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# The Human Antibody Response To *Giardia intestinalis*

A thesis presented
in partial fulfilment of the requirements,

for the degree,

of Masters of Science
in Microbiology
at
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Judy Lai Peng Chan
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# Abstract

Giardiasis is usually diagnosed in the laboratory by microscopic examination of faeces for the presence of cysts and / or trophozoites. However in principle, it is possible that *Giardia* infection could be diagnosed serologically. To investigate this possibility an Enzyme Immunoassay (EIA) was developed using *Giardia*-specific mouse serum as antibody.

The efficiency of different *Giardia* antigen preparations to detect antibody in this test was investigated. The antigens included live trophozoites, frozen and thawed trophozoites, sonicated trophozoites, trophozoite membranes and cysts. Antibody titres were low and no marked differences were detected when the four different antigens were compared. However, since following a natural infection the immune response to surface proteins probably predominates, we concluded that live trophozoites or cysts represented the most appropriate antigens to use in an EIA test to detect *Giardia*-specific antibody in human serum.

Live trophozoites adsorbed to polystyrene microtitre wells were removed by the washing procedure, thus giving an insensitive test and inconsistent results. This problem was overcome by precoating the microtitre wells with poly-l-lysine following which trophozoites and cysts adhered to the wells strongly enough to resist the washing procedures. The EIA test was optimised with *Giardia*-specific mouse serum as antibody and the same system was used to detect antibody in human serum.

IgG, IgM, and IgA antibody were assayed in "current infection", "convalescent" and "negative control" human sera. IgG antibody titres were slightly elevated in "convalescent" sera as compared to the other two groups. IgM antibody titres were slightly elevated in "current infection" sera and IgA antibody levels were not found to be elevated.

Since IgM antibody is present early in an infection but does not persist, its presence or absence could, in principle, be used to distinguish a current from a previous infection of *Giardia*. However, only slightly elevated levels of *Giardia*-specific IgM antibody in "current infection" sera were detected in our tests so this approach to diagnosis will need further development if it is to be used for diagnostic purposes.

*Giardia* is not an invasive organism and it is possible that some antigens may play a major role in eliciting an immune response in humans. Thus, potential *Giardia* antigens were investigated by "immunoblotting" total *Giardia* proteins with human sera from clinically diagnosed cases of giardiasis.

It was found that the human antibody response to *Giardia* varies between individuals and many *Giardia* proteins reacted with the immune human sera. However, IgG antibody found in many of the serum samples reacted with a 200 kDa, 62 kDa and 42 kDa protein. IgA and IgM antibodies also reacted with a 62 kDa protein which may be similar to a 55 kDa structural protein (tubulin) found in *Giardia*.

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## Figures

# 1.0 Introduction.

## 1.1 The biology of *Giardia.*

The protozoa *Giardia* is a member of the order Diplomonadiae. This includes a variety of binucleate flagellate parasites typically inhabiting the intestinal tract of mammals, birds and reptiles.

The three main species, as proposed by Filice in 1952, are *Giardia intestinalis, Giardia muris* and *Giardia agilis.* This proposition is based on the morphology of its median bodies. The median body structure is unique to *Giardia* and is composed mainly of microtubules. The function of the median body is still unknown.

*Giardia intestinalis* (syn. *Giardia lamblia, Giardia duodenalis*) is found in humans, mammals and birds and has a pointed elongate median body located transversely to the cell. It is often described as "hammer claw" in shape. *Giardia muris* found in rodents, other mammals and birds, has two small round median bodies. *Giardia agilis* is found in amphibian hosts and has a teardrop shaped median body.

Kulda and Nohynkova (1978) proposed more than forty different species based on the host animal from which *Giardia* was isolated. However this system has not been widely accepted.

Erlandsen and Bemrick (1987) proposed another species, *Giardia psittaci* which was isolated from a parakeet. This differentiation was based on evidence obtained from scanning electron microscopy. Morphological differences were observed when compared with other known species. *Giardia ardeae* isolated from a great blue heron by Erlandsen *et al* (1990) appeared to be yet another new species, based on the morphological criteria and chromosomal migration patterns.

The median body of *Giardia ardeae* appears to be pleomorphic and this observation, along with the fact that the median body of *Giardia psittaci* resembles that of *Giardia muris*, led Erlandsen *et al* (1990) to conclude that the determination of *Giardia* at species level based on median body structure should no longer be considered an adequate taxonomic tool.

