient to support a presumptive diagnosis with a compatible lesion (Frenkel, 1985b).

Toxoplasmosis in immunocompromised hosts may be accompanied by high, low and rarely an absent antibody titre. Diagnostic help should be sought from biopsy (Luft et al., 1983 and Detry et al., 1984) and isolation of parasite in the presence of antigenemia (Araujo and Remington, 1980).

**Comparative Evaluation of Serologic Tests**

As stated by Voller et al., (1976a) the frequency of toxoplasmosis is largely determined with serological investigations to ascertain the presence of antibodies. Serological methods are playing an increasingly important role in the diagnosis and epidemiological assessment of diseases. Obeirne and Cooper (1979) suggested that the ideal characteristics of a diagnostic test are speed, sensitivity, specificity, accuracy, safety, inexpensive reagents, potential for automation, long shelf life of reagents, potential for field or office use and read applicability.
The Sabin and Feldman Dye Test (1946)

The method most commonly used in the past has been the dye test (DT) of Sabin and Feldman. Although specific, sensitive and reproducible, it is technically difficult and slow, requires the use of live parasites and suitable fresh human serum as "Accessory factor". This has led to a search for less exacting but equally effective techniques (Karim and Ludlam, 1975).

IFA Test (Kelen et al., 1982)

In practice immunofluorescence is not easy to quantify for antibody assays, since it depends on subjective visual assessments of fluorescence and the results are usually expressed as the serial dilution of serum that gives the least fluorescence (Voller et al., 1976a). Serum containing antinuclear antibodies or rheumatoid factor may cause false positive reactions in IFA and IgM-IFA (Anderson and Remington, 1975 and Hughes, 1985).

IHA Test (Jacob and Lunde, 1957)

As quoted by Anderson and Remington (1975) titres in the IHA tests usually lag several days or more behind those in the DT and tend to remain at high levels even longer than the DT. IHA test frequently is negative in cases of congenital toxoplasmosis with high DT titres and therefore not recommended for the diagnosis of congenital
hazards, they are stable and have long shelf-lives. Moreover, the test is easy, simpler and more rapid to perform (Voller et al., 1976a; 1976b and Walls et al., 1977). ELISA detects antibodies synthesized early (as do IFA and DA) as well as those appearing later as detected by CFT or IHA (Carlier et al., 1980). ELISA is highly sensitive and its specificity is comparable with that of IFA and IHA (Azab et al., 1983; Dahl and Johnson, 1984). No interference was found due to the presence of either rheumatoid factor or antinuclear antibodies (Dahl and Johnson, 1984). The IgM-ELISA is more sensitive than IgM-IFA test in the diagnosis of recently acquired infection with *Toxoplasma gondii* (Naot and Remington, 1980, Naot et al., 1982 and Wieland, 1988).

**Detection of Toxoplasma Antigen**

As stated by Voller and De-Savigny (1981) antigen immunoassay could provide definite parasitological diagnosis while antibody assay provides at best only a presumptive diagnosis. Araujo and Remington (1980) noted that the detection of antigen for diagnostic purpose would be useful in those cases in which clinical findings are suggestive of acute toxoplasmosis but for which antibody response is suppressed because of serious underlying disease or immuno-suppressive therapy. ELISA is used for detection of soluble Toxoplasma antigen (Araujo and Remington, 1980 and Linderschmidt, 1985).
Complement Fixation test (CFT), Direct Agglutination test (DA), Immunosorbent Agglutination Assay (ISAGA), reverse ELISA or double sandwich ELISA and Enzyme Linked Immunofiltration Assay (ELIFA which enables differentiation of maternal from neonatal antibodies) are also used as diagnostic test for toxoplasmosis (Desmont and Remington, 1980; Foulton and Turk, 1980, Goldman, 1957 and Holliman et al., 1990).

Skin Test

Infection with Toxoplasma results in the development of cell-mediated immunity against the parasite. This may be demonstrated with the toxoplasmin skin test, which elicits delayed hypersensitivity. The skin test becomes positive several weeks or months after infection and is thus of little value in the diagnosis of acute toxoplasmosis. The test has been useful primarily in epidemiologic studies. This test can be used to support a clinical diagnosis of toxoplasmic retinochoroiditis where no serological tests are available. The test is usually standardized, such that a positive result is one that shows 5mm induration 48 to 72 hours after injection of 0.1 ml of toxoplasmin intradermally in the volar aspect of the forearm (Anderson and Remington, 1975).
Other Specialised and Research Techniques

Detection of circulating antibodies (exo-antigens and PEF Penetration Enhancing Factor) which are only present in the parasitemic phase and about which little is known (BioMerieux monograph on Toxoplasmosis, 1983). The other techniques are electron microscopy transmission and scanning of the parasite was also used as research tool. Bone marrow aspiration can be used as a diagnostic tool in acute toxoplasmosis in kidney transplant recipient (Ferron et al., 1990).

The Western Blot and Polymerase Chain Reaction (PCR) is also used in advance research laboratories to detect specific protein or sequence of DNA (Burg et al., 1989, De-la-Cruz et al., 1989 and Savva et al., 1990).

The technique which is sensitive, specific, easy, cheap take less time and safe should be used for routine diagnostic work.

TREATMENT

The pregnant women having active infection should be given 3 g of spiramycin orally for 3 weeks, which should be repeated at an interval of 2 week until term (Wilson, 1990). The early congenital toxoplasmosis cases should be advised 50-100 mg/Kg sulfadiazine and 0.5-1 mg/Kg pyrimethamine every 2-4 days, with intramuscular injection of 5 mg of folinic acid every 2-4 days in a dose of 100
mg/Kg/day between each course of therapy. Corticosteroid therapy is sometimes indicated. Treatment should be stopped at approximately one year as cellular immunity has sufficiently developed to cope with disease at this age (Hohlfeld et al., 1989). Immunodeficient patients should be given pyrimethamine but on a long term basis. In the immunologically normal person with severe disease, treatment for 4-6 weeks is recommended (Pien and Garin, 1989 and Key et al., 1990).

For active ocular toxoplasmosis, pyrimethamine and sulphadiazine are administered for one month within 10 days, the borderline retinal lesions should sharpen and vitreous haze should disappear. If response is unfavourable, repeated courses are needed, systemic corticosteroid can be administered if vision endangered by lesion involving the macula, optic nerve head, or papillomacular bundle. Occasionally vitrectomy and lensectomy may be necessary to restore visual acuity (WHO, 1989; Desmonts and Couvreur, 1974a,b and McCabe and Remington, 1985b).

The other medicines used to treat toxoplasmosis macrolides derived from erythromycin and tylosin (14- and 18-membered macrolides, respectively) of limited value. Erythromycin and azithromycin are found better and effective. Desmycosin, dirithromycin and roxithromycin had no detectable activity. Gamma interferon is also used to treat toxoplasmosis (Dutton, 1989, McCabe and Oster, 1989 and
Rayman et al., 1990). Beta interferon can also be used to inhibit *Toxoplasma gondii* (Schmitz et al., 1989).

**PREVENTION OF TOXOPLASMOsis**

Toxoplasma infection represents a prominent example of a disease which can be controlled or prevented by taking advantage of its epidemiologic characteristics (Frenkel, 1974, Feldman, 1982, Conyn-van-Spaendonck, 1989, Koskineniemi et al., 1989 and Leighty, 1990).

**Primary Prevention**

*Preventive measures for disease control*

1. Meat should be heated throughout at 66°C (150°F) before consumption.

2. Raw edibles like meat, eggs, vegetables and milk should be avoided.

3. Hands should be washed with soap and water after handling meat to remove any organism contaminating the skin.

4. Animal handling should be avoided if possible and those fond of pets should washed their hands on regular basis.

5. Indoor cats can be prevented from disease by feeding them dry, canned, or boiled food and by restraining them from eating outside.

6. The faeces of cat should be treated with 33% ammonia and 7% tincture iodine but their cost, objectionable smell and danger involved in their use would probably
Preclude their use on a daily basis. Addition of boiling water or application of dry heat (over 65°C) appear more practical.

7. Handling soil should be avoided and people should be advised not to play or handle sand, or if so washing of hand should be done with soap and water thoroughly.

8. Drinking water should be properly boiled before use.

9. Disinfectant should be used to kill insects and flies which may result in contamination of food and water (1-8 Frenkel and Dubey, 1972 and Willson and Remington, 1980).

10. Screening of blood donors for toxoplasmosis should be done at least in immunocompromised patients if not possible in all the cases (Jeannel et al., 1990).

11. Organ transplant donor should be screened for toxoplasmosis.

12. Screening of women should be performed either before marriage or before conception (Foulon et al., 1994 and Golledge and Beaman, 1990) and sero-negative women also screened during pregnancy for sero conversion (Foulon et al., 1990).

13. Pregnant sero-negative women should not be employed in laboratories which deals with life culture of Toxoplasma gondii (Chatterjee, 1980, Forestier, 1985 and Zygmunt, 1890).

14. Frenkel (1981) suggested that prevention of
congenital toxoplasmosis rather than palliation should be our aim. We only need to persuade pregnant women to wash their hand after contact with meat, cats, other animals, soil and cook their food adequately.

15. Immunization is difficult (Frenkel and Waldeland, 1983). In animals live and dead vaccine are not sufficiently protective (Hermentin and Aspock, 1988). The organism Hammondia hammondii, a near relative of Toxoplasma gondii may be of interest since it might protect against toxoplasma infection without damaging host. Human use is still doubtful (Carter et al., 1974 and Darde et al., 1982).

Secondary Prevention

The secondary prevention consist of an early diagnosis and adequate treatment. As cited by Wilson and Remington (1980) the infection of the foetus might be prevented by treatment during gestation of those women who acquire their primary infection with Toxoplasma during pregnancy. Desmonts and Couvreur (1974b) revealed that treatment of women with spiramycin who acquired infection during pregnancy, reduced the incidence of congenital toxoplasmosis by 50%.

Recent studies re-emphasize that any infant with congenital toxoplasmosis, whether symptomatic or not should be treated as treatment may decrease the frequency or severity of adverse sequelae (Willson et al., 1980 and Koppe et al., 1986).
**Tertiary Prevention**

Disability limitation are the main components of tertiary prevention. It constitutes special schooling for visually handicapped and moderately retarded children, state supported foster care for severely retarded and regular ophthalmologic follow-up care (Rudd and Peckman, 1988).
MATERIALS AND METHODS
A total of 105 women mainly of AB group and TA group who were on list of dilatation and curettage were included in this study. They were arranged in the following manner:

A) AB groups (Abortion group). 54
B) TA groups (Therapeutic abortion group). 51

These cases were collected from various hospitals and maternity homes of Karachi. Each was assigned a code number. Relevant information was recorded on performa (Appendix I) and after taking all aseptic measure 3-5 ml of blood was drawn from a superficial vein of upper limb (forearms) with the help of disposable syringe. It was then transferred to a sterile screw capped tube slowly and carefully to avoid haemolysis. It was allowed to clot then centrifuged at 3000 rpm for 15 minutes. Serum was separated and transferred with the help of a disposable pasteur pipette to another sterile tube and stored in a freezer at -20°C until processed for analysis (Appendix II). Care was taken in all samples that they were not repeatedly frozen and thawed or store for more than 6 months as prolong storage of serum (over one year in frozen state) may cause formation of lipid aggregates. These aggregates may elevate the absorbance of negative sera (Shillitoe, 1982). The study was started in January 1990 and ended in September 1992. All samples were screened for specific IgM and IgG antibodies against Toxoplasma gondii using Enzyme Immuno Assay (EIA). They were also screened for Human Immunodeficiency Viruses (HIV) by EIA. Products of conception were collected after all aseptic care into a
sterile dilatation and curettage syringe with negative pressure. The material was processed in the following manner:

i) Wet mount preparation (Appendix VII).

ii) Stained preparation with alkaline or polychrome methylene blue after preparing smear (Appendix III).

iii) Animal inoculation.

**WET MOUNT (Appendix VII)**

The wet mount preparation was made from material obtained by dilatation and curettage from patients. It was examined under ordinary microscope for the presence of parasite after placing one drop of material over glass slide and covering it with cover slip and watched.

**Stained Preparation (Appendix III)**

Smear was made from material on a glass slide which was air dried and fixed with alcohol. It was covered with polychrome or alkaline methylene blue for one minute, washed with distilled water and examined under a microscope. The parasites appeared blue (blue cytoplasm and dark blue nucleus).

**MICE INOCULATION**

The product of conception was inoculated in peritoneal cavity of mice using a wide bore needle. 0.1 ml conceptus and saline was inoculated into two sets of mice of age between 10 to 12 weeks and weight from 110 to 120 grams. The first group was sacrificed on 3rd or 4th day. The blood was drawn from heart of mice in 3.5 ml of saline. The blood after mixing with saline was centrifuged at 3000 rpm for 15 minutes and supernatant was removed and stored
in separate tube at -20°C till processed. The peritoneum was washed with 1.0 ml of saline. The material thus obtained was used for making wet mount preparation, stained slide with alkaline methylene blue and remaining material was equally poured into artificial media (monophasic & diphasic TC-199 with or without antibiotics and M-medium) in triplicate and incubated at 4-8°C, 25°C and 37°C. The material was examined on every second day for growth of Toxoplasma gondii and doubling time was noted, adopting trypan blue exclusion method for viable count. The other set of mice were sacrificed on and about 3rd week. They were processed in similar manner. All mice sacrificed were dissected, their organs and tissues were preserved in 10% formalin and processed for histopathological slide preparation (Appendix XII and XIII). The results were properly recorded.

**Enzyme Immuno Assay (EIA) For Toxoplasma IgM and IgG**

All the sera were tested in duplicate for specific IgG and IgM antibodies against Toxoplasma gondii. A solid phase enzyme immunoassay (ELISA) for the quantitative analysis of antitoxoplasma IgM and IgG class antibodies in human serum was used. For the purpose Labsystem Kits were used (Labsystems OY, Pulkstitie 8, 00880 Helsinki, Finland). In this kit 24 μg of soluble antigen prepared from the tachyzoites of the Rh strain of Toxoplasma gondii was coated on polystyrene surface of microwells. Alkaline phosphatase conjugated antihuman IgM or IgG was used as the second antibody. Patient's serum was added in the wells and anti-toxoplasma antibodies, if present, combine with the antigen.
Principle:

The principle of Labsystems Toxoplasma gondii IgM & IgG ELISA kit is based on an indirect solid phase enzyme immunoassay system with alkaline phosphatase as the marker enzyme. When present in patient's serum Toxoplasma gondii IgM or IgG class antibodies combines with the Toxoplasma antigens attached to the polystyrene surface (===) in the wells of the microstrips.

The wells were washed and a colourless substrate, p-nitrophenylphosphate (pNPP), was added. The substrate was hydrolysed by the enzyme to a coloured end product, p-nitrophenol, which was read in a spectrophotometer (Uniscan II) at 405 nm after terminating the reaction with NaOH.

The colour intensity was directly proportional to the concentration of the Toxoplasma IgM & IgG class antibodies in patient's serum. Details of the procedure are given in appendix VIII & IX.

TEST PROCEDURE

Step I

Appropriate number of coated microstrips needed for the number of samples (reagent, blank, controls, serum samples) to be tested were arranged into the microstrip frame. 100 µl of the diluted sample diluent was added in two wells. Negative and positive standard serum samples with predetermined values for IgM and IgG were similarly put in the respective wells. The serum samples were also added in duplicate. The microstrips were covered tightly with plastic sheets and incubated for 60 minutes at 37°C.
Note: Each sample and control was tested in duplicate.

Washing

The wells were emptied by shaking out the liquid and gently tapping the inverted microstrips a few times on a clean paper towel. 200 µl of washing solution was added into each well and emptied three times by shaking out the liquid. After the third washing, inverted microstrips were tapped a few times on a paper towel.

Step II

100 µl of the diluted conjugate was pipetted into each well. Microstrips were covered tightly with plastic sheets and incubated for 60 minutes at 37°C. Washing was done similarly as in Step I.

Step III

100 µl of the substrate solution was pipetted into each well and incubated exactly for 30 minutes at 37°C.

Step IV

100 µl of freshly prepared 1M NaOH was pipetted into each well and mixed carefully so as to stop the enzyme substrate reaction. Details of procedure are given in appendix VIII & IX.

Reading

The absorbance was measured at 405 nm immediately after stopping the enzymatic reaction. Uniscan II Photometer (plate or microstrip reader) was used for the measuring the absorbance.
Quality Control Values

Before proceeding to read the absorbance of the test samples it was necessary to read the absorbance of the reagent blank and the controls. If they fell within the quality control limits (as shown below) then the readings of the samples were taken.

QUALITY CONTROL VALUES FOR IgM

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>EXPECTED VALUE (IN ABSORBANCE UNITS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REAGENT BLANK</td>
<td>&lt; 0.500</td>
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<tr>
<td>NEGATIVE CONTROL</td>
<td>&lt; 0.200</td>
</tr>
<tr>
<td>POSITIVE CONTROL</td>
<td>1.000 - 1.500</td>
</tr>
</tbody>
</table>

QUALITY CONTROL VALUES OF IgG

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>EXPECTED VALUE (IN ABSORBANCE UNITS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REAGENT BLANK</td>
<td>&lt; 0.500</td>
</tr>
<tr>
<td>NEGATIVE CONTROL</td>
<td>&lt; 0.200</td>
</tr>
<tr>
<td>LOW POSITIVE CONTROL</td>
<td>0.500 - 1.000</td>
</tr>
<tr>
<td>HIGH POSITIVE CONTROL</td>
<td>1.000 - 1.500</td>
</tr>
</tbody>
</table>

Note The reagent blank absorbance has already been subtracted from these values.

Calculation of the Results

The mean absorbance was calculated for all the duplicate samples. Care was taken that the coefficient of variation (CV%) between duplicate values for positive samples was less than 10%. If it was more than 10% the test was repeated. CV% was calculated as follows:
where SD is the standard deviation.

The absorbance value of each sample was converted to Enzyme Immunoassay Units (EIU) and the levels of antibodies were reported in EIU according to the following formula:

\[
\text{EIU} = \frac{\text{A sample - Arb} \times 100}{\text{Apc - Arb}}
\]

Where

A sample = the mean absorbance of the patient sample.

Arb = the mean absorbance of the reagent blank.

Apc = the mean absorbance of the positive control.

**Interpretation of results**

**For IgM EIU**

<table>
<thead>
<tr>
<th>&lt; 20</th>
<th>Not detectable</th>
<th>(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 - 40</td>
<td>Low positive</td>
<td>(+)</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>Positive</td>
<td>(++)</td>
</tr>
</tbody>
</table>

**For IgG EIU**

<table>
<thead>
<tr>
<th>&lt; 10</th>
<th>Not detectable</th>
<th>(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 - 19</td>
<td>Uncertain positive</td>
<td>(-)</td>
</tr>
<tr>
<td>20 - 59</td>
<td>Low positive</td>
<td>(+)</td>
</tr>
<tr>
<td>60 - 130</td>
<td>Positive</td>
<td>(++)</td>
</tr>
<tr>
<td>&gt; 130</td>
<td>High Positive</td>
<td>(+++)</td>
</tr>
</tbody>
</table>
OUTLINE OF PROCEDURE FOR TOXOPLASMA IgM & IgG ANTIBODIES

STEP I
Add 100 ul of diluted sample/reagent blank/control

Incubate for 60 minutes at 37°C

Wash with Tween 20 (Diluted)

STEP II
Add 100 ul conjugate solution

Incubate for 60 minutes at 37°C

Wash with Tween 20 (Diluted)

STEP III
Add 100 ul substrate solution

Incubate for 30 minutes at 37°C

STEP IV
Add 100 ul 1M NaOH

Measure absorbance at 405 nm (On Uniscan II)
Toxoplasma IgM Positive Confirmatory Test (Appendix X)

The IgM positive results were also confirmed by IgM confirmatory kit Labsystems Finland (6106000). In this method 10 µl patient’s serum was taken and 190 µl pretreatment solution (phosphate buffered saline, pH 7.4 0.2% protein content 2.7% with 0.1% sodium azide as preservative) which neutralised other’s cross reacting antibodies, mixed on a rotator for 30 minutes. It was centrifuged at 3000 rpm. The supernatant was removed from pretreatment solution and diluted with sample diluent in 1:5 dilution. The rest of the procedure was adopted as of toxo IgM kit.

Human Immuno Deficiency Virus (HIV) by Enzyme Immuno Assay

(EIA) test Kit

Manual Procedure

PRELIMINARY PREPARATIONS

NOTE

The disposable gloves were worn throughout the procedure.
- EIA kit was brought to room temperature (RI).
- Incubator was prewarmed at +37°C.
- Washing solution was brought to room temperature.
- Reagents present in the kit were prepared (Appendix XI).
- Sample diluent was used for specimen dilution and for reagent blank.
- Controls were included in each test set run.
- Diluted specimens (1:100) were prewarmed at +37°C with sample diluent (10 µl of serum in 1.0 ml diluent) and mixed well.
**STEP I**
- Appropriate number of Microstrips were placed out for testing samples (patient sera, control sera and reagent blank) into the microstrip frame.
- 100μl of the diluted specimens were pipetted and poured into Microstrip wells.
- 100 μl of sample diluent containing protein solution was placed in two wells each (reagent blank) and 100 μl of controls (positive and negative control) in two wells each.
- Microstrips were tightly covered by plastic sheets and then incubated for 60 minutes at 37°C.

**WASHING**
- The wells were emptied by shaking out the liquid and gently tapped by inverting it and microstrips were kept few times on a clean paper towel.
- 200 μl of washing solution was added into each well. A few millimeters from the end of the tip of pipette was cut off to avoid too much pressure and foaming during pipetting.
- Wells were emptied once again by shaking out the liquid.
- Washing was done three times in total (one cycle) and after third washing microstrips were inverted few times on a paper towel.
- Approximately 10 ml of washing solution per microstrip was needed in one washing cycle.

**STEP II**
- 100 μl of the diluted conjugate was poured into each well.
- It was then incubated exactly for 30 minutes at 37°C and washed again three times with Tween 20.

**STEP III**
- Pipette 100 μl of substrate para nitrophenylphosphate (pNPP) solution was added into each well.
- It was incubated exactly for 30 minutes at 37°C and washed again three times with Tween 20.

**STEP IV**
- 100 μl of 1M NaOH was poured into each well and mixed carefully.
- This was done to stop the enzyme substrate reaction.