*Giardia intestinalis* exists in two forms in its life cycle; namely the trophozoite and the cyst. The trophozoite is binucleate, has four pairs of flagella and is bilaterally symmetrical (Filice 1952). Multiplication occurs at this stage by binary fission. The trophozoites colonise the upper two thirds of the small intestine (ie. the duodenum and jejunum) by attaching to the microvillus border with their ventral adhesive discs (Meyer and Jarroll 1980). The trophozoites detach and encyst as they travel down the intestinal tract of the host. The cyst form is subsequently excreted with the faeces.

The cyst form is the infective stage in the life cycle and infection in a new host occurs through the ingestion of *Giardia* cysts which excyst in the duodenum. The resulting trophozoite undergoes mitotic division to establish infection.

## 1.2 Pathogenicity, disease and symptoms.

The mode of pathogenicity in giardiasis is still unclear. Invasion of the mucosal and submucosal layers of the intestine by *Giardia* trophozoites are unusual in giardiasis, but damage to the microvilli does occur. Scanning electron microscopy of the intestinal epithelium from murine models for giardiasis (Erlandsen and Chase, 1974) and of human intestinal biopsies from giardiasis patients (Erlandsen and Feely, 1974) show disc shaped lesions on the microvillus border created by the ventral disc of the trophozoite. These lesions could disrupt the functional integrity of the microvillus border and cause deficiencies in dissacharidase, peptide hydrolase, enteropeptidase activity and vitamin B12 absorption (Wolfe, 1978; Burke, 1975). Mechanical obstruction to food absorption in the intestine due to the adherence of millions of trophozoites to the microvillus border has also been postulated as a disease mechanism (Wolfe 1978).

Giardiasis may range from an acute gastro-intestinal illness to being virtually asymptomatic. Symptoms vary and may include nausea, fever, abdominal cramps, anorexia, abdominal distention followed by foul fulminant diarrhoea and flatulence. Diarrhoea usually lasts for at least six weeks (Walzer *et al*, 1971; Brodsky *et al*, 1974).

Most patients recover after appropriate drug therapy although spontaneous recovery from giardiasis does occur while many individuals recover from giardiasis after appropriate drug therapy. Sometimes the condition may become chronic, lasting three years or more (Wolfe, 1978; Chester *et al*, 1985). The number of cysts excreted by patients may also vary widely. Numbers ranged from barely detectable to over ten million per gram of stool in one untreated patient (Boucher and Gillin, 1990).

## 1.3 Presence and detection of *Giardia*-specific antibody in *Giardia* infection.

Antibody responses in giardiasis are present and detectable both in sera and in other bodily fluids. The indirect immunofluorescence test and the enzyme immunoassay (EIA) are the two main methods used to investigate the nature of the *Giardia*-specific antibody response in humans (Ridley and Ridley, 1976; Radulescu *et al*, 1976; Visveswara *et al*, 1980; Table 1.).

Various researchers have reported the presence of elevated *Giardia*-specific serum IgG levels in giardiasis patients (Ridley and Ridley, 1976; Radulescu *et al*, 1976; Visveswara *et al*, 1980; Smith *et al*, 1981; Goka *et al*, 1986; Miotti *et al*, 1986; [a]Nash *et al*, 1987; Janoff *et al*, 1988) *Giardia*-specific serum IgM levels were investigated by Goka *et al* (1986), Nash *et al* (1987), and Gandhi *et al* (1989). They found *Giardia*-specific serum IgM levels to be elevated but this antibody did not persist for more than a few weeks.

There are differing reports on the presence of elevated *Giardia*-specific serum IgA antibody in *Giardia* infected individuals. Ridley and Ridley (1976) could not detect the presence of *Giardia*-specific serum IgA in giardiasis patients but Birkhead *et al* (1989), Janoff *et al* (1988), and [a]Nash *et al* (1987) have reported elevated *Giardia*-specific serum IgA levels in *Giardia* infected patients.

*Giardia*-specific secretory IgA has also been reported to be present in the breast milk of *Giardia* infected mothers (Nayak *et al*, 1987) and in the intestinal secretions of *Giardia* infected volunteers ([a]Nash *et al* 1987).

## 1.4 Diagnosis of giardiasis

There are two main techniques available for diagnosis of a *Giardia intestinalis* infection in humans. Cyst and trophozoite detection in faecal samples by microscopic examination remains the method of choice by most medical laboratories. The second option is by detecting *Giardia* antigens in the faeces of the host with a commercially available test kit. Alternative diagnostic methods, such as DNA probes, are still very much in their infancy (Butcher and Farthing, 1988).