**Artificial Culture:**

The media used were TC-199 medium monophasic with TC salt (with or without antibiotics). TC-199 medium with TC salts biphasic (liquid phase) in 10% blood agar slant (solid phase) and M-medium were used for artificially culturing *Toxoplasma gondii*. The product of conception (0.1 ml) was poured into different media in triplicate and kept at different temperatures (4–8°C, 25°C and 37°C). In similar manner pouring was done from the peritoneal wash from mice sacrificed on different days. Initial counting and counting every second day was done to check the growth and doubling time in Neubeur modified counting chamber adopting trypan blue exclusion method for viable count (Appendix XIX). The doubling time was time in which number of organisms (parasites) double in known quantity in how many days or hours. Growth from artificial media was used as follows:
1. **Wet mount** was used for direct microscopy and video recording.

2. **Alkaline or Polychrome Loeffler's methylene blue**

3. **Other staining methods**
   a) Giemsa stain.
   b) Carbol Fuchsin stain.
   c) Safranin Stain.

**Giemsa Stain (Appendix IV)**

**Method**

1. The film was first fixed with pure alcohol for 3 to 5 minutes and allowed to dry.

2. Giemsa's stain was diluted by adding one drop to each one ml of distilled water and brought to neutral or faintly alkaline (pH 7.2).

3. The diluted stain was poured over the film (about 5 ml per film was used) and kept for 30 to 45 minutes.

4. The slide was then flushed in a gentle flow of tap water, after which it was placed in an upright position with the film side inwards to drain and dry.

5. The stained film was examined under 1/12 inch oil immersion lens.

**Carbol Fuchsin Stain**

**Method**

1. The smear was made and allowed to dry in air (fix with alcohol).

2. Carbol fuchsin was poured over the smear for 2 minutes.

3. It was then washed with distilled water.
4. It was left at water drainer for drying and then it was watched under microscope.

**Safranin Stain**

**Method**

1. The smear was made from sample and dried in the air (fixed with alcohol).
2. Safranin was poured over smear for 2 minutes.
3. It was then washed with distilled water.
4. It was left at water drainer for drying and then was watched under microscope.

**ELECTRON MICROSCOPY TRANSMISSION:**

The Toxoplasma used in this investigation was derived from the artificial culture grown in our laboratory. After recovering it from white mice, it appears to be identical with RH strain of *Toxoplasma gondii*. Material from artificial media was tried as vehicles for the Toxoplasma to be sectioned. The fluid appears to have definite advantages in both fixation and concentration and has been used exclusively for the material reported here. Two variations in preparation for fixation were found useful. At first, sediment from the centrifuged fluid was imbedded in melted 10% gelatin in saline, which was cooled, cut into cubic millimeter blocks, and immersed in the fixative. Portions of the block were excellent but fixation was inconsistent and concentration of the Toxoplasma was sometime inadequate. The other method adopted was
the uniformity of the material and produced a flocculent sediment easily concentrated.

The fixative used in all cases was an aqueous solution of osmium tetraoxide buffered at pH 7.4 with veronal-acetate. Variations of time and concentration were compared and an apparent optimum was excellently used 0.5% OsO₄ for two hours at 4°C. The addition of 0.32% NaCl used as the buffered fixative to decreased vacuolization of the cells. After washing and dehydration with graded alcohols, the sediment was impregnated in a mixture of 4 parts butyl methacrylate monomer and 1 part isobutyl methacrylate monomer. Polymerization in No.2 gelatin capsules was carried out at 40°C with the aid of a catalyst. Thin sectioning was accomplished with a modified spencer rotary microtome 50% acetone water reservoir behind the knife edge and picked up directly on formvar coated 200-mesh stainless steel grids. The sections were screened by microscopic examination under reflected light; those showing interference colours indicating a thickness of approximately 0.05 micron or less were chosen for study. The sections were studied in electron microscope equipped with a self based specimens were photographed routinely at an initial magnification of 6500 X. and further. Enlargements were made from these negatives.

**ELECTRON MICROSCOPY (SCANNING):**

Samples were taken from artificial medium in two tubes and were washed several times with 100% ethanol. Tubes were filled with material with 100% ethanol. After balancing tubes were centrifuged at 5000
G for 20-30 minutes. Supernatant was discarded. This procedure was repeated several times and remaining material was critical point-dried using CO₂ as drying agent. Specimen was mounted on stub, gold coated and palladium alloy in sutter coater. It was placed at examining unit after coating and examined in Jeol scanning electron microscope at different magnifications.
RESULTS
The present study involved the analysis of serum samples and product of conception in 105 females. The serum samples were processed for IgM and IgG antibodies specific to *Toxoplasma gondii* by EIA. Sero-positive cases of IgM were confirmed by IgM confirmatory Kit (EIA). All humans sera were also screened for HIV by EIA. The product of conception was used for making wet mount, stained preparation and inoculation in mice. The mice were sacrificed, one group on 3rd or 4th day and other on or about 3rd week. They were subjected to wet mount and staining preparation. Serological study for IgM and IgG antibodies was also done by EIA. The remaining material was poured into artificial media.

**WET MOUNT AND STAINED (DIRECT) PREPARATION**

The results of wet mount and alkaline methylene blue stain were made from conceptus material of women were having parasites in 02 (3.7%) slides examined out of 54 in AB group. There was no parasite seen in TA group out of 51 checked. From a total of 105 only 02 (1.9%) placental samples had parasite both on wet mount and stained preparation (Table 13).

**EIA IgM and IgG antibodies specific to *Toxoplasma gondii***

All samples of women were tested for IgM and IgG type of antibodies specific to *Toxoplasma gondii* by EIA. We have tested 105 specimen for IgM antibodies from which 16 (15.2%) were sero-positive. In AB group we have tested 54 women from which 14 (25.9%) were
positive, whereas 02 (3.9%) were sero-positive in TA group out of 51 screened (Table 1).

The results of IgM type of antibodies according to degree of positivity were 09 (16.7%) borderline positive, 05 (9.3%) positive in AB group and in TA group there was 01 (1.9%) borderline positive and 01 (1.9%) positive (Table 2). IgM sero-positivity in different age groups were in 15-24 years (20%), in 25-34 years (14%) and in 35-44 years (10%) in overall group (Table 5, Fig. 8 & Fig.9).

IgM sero-positivity in different age group females were in 15-24 years (30%), in 25-34 years (25.1%) and in 35-44 years (20%) in AB group (Table 7, Fig. 12 & Fig. 13). Sero-positivity in different age group women for IgM were in 15-24 years (6.7%) age group, in 25-34 years (3.8%) age group and non in 35-44 years age group in TA group (Table 9, Fig.16 & Fig. 17).

IgM sero-positive cases from 54 screened in AB group (sub groups), there were 08 (46%) threatened abortions, inevitable abortions 03 (21.4%) incomplete abortions and in missed abortions 02 (18.2%) (Table 11).

**IgM Confirmatory**

All IgM sero-positive cases were confirmed by IgM confirmatory kit. One uncertain positive case gave negative result with this kit, which was later recorded as negative after IgM confirmatory kit result.

Screening for IgG type antibodies specific to *Toxoplasma gondii* in 105 cases, there were 35 (33.3%)
sero-positive in overall groups. In AB group there were 27 (50.0%) sero-positive cases out of 54 screened and in TA group from 51 cases 08 (15.7%) were sero-positive (Table 3). According to degree of positivity in AB group there were 08 (14.8%) low positive, 03 (5.5%) positive, 03 (5.5%) borderline positive. In TA group there were 03 (5.9%) borderline positive. 02 (3.9%) low positive, 02 (3.9%) positive, 01 (1.9%) high positive (Table 4). IgG sero-positive cases according to age in overall group were 05 (14.3%) in 15-24 years age group and 11 (55.0%) in 35-44 years age group (Table 6. Fig. 10 & Fig. 11). In AB group out of 54 cases tested, there were 04 (20.0%) in 15-24 years age group, 15 (62.5%) in 25-34 years age group and 08 (80.0%) in 35-44 years age group (Table 8. Fig. 14 & Fig. 15). In TA group IgG sero-positive cases from 51 tested, there were 01 (6.7%) in 15-24 years age group, 04 (15.4%) in 25-34 years age group and 03 (30.0%) in 35-44 years age group (Table 10. Fig. 18 & Fig. 19). In different subgroups of AB group screened for IgG antibodies there were 13 (65.0%) in threatened abortion, 03 (33.3%) in inevitable abortion, 07 (50.0%) in incomplete abortion and 04 (36.4%) in missed abortion subgroups (Table 12).

**IgM Study in Mice**

Sero-positive case was nil in mice sample tested for IgM type of antibodies specific for Toxoplasma gondii from mice sacrificed on 3rd or 4th
day. In mice sacrificed on or about 3rd week there were 02 (3.0%) sero-positive in AB group out of 54 tested (Table 15). There was no positive case in TA group.

**IgG study in Mice**

There was not a single sero-positive case for IgG type of antibodies in mice sacrificed on 3rd or 4th day. In mice sacrificed on or about 3rd week there were 02 (3.7%) sero-positive case for IgG type of antibodies specific to *Toxoplasma gondii* in AB group out of 54 tested (Table 15). There was no sero-positive case in TA group.

**Human Immuno Deficiency Virus by EIA Test (Appendix Xii)**

All the human sera of AB group and TA group were screened for HIV by EIA. There was not a single case found positive in both AB and TA groups.

**Wet Mount and Stained Preparation From Mice**

Wet mount and stained preparation (alkaline methylene blue) from peritoneal wash of mice group sacrificed on 3rd or 4th day and mice group sacrificed on or about 3rd week. The parasite were seen in both wet mount and stained preparation made from peritoneal wash of mice sacrificed on 3rd or 4th day in AB group 02 (3.7%) out of 54 slides and non in TA group out of 51 cases (Table 14). In mice sacrificed on or about 3rd week there was no parasite (*Toxoplasma gondii*) seen on slide in both wet mount and stained preparation (Appendix III).
Artificial Culture of Toxoplasma gondii (Appendices XIV, XV, XVI and XVII)

Peritoneal wash from all the mice sacrificed on 3rd or 4th day and on or about 3rd week were placed in equal amount in tubes of each medium. The media used for pouring were M.Medium, TC-199 Medium with or with antibiotic with TC salt solution of Hanks and 10% blood agar slant. TC-199 media monophasic and diphasic preparation were used. TC-199 medium diphasic medium in 10% blood agar slant was used. All media in tubes were kept at 37°C, 25°C and 4-8°C in refrigerator. Best growth was seen on TC-199 without antibiotic in liquid phase (monophasic) at 25°C and 37°C. The M.medium has been found ideal for keeping organism for longer period at 4-8°C. In TC-199 medium doubling time observed was 04 days at 25°C and 37°C 04 days. In a bottle having M.Medium about 100 ml poured with parasite. It was observed that parasite (Toxoplasma gondii) remained viable in adequate number for more than 02 years. It was also observed that the medium exhaust earlier at higher temperature. The medium must be changed at 37°C before 10th day of pouring and at 25°C before 18th day. The growth of Toxoplasma gondii and longer survival time at 4-8°C gave us opportunity for extensive study of Toxoplasma gondii from different angle.