Cyst and trophozoite detection in faecal samples is tedious, requiring microscopic examination of faecal smears and a well trained technician to detect *Giardia* cysts and possibly trophozoites in diarrhoeic stools.

The sensitivity of cyst detection in faecal samples by microscopic examination is only twenty to fifty percent in a single sample (Burke, 1977; Wolfe, 1978; Healy, 1979) or fifty to seventy percent in multiple samples (Burke, 1975; Burke, 1977; Healy, 1979).

Examination of at least three separate faecal samples are recommended before a final diagnosis is made. This is because *Giardia* cysts are shed intermittently in faeces. Examination of faecal samples on alternate days have been shown to give an increased positive yield over specimens collected on three consecutive days (Wolfe, 1984).

Two types of *Giardia*-specific antigen detection tests are available commercially. The indirect immunofluorescence test detects *Giardia* cysts present in faeces and environmental samples using monoclonal antibodies directed against the *Giardia* cyst. This test aids in the identification of *Giardia* cysts and one commercial test kit boasts of a test sensitivity of 97.4% (Merifluor™, Meridian Diagnostics Inc.).

The ProSpecT/*Giardia*® test (Alexon Biomedical Inc.) is an enzyme immunoassay available commercially. It detects a *Giardia*-specific antigen of 65 kilodaltons (kDa) in faecal samples and is reported to be 30% more sensitive than conventional microscopic detection of cysts and trophozoites in faecal smears (Rosoff *et al*, 1989).

Less common techniques include the duodenal biopsy, duodenal aspirate and the string test (Enterotest®). These procedures may be necessary if other methods give an inconclusive diagnosis.

Two methods have been used to detect cyst antigens using the EIA test. The cyst antigens are detected by either capturing the whole *Giardia* cyst or by detecting soluble *Giardia*-specific cyst antigens in the faecal sample (Ungar *et al*, 1984; [b]Nash *et al*, 1987; Stibbs *et al*, 1988; Green *et al*, 1988; Knisley *et al*, 1989; Janoff *et al*, 1989; Rosoff *et al*, 1989).

The sensitivity of the EIA capture technique ranges from eighty seven to ninety eight percent for antigen detection in faeces (Table 1.) and ninety six percent for direct EIAs detecting *Giardia*-specific antibodies in sera (Table 2.)

## 1.5 *Giardia* antigens and EIAs in research.

Two approaches have been employed in the development of enzyme immunoassays (EIA) for *Giardia* diagnosis and research. EIAs have either been used to detect *Giardia*-specific trophozoite antibodies in sera or *Giardia*-specific cyst antigens in faecal samples. The work by different researchers has been tabulated (Table 1 and 2).

*Giardia intestinalis* proteins which are potential antigens in a natural infection have been investigated by Smith *et al* (1982) and

Moore *et al* (1982). Smith *et al* (1982) used strains from four different geographical locations. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) patterns were found to be similar in the four strains and the molecular weights of the protein bands ranged between 12 kDa to 140 kDa. The electrophoretic migration patterns in the SDS-PAGE showed twenty six protein bands. Moore *et al* (1982) demonstrated a minimum of 20 distinct protein bands following SDS-PAGE separation of total *Giardia* trophozoite protein.

Most reports on the study of *Giardia intestinalis* antigens have found *Giardia* surface proteins to provoke an immune response. (Edson *et al*, 1986; Ortega Pierres *et al*, 1988; Wenman *et al*, 1986; Taylor and Wenman, 1987)

There have been two approaches to this area of antigen determination in *Giardia* research. The antigens specific to *Giardia intestinalis* have been elucidated either by using human anti-*Giardia* sera from giardiasis patients, or by using immune anti-*Giardia* sera from laboratory animals.

There seems to be a heterogeneous collection of *Giardia* proteins of different molecular weights which were reactive with Giardia-specific antibody in sera.. *Giardia* proteins of approximately 31, 55, 63 and 88 kDa appear to be the main antigens which elicited an immune response (Torian *et al*, 1984; Edson *et al*, 1986; Wenman *et al*, 1986; Taylor and Wenman, 1987; Nash *et al*, 1988; Ortega Pierres *et al*, 1988; Reiner *et al*, 1989) A survey of the work by other investigators on the identification of *Giardia*

antigens which elicited an immune response in humans and animals are shown in Tables 3 and 4.