The parasite was studied as follows:

a) Wet mount preparation from M.medium and TC-199 medium.
b) Stained preparations from M. medium and TC-199 medium.
   i) Alkaline methylene blue stain.
   ii) Giemsa stain.
   iii) Carbol fuchsin stain.
   iv) Safranin stain.

c) Electron Microscopy Transmission.

d) Electron Microscopy Scanning.

a) Wet Mount Preparation from artificial media

One drop from artificial media was taken and placed on a clean new slide which was covered with a new coverslip. The slide was observed under a microscope. We have observed different sizes and shapes of Toxoplasma gondii. In different sizes we observed small (Dwarf), medium (Intermediate) and large (Giant) forms. Different shapes were observed from round to crescent shapes. The parasite has been observed to move in different directions. Video film has been made to record above mentioned observations.

b) Stained Preparation from M. Medium and TC-199 medium

Many smears were made from M. Medium and TC-199 medium stained with different staining techniques.

i) Alkaline Methylene Blue Stain (Appendix III)

Toxoplasma gondii was observed under microscope (cell was light blue and nucleus dark blue).

ii) Giemsa Stain (Appendix IV)

The parasite observed by Giemsa stain
(cytoplasm light blue and nuclei light red).

iii) Carbol Fuchsin Stain (Appendix XIII)

The parasite appeared dark pink without nuclear demarcation.

iv) Safranin Stain (Appendix XV)

The parasite appeared light pink without nuclear demarcation.

c) Electron Microscopy Transmission

Electron microscopy transmission performed from artificial medium gave us information about size, shape and ultra structure of Toxoplasma gondii. Different size and shapes were also seen on electron microscopy transmission at different magnifications (Photomicrographs 1-6). In ultra structures following structures were noted in electron microscopy transmission:

i) Nucleus.

ii) Nucleolus.

iii) Vacuoles.

iv) Golgi body.

v) Endoplasmic reticulum.

vi) Toxonemes (Micronemes)

vii) Conoid.

viii) Mitochondria.

ix) Rhoptries.

x) Membrane.
d) **Electron Microscopy Scanning**

Electron microscopy scanning showed us *Toxoplasma gondii* at different magnifications (Photomicrographs 7-13).
TABLE 1

HUMAN SERUM TOXOPLASMA IgM SERO-POSITIVE CASES - BY EIA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Tested</th>
<th>IgM Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>54</td>
<td>14</td>
<td>25.9</td>
</tr>
<tr>
<td>TA</td>
<td>51</td>
<td>02</td>
<td>3.9</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>16</td>
<td>15.2</td>
</tr>
</tbody>
</table>

P < 0.05 significant.
**TABLE 2**

IgM SERO-POSITIVE CASES OF TABLES ARRANGED ACCORDING TO LEVEL OF POSITIVITY

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Tested</th>
<th>IgM Sero-positive with level of positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>54</td>
<td>14 (25.9) [ UNP 09 (16.7) PO 05 (9.3) ]</td>
</tr>
<tr>
<td>TA</td>
<td>51</td>
<td>02 (3.9) [ UNP 01 (1.9) PO 01 (1.9) ]</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>16 (15.2)</td>
</tr>
</tbody>
</table>

Fig. In parenthesis are percentages.

P < 0.05 significant.

P > 0.05 insignificant (between UNP and PO in each group)

**INTERPRETATION OF RESULTS**

<table>
<thead>
<tr>
<th>Status</th>
<th>Range</th>
<th>EIU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt; 20</td>
<td>EIU</td>
</tr>
<tr>
<td>Uncertain positive</td>
<td>20-40</td>
<td>EIU</td>
</tr>
<tr>
<td>Positive</td>
<td>&gt; 40</td>
<td>EIU</td>
</tr>
</tbody>
</table>
TABLE 3

HUMAN SERUM TOXOPLASMA IgG SERO-POSITIVE CASES
BY EIA

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Tested</th>
<th>IgG Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>54</td>
<td>27</td>
<td>50.0</td>
</tr>
<tr>
<td>TA</td>
<td>51</td>
<td>08</td>
<td>15.7</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>35</td>
<td>33.3</td>
</tr>
</tbody>
</table>

P < 0.05 significant.
TABLE 4

IgG SERO-POSITIVE CASES OF TABLE 3 ARRANGED ACCORDING TO LEVEL OF POSITIVITY

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Tested</th>
<th>IgM Sero-positive with level of positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>54</td>
<td>27 (50.0) [ UNP 13 (24.1) LP 08 (14.8) PO 03 (5.5) HP 03 (5.5)]</td>
</tr>
<tr>
<td>TA</td>
<td>51</td>
<td>08 (15.7) [ UNP 03 (5.9) LP 02 (3.9) PO 01 (1.9) HP 01 (1.9)]</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>35 (33.3)</td>
</tr>
</tbody>
</table>

Fig. in parenthesis are percentages.

P < 0.05 significant (Between main group and some groups of level of positivity case).
P > 0.05 insignificant (In some cases of degree of positivity)

INTERPRETATION OF RESULTS

<table>
<thead>
<tr>
<th></th>
<th>EIU</th>
<th>EIU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt; 10</td>
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<tr>
<td>Uncertain positive</td>
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<td>Low Positive</td>
<td>20-59</td>
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<tr>
<td>Positive</td>
<td>60-130</td>
<td>EIU</td>
</tr>
<tr>
<td>Highly positive</td>
<td>&gt; 130</td>
<td>EIU</td>
</tr>
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</table>
**TABLE 5**

**TOXOPLASMA IgM SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE (ALL GROUPS)**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total tested</th>
<th>IgM Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-24 years</td>
<td>35</td>
<td>07</td>
<td>20.0</td>
</tr>
<tr>
<td>25-34 years</td>
<td>50</td>
<td>07</td>
<td>14.0</td>
</tr>
<tr>
<td>35-44 years</td>
<td>20</td>
<td>02</td>
<td>10.0</td>
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</table>
Fig. 8 TOXOPLASMA IgM SERO-POSITIVE CASES ACCORDING TO AGE (ALL GROUP)
Fig 9  TOXOPLASMA IgM SERO-POSITIVE CASES ACCORDING TO AGE IN OVERALL GROUP (HISTOGRAM)
TABLE 6

TOXOPLASMA IgG SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE (ALL GROUPS)

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total tested</th>
<th>IgG Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-24 years</td>
<td>35</td>
<td>05</td>
<td>14.3</td>
</tr>
<tr>
<td>25-34 years</td>
<td>50</td>
<td>19</td>
<td>38.0</td>
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<tr>
<td>35-44 years</td>
<td>20</td>
<td>11</td>
<td>55.0</td>
</tr>
</tbody>
</table>
Fig 10 TOXOPLASMA IgG SERO-POSITIVE CASES ACCORDING TO AGE IN OVERALL GROUP
Fig 11 IgG TOXOPLASMA SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE IN OVERALL GROUPS (HISTOGRAM)
TABLE 7

TOXOPLASMA IgM SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE (AB GROUP)

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total tested</th>
<th>IgM Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-24 years</td>
<td>20</td>
<td>06</td>
<td>30.0</td>
</tr>
<tr>
<td>25-34 years</td>
<td>24</td>
<td>06</td>
<td>25.0</td>
</tr>
<tr>
<td>35-44 years</td>
<td>10</td>
<td>02</td>
<td>20.0</td>
</tr>
</tbody>
</table>
Fig 12  TOXOPLASMA IgM SERO-POSITIVE CASES ACCORDING TO AGE (AB GROUP)
Fig 13  TOXOPLASMA IgM SERO-POSITIVE CASES ACCORDING TO AGE IN AB GROUP (HISTOGRAM)
**TABLE 8**

**TOXOPLASMA IgG SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE (AB GROUP)**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total tested</th>
<th>IgG Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-24 years</td>
<td>20</td>
<td>04</td>
<td>20.0</td>
</tr>
<tr>
<td>25-34 years</td>
<td>24</td>
<td>15</td>
<td>62.5</td>
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<tr>
<td>35-44 years</td>
<td>10</td>
<td>08</td>
<td>80.0</td>
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</table>
Fig 14  TOXOPLASMA IgG SERO-POSITIVE CASES ACCORDING TO AGE (AB GROUP)
Fig 15  TOXOPLASMA IgG SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE IN AB GROUP (HISTOGRAM)
**TABLE 9**

**TOXOPLASMA IgM SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE (TA GROUP)**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total tested</th>
<th>IgM Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-24 years</td>
<td>15</td>
<td>01</td>
<td>6.7</td>
</tr>
<tr>
<td>25-34 years</td>
<td>26</td>
<td>01</td>
<td>3.8</td>
</tr>
<tr>
<td>35-44 years</td>
<td>10</td>
<td>00</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Fig 16  TOXOPLASMA IgM SERO-POSITIVE CASES
ACCORDING TO AGE (TA GROUP)
Fig 17  TOXOPLASMA IgM SERO-POSITIVE CASES
ACCORDING TO AGE IN TA GROUP (HISTOGRAM)
### TABLE 10

**TOXOPLASMA IgG SERO-POSITIVE CASES ARRANGED ACCORDING TO AGE (TA GROUP)**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total tested</th>
<th>IgG Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-24 years</td>
<td>15</td>
<td>01</td>
<td>6.7</td>
</tr>
<tr>
<td>25-34 years</td>
<td>26</td>
<td>04</td>
<td>15.4</td>
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<tr>
<td>35-44 years</td>
<td>10</td>
<td>03</td>
<td>30.0</td>
</tr>
</tbody>
</table>
Fig 18  TOXOPLASMA IgG SERO-POSITIVE CASES ACCORDING TO AGE (TA GROUP)
Fig 19 TOXOPLASMA IgG SERO-POSITIVE CASES ACCORDING TO AGE IN TA GROUP (HISTOGRAM)
**TABLE 11**

IgM SERO-POSITIVE SUB-GROUPS FROM AB GROUP

<table>
<thead>
<tr>
<th>Sub-Groups</th>
<th>Total Tested</th>
<th>IgM Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threatened abortion</td>
<td>20</td>
<td>08</td>
<td>40.0</td>
</tr>
<tr>
<td>Inevitable abortion</td>
<td>09</td>
<td>01</td>
<td>11.1</td>
</tr>
<tr>
<td>Incomplete abortion</td>
<td>14</td>
<td>03</td>
<td>21.4</td>
</tr>
<tr>
<td>Missed abortion</td>
<td>11</td>
<td>02</td>
<td>18.2</td>
</tr>
</tbody>
</table>


**TABLE 12**

<table>
<thead>
<tr>
<th>Sub-Groups</th>
<th>Total Tested</th>
<th>IgG Sero-positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threatened abortion</td>
<td>20</td>
<td>13</td>
<td>65.0</td>
</tr>
<tr>
<td>Inevitable abortion</td>
<td>09</td>
<td>03</td>
<td>33.3</td>
</tr>
<tr>
<td>Incomplete abortion</td>
<td>14</td>
<td>07</td>
<td>50.0</td>
</tr>
<tr>
<td>Missed abortion</td>
<td>11</td>
<td>04</td>
<td>36.4</td>
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</table>
**TABLE 13**

WET MOUNT AND STAINED FILM MICROSCOPY FROM PRODUCT OF CONCEPTION

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Tested</th>
<th>Toxoplasma</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>54</td>
<td>02</td>
<td>3.7</td>
</tr>
<tr>
<td>TA</td>
<td>51</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>02</td>
<td>1.9</td>
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**TABLE 14**

**MICE SACRIFICED ON 3RD OR 4TH DAY, WET MOUNT AND STAINED FILM MICROSCOPY**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Tested</th>
<th>Toxoplasma</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>54</td>
<td>02</td>
<td>3.7</td>
</tr>
<tr>
<td>TA</td>
<td>51</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>02</td>
<td>1.9</td>
</tr>
</tbody>
</table>
PHOTOMICROGRAPHS
Photomicrograph 1: Electron micrograph transmission 8,500 X.

of Toxoplasma gondii
Photomicrograph 2: Electron micrograph transmission 6,500 X. of *Toxoplasma gondii*
Photomicrograph 3: Electron micrograph transmission 6,500 X. of *Toxoplasma gondii*
Photomicrograph 4: Electron micrograph transmission 17,500 X. of *Toxoplasma gordii*
Photomicrograph 5: Electron micrograph transmission 30,000 X. of *Toxoplasma gondii*
Photomicrograph 6: Electron micrograph transmission 30,000 X. of *Toxoplasma gondii*
Photomicrograph 7: Electron micrograph scanning 5,000 X.

of Toxoplasma gondii
Photomicrograph 8: Electron micrograph scanning 5,000 X. of *Toxoplasma gondii*
Photomicrograph 9: Electron micrograph scanning 6,500 X.

of Toxoplasma gondii
Photomicrograph 10: Electron micrograph scanning 7,500 X.

of *Toxoplasma gondii*
Photomicrograph 11: Electron micrograph scanning 10,000 X.

of *Toxoplasma gondii*
Photomicrograph 12: Electron micrograph scanning 10,000 X.

of *Toxoplasma gondii*
Photomicrograph 13: Electron micrograph scanning 10,000 X.

of *Toxoplasma gondii*
Photomicrograph 14: *Toxoplasma gondii* in direct smear (From product of conception) stained by Alkaline methylene blue 400X.
Photomicrograph 15: *Toxoplasma gondii* in direct smear (from product of conception) stained by Giemsa stain 400X.
Photomicrograph 16: *Toxoplasma gondii* from artificial culture stained with Alkaline methylene blue 400X.
Photomicrograph 17: *Toxoplasma gondii* from artificial culture stained with Alkaline methylene blue 100X.
Photomicrograph 18: *Toxoplasma gondii* from artificial culture stained with Giemsa 400X.
Photomicrograph 18: *Toxoplasma gondii* from artificial culture stained with Giemsa 100X.
Photomicrograph 20: *Toxoplasma gondii* from artificial culture stained with Carbol fuchsin 400X.
Photomicrograph 21: *Toxoplasma gondii* from artificial culture stained with Carbol fuchs in 100X.
Photomicrograph 22: *Toxoplasma gondii* from artificial culture stained with Safranin 400X.
Photomicrograph 23: *Toxoplasma gondii* from artificial culture stained with Safranin 100X.
Photomicrograph 24: *Toxoplasma gondii* from artificial culture stained with *Giemsa* 1000X.
DISCUSSION
It has been established that Toxoplasma infection has a worldwide distribution in human and other warm-blooded vertebrates but the frequency of the infection varies from one country to another and also in different parts of a given country (Feldman, 1982). One half billion human infection is a conservative estimate (Kean, 1972).

The population which was sampled for this study is rather restricted and signifies only females in the reproductive age (15-45 years) of a particular area are represented. In view of the small area, the prevalence observed gave only some idea with regard to prevalence of infection and its correlation. The prevalence of IgM type of antibodies in total group were 16 (15.2%) out 105 tested. In AB group out of 54 there were 14 (25.9%) sero-positive whereas in TA group there were 02 (3.9%) out of 51 tested. P value < 0.05 which is significant. The prevalence of IgG type of antibodies in child bearing age women is quite high and much more in AB group (Abortion group). The prevalence of IgG of antibodies specific in Toxoplasma gondii is 33.3% in over all child bearing women which is much more in AB group (50.0%). The prevalence of Toxoplasma infection among women of child bearing is quite substantial as compared with 5% in pregnant Navajo Indians (Feldman, 1982), 22% in London Survey (Ruos and Bourne, 1972), 25% in Egypt (Fikry at al., 1980), 31.2% in Saudi Arabia (Basalamah and Sarebour, 1981) and 10% in Montreal (Vie et al., 1977).

However, this prevalence appears to be low when compared with 51.5% in Morocco (Idrissi et al., 1984), 58% in Kuwait (Al-Nakib et al., 1983) and 84% in France (Desmots and Conveur 1974b). The prevalence found
in this study was similar to one reported by Mehta (1987) and Ahmed et al. (1990). EIA was the most sensitive and specific techniques used in this study.

AB group which was comprised of the following subgroups threatened abortion group, inevitable abortion, incomplete abortion and missed abortion. Highest percentage of sero-positive cases for IgM antibodies specific to *Toxoplasma gondii* were in threatened abortion subgroup 40.0%, followed by incomplete abortion 21.4%, missed abortion 18.2% and inevitable abortion 11.1%. Sero-positive for IgG was 65% in threatened abortion subgroup, followed by incomplete abortion 50.0%, missed abortion 36.4% and inevitable abortion 33.3%. Out of all subgroups highest sero-positivity for IgM and IgG was seen in threatened abortion group.

The present study reveals that IgM type antibodies sero-positive cases decreases as age increases whereas IgG sero-positive cases increases with increase in age of women which is similar to study by Flack and Kwantes (1980). We have found out inverse correlation between IgM type of antibodies to age and direct correlation of IgG type of antibodies to age.

There have been controversial reports regarding the extent to which *Toxoplasma* infection causes abortions (Stray-Pedersen and Lorentzen Styr, 1979; McCabe and Remington, 1984; Tan and Mak, 1985).

As stated by Frenkel (1984) and Fechere et al. (1984) there seems to be no doubt that the parasite may invade the placenta and foetus and leads to spontaneous abortion if a woman acquires an acute infection during her first
few gestational weeks. Desmontes and Couvreur (1971 a,b) suggested that acute toxoplasmosis may not be a frequent cause of abortion. Different research workers gave different view regarding Toxoplasma gondii associated with abortion. Our results lend support to the hypothesis that Toxoplasma infection plays an important role in abortion.

All the humans sera were screened for HIV by EIA and non were found reactive for HIV, which indicates that Toxoplasma infection was not opportunistic infection due to immuno suppressive caused by HIV infection.

Serological study in mice sacrificed on 3rd or 4th day gave no sero-positive cases for IgM and IgG type of antibodies. whereas mice sacrificed on or about 3rd week there were 02 (3.7%) sero-positive for IgM and IgG out of 54 (AB 4 and AB 6). Sero-positivity was not seen in other group. Case number AB 4 and AB 6 were sero-positive from AB groups.

Direct Microscopy (Wet mount) gave positive results in two cases of sacrificed on 3rd or 4th day. The other slides were without any parasite in both group sacrificed on 3rd or 4th day and or about 3rd week.

In this study we have managed to culture Toxoplasma gondii artificially for the first time in different media but the best possible results were in monophasic TC-199 with salt and without antibiotics. Artificial media growth helped us in studying Toxoplasma in wet mount preparation, which reveals that Toxoplasma is quite a mobile parasite which move in different directions. Wet mount preparation was recorded on video film and motions of Toxoplasma was re-studied. Doubling time was also noted, which was found 4 days in present study.
Different stained preparations were made from artificial media but the best results were by alkaline methylene blue and Giemsa stain with other stains we only managed to give color to *Toxoplasma* without nuclear markings, which proves that the alkaline methylene blue and Giemsa are the best stain for *Toxoplasma gondii*.

Scanning electron microscopy was also done from artificial culture media gave us excellent results in different magnification which are present in photomicrograph section. Electron microscopy transmission was also performed which revealed ultrastructures of *Toxoplasma gondii* such as nucleus, nuclear membrane, nucleolus, attenuated upper end, golgi body, globular lower end, coniod, rhoptries, vacuole, mitochondria, membrane, endoplasmic reticulum and toxoneme etc.

In this study *Toxoplasma gondii* was kept in artificial culture media in large bottle (with sufficient amount of media) for about 02 years unchanged at 4-8°C. The slides were made off and on from this bottle for wet mount and stained preparation have shown Toxoplasma gondii.

Wet Mount preparation is ideal for observing parasites movements. Best media for obtaining maximum growth of *Toxoplasma gondii* was monophasic TC-199 medium with salt & without antibiotics. The slides were made off and on from this bottle for wet mount and stained preparation have shown *Toxoplasma gondii*. Wet mount preparation is ideal for observing parasite movements.

Histopathological study did not reveal any special finding to be mentioned here.
CONCLUSIONS AND RECOMMENDATIONS
I have drawn following conclusions from present study:

1. *Toxoplasma gondii* is quite prevalent in our population in women of child bearing age.

2. IgM sero-positivity decreases with increase in age (inverse correlation).

3. IgG sero-positivity increases with increase in age (positive correlation).

4. Results of EIA techniques are highly sensitive and specific, which can be used as serodiagnostic tool as a routine.

5. Direct microscopy with wet mount and stained preparations will help in diagnosis, which are comparatively cheap and easy technique should be adopted as a routine.

6. Artificial culture of *Toxoplasma gondii* can be done with recomended technique.

7. Artificial culture of *Toxoplasma* helped us in studying parasites in greater detail and also provide us chance to do electron microscopy (Transmission and scanning).

8. It can be stained with other stains too.

9. Three forms of *Toxoplasma gondii* has been observed large (Gaint form), medium (Intermediate) and small (Dwarf).
10. Morbidity was observed in *Toxoplasma gondii*, which was more in small (dwarf) form as compared with medium (intermediate) and large (Gaint) forms.

11. Histopathology of different specimen taken from mice did not reveal any thing significant to report.

12. Screening of women of child bearing age is advisable. It is also advisable to perform direct microscopy of product of conception in aborting females.

It is recommended that a large sample size study should be conducted with financial assistance from agencies to artificial culture and to perform PCR on *Toxoplasma gondii*.

It is also advisable to move in direction of preparing vaccine against *Toxoplasma gondii* to overcome serious consequences caused by *Toxoplasma gondii* to mothers and their babies.
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APPENDICES
APPENDIX I

DEPARTMENT OF PATHOLOGY
DOW MEDICAL COLLEGE AND
DEPARTMENT OF MICROBIOLOGY
BASIC MEDICAL SCIENCES INSTITUTE
JINNAH POSTGRADUATE MEDICAL CENTRE
KARACHI

CASE NO..........................DATE OF SAMPLE

COLLECTION..........................

HOSPITAL..........................WARD NO..............BED

NO..................CR.NO.......... 

NAME..............................W/O........................SEX........AGE...........

ADDRESS.................................................. 

OCCUPATION.............................................. 

GRAVIDA....................................................

HISTORY OF ABORTION

1.Threatened..............................

2.Inevitable..............................

3.Incomplete............................

4.Missed...................................

5.Therapeutic............................

DESCRIPTION.................................

ANY OTHER INFORMATION...........................

SPECIMEN REQUIRED         BLOOD 5 c.c.

PRODUCT OF CONCEPTION       IN STERILE CONTAINER
APPENDIX II

COLLECTION AND STORAGE OF BLOOD SAMPLES:

The blood samples were collected from suitable superficial vein of upper limb after taking all aseptic measures. The blood was allowed to clot in small sterile test tube. After that serum was separated from blood by centrifuging at 3000 G for 10 - 15 minutes and stored in a small plastic vials with a covering lid at -20°C. The above procedure was performed near flame so as to avoid airborne contamination of serum and in shortest possible time.

Plasma samples were not used for test because they may give agglutination. In case testing of occupational plasma sample was unavoidable they were pretreated by following methods:

a) Method No. 1

1) Make 2.7% solution of calcium chloride in water.
2) Take 0.9 ml of plasma add 0.1 ml of 2.7% calcium chloride.
3) Keep at 35°C for two hours in an incubator.
4) Centrifuge at 1000 G for 10 minutes.
5) Remove serum from clot.

b) Method No. 2

1) Prepare 10 units per ml solution of thrombin in water.
2) Take 1 ml of plasma and add 0.05 ml of thrombin solution.
3) Keep it for 30 minutes at room temperature.
4) Centrifuge it at 1000 G at least for 10 minutes.
5) Serum is removed from clot.

PRODUCT OF CONCEPTION:

Product of conception was collected with all aseptic measure in a sterile container.
APPENDIX III

POLYCHROME LOEFFLER METHYLENE BLUE STAIN:

To make about 130 ml:

Methylene Blue approx. 0.5 gm
Ethanol (ethyl alcohol) absolute 30 ml
Potassium Hydroxide 200 g/l (20% w/b) 0.1 ml
Distilled water 100 ml

1. Methylene blue was weighed on a piece of a clean paper (preweighed) dissolved in about 30 ml of the water. Stain was made saturated by adding more stain.

2. Stain was transferred to a clean brown bottle.

3. Alcohol and potassium hydroxide solution was added to stain.

Caution: The potassium hydroxide solution is highly corrosive, therefore handle with care. Do not mouth pipette. Ethanol is highly flammable, therefore use well away from an open fire.

4. Label the bottle and store in a dark place at room temperature. The stain is stable for several months.

For use: Transfer a small amount of the stain to a brown bottle with a cap into which a dropper can be inserted.

Method:

1. Smear was fixed with alcohol and dried.

2. It was covered with stain for 1 minutes.

3. Slide was washed with clean or tap water.

4. Slide was air dried placing in draining rack.

5. Smear was examined under microscope in different magnifications for parasite.

Results:

Cells.........................................................................................Blue (Light)
Nuclei.......................................................................................Blue (Dark)
APPENDIX IV

GIEMSA STAIN

The composition of the stain is the same as that used to stain blood parasites.

To make about 500 ml:

Giemsa powder.................................................................3.8 gm
Glycerol (glycerin)............................................................250 ml
Methanol (methyl alcohol)............................................................250 ml

1. Weigh the Giemsa on a piece of clean paper (preweighed), and transfer to a dry brown bottle of 500 ml capacity which contains a few glass beads.

Note: Giemsa stain will be spoiled if water enters the stock solution during its preparation or storage.

2. Using a dry cylinder, the methanol and add to the stain, mix well.

Caution: Methanol is toxic and highly flammable, therefore handle with care and use well away from an open flame.

3. Glycerol was measured in cylinder and added to the stain. It was mixed well.

4. Bottle of stain was placed in a water bath at 50-60°C, or if not available at 37°C, for up to two hours to help the stain to dissolve (mix well at intervals).

5. It was labeled and marked flammable and toxic, then stored at room temperature in the dark and kept well stoppered, (stain is stable for several months).

For use: Filter a small amount of the stain into a stain dispensing container (Make working solution in 1:10 or 1:25 dilution as required).

Method:

1. The film was first fixed with pure methyl alcohol or ethyl alcohol for 3 to 5 minutes and allowed to dry.
2. Giemsa's stain was diluted by adding 1 drop to each 1ml of distilled water, neutral or faintly alkaline (pH 7-7.2).

3. The diluted stain was poured over the film (about 5 ml per film if required) and kept for 30 to 45 minutes.

4. The slide was then flushed in a gentle flow of tap water, after which it was placed in an upright position with a film side inwards to drain and dry.

5. The stained film was examined under 1/12 inch oil immersion lens.
APPENDIX V

Carbol fuchsin stain:

To make about 1100 ml:

Basic fuchsin ................................................................. 10 gm
Ethanol of methanol, absolute ........................................ 100 ml
Phenol ........................................................................... 50 gm
Distilled water ................................................................ 1 liter

1. Weigh the basic fuchsin on a piece of clean paper (preweighed), and transfer to a bottle of at least 1.5 litre capacity.

2. Measure the ethonal (ethyl alcohol) or methanol (methyl alcohol), and add to the bottle. Mix at intervals until the basic fuchsin was fully dissolved.

Caution: Methonal and ethonal are highly flammable, therefore use away from an open flame.

3. With great care, weigh the phenol in a beaker. Measure the water, and add some of it to the beaker to dissolve the phenol. Transfer to the bottle of stains, and mix well.

Caution: Phenol is a highly corrosive, toxic, hygroscopic chemical, therefore handle with great care. To avoid spilling phenol on the balance pan, remove the beaker when adding or subtracting the chemical.

4. Add the remainder of the water, and mix well.

5. Label and store at room temperature. The stain is stable indefinitely and was used as and when required.

For use: Filter a small amount of the stain in to a dropper bottle of other suitable stain dispensing container.

Method:

1. Make the smear and let it dry in the air.

2. Put carbol fuchsin over smear for 2 minutes.
3. Wash with distilled water.

4. Leave it at water drainer to dry and then watch under microscope.
APPENDIX VI

Safranin stain:

To make 100 ml:

Safranin grinded ................................................................. 0.5 gm
Alcohol ................................................................. 10 ml
Distilled water ................................................................. upto 100 ml

Method:

1. Make the smear and let it dry in the air fix it with alcohol.

2. Put safranin over smear for 2 minutes.

3. Wash with distilled water.

4. Leave it at water drainer to dry and then watch under microscope
APPENDIX VII

WET MOUNT:

1. One drop from artificial medium/\textit{conceptus} was poured on slide.

2. It was covered with coverslip.

3. It was watched under microscope for parasite.

4. It was sealed from side to watch activity for longer time.

5. Video film was also made from wet mount.
APPENDIX VIII

A SOLID-PHASE ENZYME IMMUNOASSAY FOR THE DETERMINATION OF IgM-CLASS
ANTIBODIES TO TOXOPLASMA GONDII IN HUMAN SERUM

KIT CONTENTS AND REAGENTS PREPARATION:

* Reagents was stored between +2°C and +8°C.
* The expiry date was printed on each component and on the package.
* Avoid unnecessary exposure to light. This was merely a precaution. The only sensitive reagents
  was the substrate tablets (pNPP), which was packaged in brown glass vial for protection.
* Once opened, the components must be sealed tightly e.g. with parafilm or tape.

ADDITIONAL MATERIAL REQUIRED:

* Distilled or deionized water, preferably sterile.
* 1 M NaOH (4 gm NaOH in 100 ml of distilled water).
* Graduated cylinders, up to 50 ml, for reagent dilution.
* Test tubes, 5 ml, for specimen dilutions.
* Tubes or bottles to store the diluted and reconstituted reagents.
* Micropipettes of different sizes, 10 μl, 100 μl, 1000 μl and 5000 μl.
* Paper towels or absorbent papers.
* Timer, 60 minutes range.
* Incubator, plus 37°C.
* Photometer (plate or microstrip reader), 405 ml, Uniscan IL

1. TOXOPLASMA GONDII COATED MICROSTRIPS

(Inactivated parasites from mouse peritoneal fluid)

Contains:

Microstrips coated with inactivated parasites from mouse peritoneal fluid.
Preparation:

Ready for use.

Note:

Deterioration was indicated by significant decrease in the absorbance levels of controls.

2 SAMPLE DILUENT

Contents:

Phosphate buffered saline, pH 7.4 ± 0.2, with espcial ingredients and 0.1% sodium azide as preservative, 4 X concentrated.

Preparation:

Dilute the amount needed 1:3 (1:4) with distilled water, preferably sterile. Add protein solution (see 9) to make a 5% solution, example 0.5 ml protein solution in 9.5 ml sample diluent. Mix well and prewarm to +35°C before use.

Storage:

Discard unused buffer containing protein solution.

Stability:

The diluted buffer without protein solution was stable for 1 month as refrigerated. Signs of deterioration were microbial contamination, a change in pH or the presence of particulate matter.

Note:

Salt crystals may form in the diluent concentrate when kept at refrigerated temperatures. If necessary, redissolve the salt crystals by warming the solution to +37°C. During redissolving the concentrate may turn opalescent. This did not indicate microbial contamination.

3a NEGATIVE CONTROL SERUM (HUMAN):

Contents:

Human serum with 0.1% sodium azide as preservative.
Preparation:

Ready for use. Prewarm the amount needed to +37°C before use.

Storage:

Once opened the control should be divided into aliquots and stored frozen at -20°C or below. Repeated freezing and thawing is not recommended.

Stability:

Divide into aliquots and keep frozen is stable for 3 months.

3b. **Positive Control Serum (Human):**

Contents:

Human serum with 0.1% sodium azide as preservative.

Preparation:

Ready for use. Prewarm the amount needed to +37°C before use.

Storage:

Once opened the control should be divided into aliquots and stored frozen at -20°C or below. Repeated freezing and thawing is not recommended.

Stability:

Divided into aliquots and keep frozen was stable for 3 months.

4. **Conjugate Diluent**

Contents:

Phosphate buffered saline pH 7.4±0.2, special ingredients and 0.1% sodium azide as preservative.

Preparation:

Conjugate diluent was ready for use after adding protein solution. Add protein solution to make a 5% solution e.g. 0.5 ml protein solution + 5.5 ml conjugate diluent. Only the amount needed was prepared. Mixed well and prewarm to +37°C before use.
Storage:
Discard unused buffer containing protein solution.

Stability:
The buffer without protein solution was stable for 1 month as refrigerated after opening the vial.

Notes:
Signs of deterioration were microbial contamination, a change in pH or the presence of particulate matter.

5. ANTI-HUMAN IgM-AP CONJUGATE (RABBIT)

Contents:
Concentrate of alkaline phosphate conjugated anti-human IgM, 0.1% sodium azide as preservative.

Preparation:
Prepare only the volume needed for one test run just prior to use. Mix the concentrate well by turning the vial upside down a few times. Dilute carefully 1+9 (1:10) with prewarmed (+37°C) conjugate diluent.

Storage:
Discard unused diluted conjugate solution.

6. SUBSTRATE DILUENT

Contents:
1.01 M diethanolamine (DEA) and 0.505 mM MgCl₂, pH 9.9±0.2.

Preparation:
Ready for use. Prewarm the amount needed to +37°C before use.
Note:

Signs of deterioration were microbial contamination, a change in pH or the presence of particulate matter.

7. **pNPP-SUBSTRATE**

**Contents:**

5 mg para-Nitrophenyl phosphate (pNPP) in one tablet.

**Preparation:**

Ready for use in substrate solution.

**SUBSTRATE SOLUTION:**

Reconstitute 1 tablets in 2.5 ml prewarmed (+37°C) substrate diluent about 10 minutes before use in assay.

**Storage:**

Discard unused reconstituted substrate solution.

8. **TWEEN 20, WASHING SOLUTION**

**Contents:**

Concentrate of tween 20, 0.1% sodium azide as preservative.

**Preparation:**

Dilute 1+499 (1:500) with distilled water, preferably sterile.

**Storage:**

Store the diluted and refrigerated for one week. Signs of deterioration are microbial contamination, or the presence of particulate matter.

9. **PROTEIN SOLUTION**

**Contents:**

Protein solution with 0.1% sodium azide as preservative.
Preparation:

Ready for use.

Note:

Mix the contents well before pipetting.

INCUBATION COVERS

Plastic sheets to cover the microstrips during incubation.
APPENDIX IX

A SOLID-PHASE ENZYME IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF
IgG-CLASS ANTIBODIES TO TOXOPLASMA GONDII IN HUMAN SERUM AND MICE SERUM

KIT CONTENTS AND REAGENT PREPARATION:

- Reagents were stored between +2°C and +8°C.
- The expiry date was printed on each component and on the package.
- Avoid unnecessary exposure to light. This was merely as precaution. The only sensitive reagents were the substrate tablets (pNPP), were packed in brown glass vials for protection.
- Once opened, the components must be sealed tightly e.g. with parafilm or tape.

ADDITIONAL MATERIALS REQUIRED:

- Distilled or deionized water, preferably sterile.
- 1 M NaOH (4 gm NaOH in 100 ml distilled water).
- Graduated cylinders, up to 50 ml, for reagent dilutions.
- Test tubes, 5 ml, for specimen diluted and reconstituted reagents.
- Micropipettes of different sizes 10 ul, 100 ul, 1000 ul and 5000 ul.
- Paper towels or absorbent paper.
- Timer, 60 minutes range.
- Incubator, +37°C.
- Photometer (plate or microstrip reader), 405 nm, Uniscan IL

1. **TOXOPLASMA GONDII COATED MICROSTRIPS (96 WELLS)**:

   Contents:

   Microstrips coated with inactivated parasites from mouse peritoneal fluid.

   Preparation:

   Ready to use.
Note:

Deterioration was indicated by a significant decrease in the absorbance levels of controls.

2. IgG-SAMPLES DILUENT

Contents:

Phosphate buffered saline, pH 7.4± 0.2, with special ingredients and 0.1% sodium azide as preservative, 4x concentrated.

Preparation:

Dilute the amount needed 1+3 (1:4) with distilled water, preferably sterile. Mix well and prewarm to +37°C before use.

Storage:

The constituted buffer at +2°C to +8°C.

Stability:

As reconstituted and refrigerated for 1 month. Signs of deterioration were a change in pH, microbial contamination and the presence of particulate matter.

Note:

Salt crystals may form in the diluent concentrate when kept at refrigerated temperatures. If necessary redissolve the salt crystals by warming the buffer solution to +37°C. During redissolution the concentrate may turn opalescent. This did not indicate microbial contamination.

3a. NEGATIVE CONTROL SERUM (HUMAN):

Contents:

Human serum with 0.1% sodium azide as preservative.

Preparation:

Mix well by inverting the container.
Storage:

Once opened the control serum was divided into aliquots and stored frozen at -20°C or below. Repeated freezing and thawing not recommended.

Stability:

Divided into aliquots and frozen for 3 months.

3b. HIGH POSITIVE CONTROL SERUM (HUMAN):

Contents:

Human serum 0.1% sodium azide as preservative.

Preparation:

Mix well by inverting the container. Prewarm the amount needed to +37°C before use.

Storage:

Once opened the control serum should be divided into aliquots and stored frozen at -20°C or below. Repeated freezing and thawing is not recommended.

Stability:

Divided into aliquots and frozen for 3 months.

3c. LOW POSITIVE CONTROL SERUM (HUMAN):

Contents:

Human serum with 0.1% sodium azide as preservative.

Preparation:

Mix well by inverting the container. Prewarm the amount needed to +37°C before use.

Storage:

Once opened the control serum was divided into aliquots and stored frozen at -20°C or below. Repeated freezing and thawing is not recommended.
Stability:

Divided into aliquots and frozen for 3 months.

4 IgG-CONJUGATE DILUENT:

Contents:

Phosphate buffered saline pH 7.4 ± 0.2, special ingredients and 0.1% sodium azide as preservative.

Preparation:

Ready for use. Prewarm the amount needed to +37°C before use.

Notes:

Signs of deterioration were a change of pH, microbial contamination and the presence of particulate matter.

5 ANTI-HUMAN IgG-AP CONJUGATE (SHEEP):

Contents:

Concentrate of alkaline phosphate conjugated anti-human IgG, 0.1% sodium azide as preservative.

Preparation:

Prepare only the volume needed for once test run just prior to use. Mix the concentrate well by turning the vial upside down a few times. Dilute carefully 1+9 (1:10) with prewarmed (=37°C) conjugate diluent.

Storage:

Discard unused diluted conjugate solution.

6. SUBSTRATE DILUENT:

Contents:

1.01 M diethanolamine (DEA) and 0.505 mM MgCl2, pH 9.9±0.2.
Preparation:

Ready for use. Prewarm the amount needed to +37°C before use.

Note:

Signs of deterioration were a change of pH, microbial contamination and the presence of particulate matter.

7. pNPP-SUBSTRATE

Contents:

5 mg para-nitrophenyl phosphate (pNPP) in one tablet.

Preparation:

Ready for use to substrate solution.

SUBSTRATE SOLUTION:

Reconstitute 1 tablet in 2.5 ml prewarmed (+37°C) substrate diluent about 10 minutes before use in assay.

Storage:

Discard unused reconstituted substrate solution.

8. TWEEN 20, WASHING SOLUTION

Contents:

Concentrate of Tween 20, 0.1% sodium azide as preservative.

Preparation:

Dilute 1 ml 499 ml (1:500) with distilled water, preferably sterile.

Storage:

Store the diluted Tween 20 between +2°C and +8°C.

Stability:

After dilution it was one week in refrigerator. Signs of deterioration were microbial contamination and the presence of particulate matter.
INCUBATION COVERS

Microstrips were covered with plastic sheets during incubation.
APPENDIX X

IgM CONFIRMATORY KIT

REAGENTS:

Pretreatment Solution:
Preparation: The reagent provided in the Labsystems IgM confirmatory kit was ready for use. Mix well and prewarm the amount needed to 37°C before use in the assay.

Note: Store the solution between +2°C and +8°C.

Avoid unnecessary exposure to light.

Contents:

Phosphate buffered saline, pH 7.4±0.2, protein content 2.7% with 0.1% sodium azide as preservative.

ADDITIONAL MATERIAL REQUIRED:

a. Micropipettes:

10 μl, 40-200 μl and 1000 μl.

b. Mixer for pretreatment solution tubes.

c. Test tubes with caps, 1-3 ml and 5-10 ml.
APPENDIX XI

HUMAN IMMUNODEFICIENCY VIRUS (HIV) BY ENZYME IMMUNOASSAY (EIA):

Kit's Contents and Preparation:

1. HIV COATED MICROSTRIPS

Contents: Microstrips coated with detergent inactivated HIV antigen.

Preparation: Ready for use.

Note: Deterioration was indicated by a significant decrease in the absorbance level of positive controls and/or increase in the absorbance level of negative control.

2. SAMPLE DILUENT

Contents: The concentrate contains phosphate buffered saline, pH 7.4±0.2, with special ingredients and 0.1% sodium azide as preservative.

Preparation: Dilute the amount need 1+3 (1:4) with distilled water preferably sterile, and add protein solution (reagent 9) to make a 10% solution, e.g. 2.25 ml diluent buffer +6.75 ml distilled water + 1.0 ml protein solution. Mix well and prewarm to +37°C before use in the manual assay.

Storage: Discard unused buffer which contains protein solution.

Stability: Stable as diluted and without protein solution at +2°C to +8°C for 1 month. Signs of deterioration were a change in pH, microbial contamination and the presence of particulate matter.

Note: Salt crystals were from in the sample diluent buffer concentrate when kept at refrigerated temperatures. If necessary, re-dissolve the salt crystals by warming the buffer solution to +37°C. During re-dissolution the concentrate may turn opalescent. This did not indicate microbial contamination.
3a. HIV NEGATIVE CONTROL SERUM (HUMAN, INACTIVATED) 2.0 ml

Contents: Detergent inactivated HIV negative human serum with 0.1% sodium azide as preservative.

Preparation: Ready for use. Prewarm the amount needed to +37°C before use in the manual assay.

Stability: Once opened, stable as refrigerated at +2°C to +8°C for 1 month.

3b. HIV POSITIVE CONTROL SERUM (HUMAN INACTIVATED) 2.0 ml

Contents: Detergent inactivated HIV positive human serum with 0.1% sodium azide as preservative.

Preparation: Ready for use. Prewarm the amount needed to +37°C before use in the manual assay.

Stability: Once opened, stable as refrigerated at +2°C to +8°C for 1 month.

4. CONJUGATE DILUENT 50 ml

Contents: Phosphate buffered saline, pH 7.4±0.2, with special ingredients and 0.1% sodium azide as preservative.

Preparation: Ready for use. Prewarm the amount needed to +37°C before use in the manual assay.

Note: Signs of deterioration were a change in pH, microbial contamination and the presence of particulate matter.

5. ANTI-HUMAN IgG/IgM-AP CONJUGATE (SWINE/RABBIT) 5.0 ml concentrate

Contents: Concentrate of alkaline phosphate-conjugated anti-human IgG/IgM, 0.1% sodium azide as preservative.

Preparation: Mix the concentrate well by turning the vial upside down a few times. Prepare only the volume needed for one test run.
MANUAL ASSAY:

Dilute carefully the amount needed 1+9 (1:10) with prewarmed (+37°C) conjugate diluent about 10 minutes before use in assay.

Storage: Discard unused diluted conjugate solution.

6 SUBSTRATE DILUENT

30 ml

Contents: 1.01 M diethanolamine (DEA) and 0.505 mM MgCl₂, pH 10.0 ± 0.2.

Preparation: Ready for use. Prewarmed the amount needed to +37°C before use in the manual assay.

Note: Science of deterioration were a change in pH, microbial contamination and the presence of particulate matter.

7. pNPP-SUBSTRATE

Contents: 5 mg para-Nitrophenyl phosphate (pNPP) in one tablet.

Preparation: Ready for use in substrate solution.

SUBSTRATE SOLUTION

12 Tablets

MANUAL ASSAY:

Reconstitute one tablet in 2.5 ml prewarmed substrate diluent (+37°C) within 10 minutes of use.

Storage: Discard unused reconstituted substrate solution.

8. TWEEN20, WASHING SOLUTION

5 ml concentrate

Contents: Concentrate of Tween 20, 0.1% sodium azide as preservative.

Preparation: Dilute 1+99 (1:500) with distilled water, preferably sterile.

Storage: The diluted Tween 20 between +2°C and +8°C.

Stability: Stable as diluted and refrigerated for 1 week. Signs of deterioration were microbial contamination and the presence of particulate matter.
9. **PROTEIN SOLUTION**  
2 x 10 ml

**Contents:** Protein solution and 0.1% sodium azide as preservative.

**Preparation:** Ready for use in sample diluent buffer.

**Note:** Mix the contents well before pipetting.

**INCUBATION COVERS (FOR MANUAL ASSAY)**  
2 pcs

Plastic foils to cover the Microstrips during incubation.
HISTOLOGICAL PREPARATION:

Tissue (3-5 mm in dimension)

Fixation (formalin for 24 hours)

Dehydration (1 hour 30%, 50%, 70%, 90% soluble alcohol)

Cleaning (1 hour each in xylol, I and xylol II)

Infiltration and embedding (1/2 to 1 hour each in 50:50 xylol wax, wax I, wax II at 60°C in incubator)

Blocks

Sectioning (after obtaining the section on slide leave for 24 hours)

Staining (H & E)

Slide (2 min each in xylol I and II)

Keep for 1 minute each in descending grades of (absolute 90%, 70%, 50%, 30%) alcohol.

Dip in water

Pour haematoxylin in (3-5 minutes)

Wash in water

Overstained (give dips
acid water 5% HCl) → → Stained → Understained (give dips
of alkaline water, Na2HCO3)

Put Eosin 1% (2-3 minutes) wash with water

Keep for 1 minute each in ascending grades of alcohol
(50%, 70%, 90% Absolute alcohol)

3-5 minutes in xylol

Mount in DPX
APPENDIX XIII

HAEMOTOXYLIN AND EOSIN STAINS:

Composition:

Two solutions are prepared (Harris)

Solution I:

Haematoxylin 1 gm
Alcohol 90% 10 ml

Solution II:

Potash alum 20 gms
Distilled water 200 ml

The two solutions are mixed and to this are added:

- Mercuric oxide 0.5 gms
- Glacial acetic acid 10 ml

Boiling was done for 2 hours followed by rapid cooling.

Haematoxylin stains the nucleus blue. Depending upon the chemicals used for solution, haematoxylin can be of various types such as

- Harris
- Ehrlich's
- Mayer's

EOSIN STAINS:

Eosin stains the cytoplasm red. It is a synthetic dye used as 1% aqueous or alcoholic solution.
APPENDIX XIV

TC MEDIUM NO. 199 w/o BICARBONATE

TC Medium No. 199 w/o Bicarbonate is the same as TC Medium No. 199 except carbonate has not been added. It is prepared in 1 liter bottles. When this modified medium is to be used the reaction is adjusted by the addition of sterile sodium bicarbonate in the following manner: Adjust the temperature of the medium to 37°C. Filter sterilize a 10% solution of Sodium Bicarbonate (reagent grade) in triple distilled water, and add with agitation 3.5 ml to each liter. The final reaction of the medium will be pH 7.2-7.4 producing an orange red colour (noterise).

<table>
<thead>
<tr>
<th>Ingredients Per Liter</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>l-Arginine</td>
<td>70.0 mg</td>
</tr>
<tr>
<td>l-Histidine</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>l-Lysine</td>
<td>70.0 mg</td>
</tr>
<tr>
<td>l-Tyrosine</td>
<td>40.0 mg</td>
</tr>
<tr>
<td>dl-Tryptophane</td>
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</tr>
<tr>
<td>dl-Phenylalanine</td>
<td>50.0 mg</td>
</tr>
<tr>
<td>l-Cystine</td>
<td>20.0 mg</td>
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<tr>
<td>dl-Methionine</td>
<td>30.0 mg</td>
</tr>
<tr>
<td>dl-Serine</td>
<td>50.0 mg</td>
</tr>
<tr>
<td>dl-Threonine</td>
<td>60.0 mg</td>
</tr>
<tr>
<td>dl-Leucine</td>
<td>120.0 mg</td>
</tr>
<tr>
<td>dl-Isoleucine</td>
<td>40.0 mg</td>
</tr>
<tr>
<td>dl-Valine</td>
<td>50.0 mg</td>
</tr>
<tr>
<td>dl-Glutamic Acid</td>
<td>150.0 mg</td>
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<tr>
<td>dl-Aspartic Acid</td>
<td>60.0 mg</td>
</tr>
<tr>
<td>l-Alanine</td>
<td>50.0 mg</td>
</tr>
<tr>
<td>l-Proline</td>
<td>40.0 mg</td>
</tr>
<tr>
<td>l-Hydroxyproline</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Cysteine</td>
<td>0.10 mg</td>
</tr>
<tr>
<td>Adenine</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Guanine</td>
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</tr>
<tr>
<td>Xanthine</td>
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</tr>
<tr>
<td>Hypoxanthine</td>
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</tr>
<tr>
<td>Thymin</td>
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<td>Uracil</td>
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</tr>
<tr>
<td>Thiamin</td>
<td>0.010 mg</td>
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<tr>
<td>Riboflavin</td>
<td>0.010 mg</td>
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<tr>
<td>Pyridoxine</td>
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<tr>
<td>Pyridoxal</td>
<td>0.025 mg</td>
</tr>
<tr>
<td>Niacin</td>
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<tr>
<td>Niacinamide</td>
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<td>Pantothenate</td>
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<tr>
<td>Biotin</td>
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<td>Folic Acid</td>
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<td>p-Aminobenzoic Acid</td>
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<tr>
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<tr>
<td>(as ferrocitrate)</td>
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<td>Tween 80 (oleic acid)</td>
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<tr>
<td>Calcium Chloride</td>
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<td>Magnesium Sulfate</td>
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<td>Disodium Phosphate</td>
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<td>Monopotassium phosphate</td>
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<td>Bacto-Dextrose</td>
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<tr>
<td>Bacto Dextrose</td>
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<tr>
<td>Bacto Phenol Red</td>
<td>0.02 g</td>
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<tr>
<td>Carbon Dioxide</td>
<td>to pH 7.2</td>
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</tbody>
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APPENDIX XV

TC MEDIUM NO. 199 w/o BICARBONATE WITH ANTIBIOTICS

TC Medium No. 199 w/o Bicarbonate is the same as TC Medium No. 199 except carbonate has not been added. It is prepared in 1 liter bottles. When this modified medium is to be used the reaction is adjusted by the addition of sterile sodium bicarbonate in the following manner: Adjust the temperature of the medium to 37°C. Filter sterilize a 10% solution of Sodium Bicarbonate (reagent grade) in triple distilled water, and add with agitation 3.5 ml to each liter. The final reaction of the medium will be pH 7.2-7.4 producing an orange red colour (not cerise).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>70.0 mg</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>70.0 mg</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>40.0 mg</td>
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<tr>
<td>l-L-Tryptophane</td>
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<td>l-Phenylalanine</td>
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<tr>
<td>l-Cysteine</td>
<td>20.0 mg</td>
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<tr>
<td>l-Methionine</td>
<td>30.0 mg</td>
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<tr>
<td>l-Serine</td>
<td>50.0 mg</td>
</tr>
<tr>
<td>l-Threonine</td>
<td>60.0 mg</td>
</tr>
<tr>
<td>l-Leucine</td>
<td>120.0 mg</td>
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<tr>
<td>l-Isoleucine</td>
<td>40.0 mg</td>
</tr>
<tr>
<td>l-Valine</td>
<td>50.0 mg</td>
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<tr>
<td>dl-Glutamic Acid</td>
<td>150.0 mg</td>
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<tr>
<td>dl-Aspartic Acid</td>
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<tr>
<td>l-Alanine</td>
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<tr>
<td>l-Proline</td>
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<tr>
<td>l-Hydroxyproline</td>
<td>10.0 mg</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Oxytetracycline</td>
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<td>Adenine</td>
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<tr>
<td>Guanine</td>
<td>0.30 mg</td>
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<tr>
<td>Xanthine</td>
<td>0.30 mg</td>
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<tr>
<td>Hypoxanthine</td>
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<tr>
<td>Thymine</td>
<td>0.30 mg</td>
</tr>
<tr>
<td>Ursulose</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>Thiamin</td>
<td>0.010 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.010 mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.025 mg</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>0.025 mg</td>
</tr>
<tr>
<td>Nicin</td>
<td>0.025 mg</td>
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<tr>
<td>Penicillin</td>
<td>10^4 IU/ml Streptomycin</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>0.025 mg</td>
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APPENDIX XVI

TC SALT SOLUTION, HANKS

TC Salt Solution, Hanks, is a sterile balanced salt solution certified for use in tissue culture procedures, prepared according to the formula of Hanks, and has the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>0.14 g</td>
</tr>
<tr>
<td>Magnesium Sulfate (MgSO₄·7H₂O)</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl₂·6H₂O)</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Disodium Phosphate (Na₂HPO₄·2H₂O)</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Monopotassium Phosphate</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Triple Distilled Water</td>
<td>1000.00 ml</td>
</tr>
</tbody>
</table>

TC Salt Solution, Hanks, has a reaction of pH 7.0. It is supplied in 100 ml containers, singly or in packages of six.
APPENDIX XVII

10% BLOOD AGAR SLANTS PREPARATION

Blood Agar Base

FORMULA

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>grams per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Lab-Lamco', Powder</td>
<td>10</td>
</tr>
<tr>
<td>Peptone</td>
<td>10</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

pH 7.3 ± 0.2

DIRECTIONS

Powder: Suspend 40 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Tablets: Add 1 tablet to 5 ml of distilled water and soak for 5 minutes. Sterilize by autoclaving for 15 minutes at 121°C.

For blood agar, cool the base to 50°C and add 10% of Defibrinated Sheep Blood SR50. Mix with gentle rotation and pour into screw capped tube or other containers.
APPENDIX XVIII

M-MEDIUM:

Human ascitic fluid (sterilized) .................................................. 50.0 ml
Boric acid ................................................................................. 3.0 gm
Sodium chloride ........................................................................ 7.0 gm
Normal sodium hydroxide ........................................................ 24.0 ml
Bovine albumin 22% ................................................................. 8.0 ml
Sodium azide .............................................................................. 1.0 gm
Distilled water water ............................................................... 1000.0 ml
pH ....................................................................................... 8.5 to 9.0
APPENDIX XIX

TRYPAN BLUE EXCLUSION METHOD FOR VIVABLE CELL COUNT

Certain dyes stain only dead cells, while viable cells exclude the dye and remain unstained. Trypan blue is the dye generally used for determination of viable cell counts. One volume of 1% trypan blue solution in Hanks BSS is added to 9 volumes of cell suspension, and the mixture is held at room temperature for 10 minutes. The suspension is then centrifuged at 1000 rpm for 10 minutes, the supernatant fluid is removed, and the cells are resuspended in Hanks BSS to the original volume of the cell suspension. Further dilution may be required for counting if the cell suspension is very heavy. The cells are then counted in a hemocytometer as described above, enumerating only the unstained viable cells. Both stained and unstained cells can be counted if the relative proportions of viable and nonviable cells in the preparation need to be determined.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Case</th>
<th>HIV</th>
<th>IgM</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG</th>
<th>Results</th>
<th>IgM</th>
<th>IgG</th>
<th>IgM</th>
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<th>Results</th>
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<td>11.0</td>
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<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
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**INTERPRETATION OF IgM RESULTS:**

- < 20 EIU = Negative
- 20-40 EIU = Uncertain positive
- > 40 EIU = Positive

**INTERPRETATION OF IgG RESULTS:**

- < 10 EIU = Negative
- 10-20 EIU = Uncertain positive
- 20-60 EIU = Low positive
- 60-130 EIU = Positive
- > 130 EIU = High positive
## APPENDIX AXX

### SUMMARY OF AB GROUP

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### APPENDIX VIII CONTD.

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**INTERPRETATION OF IgM RESULTS:**

- < 10 HIU = Negative
- 20-40 HIU = Uncertain positive
- > 40 HIU = Positive

**INTERPRETATION OF IgG RESULTS:**

- < 10 HIU = Negative
- 20-40 HIU = Uncertain positive
- 20-60 HIU = Low positive
- 60-130 HIU = Positive
- > 130 HIU = High positive