Table 1. A survey of enzyme immunoassays developed by other investigators which used *Giardia intestinalis* cyst as antigen.

| Reference. | *Giardia* strain. | Method. | Anti-*Giardia* antibody source. | %Sensitivity/ Specificity. | Type of antigen |
|---|---|---|---|---|---|
| 1) Ungar *et al*, 1984 | Portland-1 WB | Indirect double antibody EIA. | Goat and rabbit anti-*Giardia* trophs. sera (P-1 and WB) | 92/- | Whole cyst and soluble ag. in S/N. |
| 2) [b]Nash *et al*, 1987 | GS/M and Isr WB strain | Indirect double antibody EIA. | Goat polyclonal anti-*Giardia* WB sera (cyst or trophs.?) | 94.5/- | Soluble *Giardia* ag. in faeces solution (S/N). |
| 3) Stibbs *et al*, 1988 | H-2 and H-3 | Ag. capture. | 1° antibody -mouse anti-*Giardia* cyst. 2° antibody -rabbit anti-*Giardia* cyst. | 92/- | Ag. in cell free stool eluate. |
| 4) Green *et al*, 1988 | Human strain. | Ag. capture. | Pooled rabbit anti-trophs. and cyst sera | 98/100 | Soluble *Giardia* ag. in faeces supernatant |

Abbreviations: Ag. = *Antigen*. 1° = *Primary*. 2° = *Secondary*. S/N = *Supernatant*. Trophs. = *Trophozoites*.

Table 1. A survey of enzyme immunoassays developed by other investigators which used *Giardia intestinalis* cyst as antigen. (cont.)

| Reference. | *Giardia* strain. | Method. | Anti-*Giardia* antibody source. | %Sensitivity/ Specificity. | Type of antigen |
|---|---|---|---|---|---|
| 5) Knisley *et al*, 1988 | Portland-1 | Ag. capture. | Rabbit anti-*Giardia* troph. sera | 92/87 | |
| | | | Goat anti-*Giardia* troph. sera | 87/91 | Soluble *Giardia* ag. in S/N. |
| 6) Stibbs 1989 | H-2 and H-3 | Ag. capture. | 1° antibody -mouse monoclonal. | 97/- | Cyst wall. in formalinised stool |
| | | | 2° antibody -rabbit anti-*Giardia* cyst IgG | 82/- | Cyst in non-formalinised stool. |
| 7) Janoff *et al*, 1989 | Portland-1 | Ag. capture | Rabbit anti-*Giardia* troph. sera | 94/95 | Soluble *Giardia* ag. in S/N. |
| 8) Rosoff *et al*, 1989 | ------ | ProSpecT/*Giardia*® diagnostic test. (Ag. capture) | *Giardia* specific antigen 65 kDa. (GSA 65) | 96/100 | GSA 65 in stool samples. |

Abbreviations: Ag. = *Antigen.* 1° = *Primary.* 2° = *Secondary.* S/N = *Supernatant.* Trophs. = *Trophozoites.*

Table 2. A survey of enzyme immunoassays developed by other investigators which used *Giardia intestinalis* trophozoite as antigen.

| .Reference. | *Giardia* strain. | Method. | Anti-*Giardia* antibody source. | %Sensitivity/ Specificity. | *Giardia*-specific antibody levels. |
|---|---|---|---|---|---|
| 1) Smith *et al*, 1981 | 1 Human strain | Wells coated with intact trophozoites. | Human sera from patients 2 weeks to 15 months from disease. | ------ | ↑ serum IgG in 80% of patients. |
| 2) Miotti *et al*, 1986 | Not stated | Wells coated with live trophs ($2x10^4$ per well). | Human sera from general population. | ------ | ↑ *Giardia* specific IgG in some sera. |
| 3) Goka *et al*, 1986 | Portland-1 | Wells coated with prefrozen $2x10^4$ trophs/well. | Human sera from patients < 6 weeks from onset of disease. | 96/96 | ↑ serum IgM. |

Abbreviations: Ag. = *Antigen* 1° = *Primary* 2° = *Secondary*. S/N = *Supernatant*. Trophs. = *Trophozoites*.

Table 2. A survey of enzyme immunoassays developed by other investigators which used *Giardia intestinalis* trophozoite as antigen (cont.).

| .Reference. | *Giardia* strain. | Method. | Anti-*Giardia* antibody source. | %Sensitivity/ Specificity. | *Giardia*-specific antibody leveis. |
|---|---|---|---|---|---|
| 4) [a]Nash *et al*, 1987 | GS/M and Isr (Human strains) | Wells coated with- a)$10^5$ Frozen / thawed trophozoites. | a)Human sera 14-21 days from infection. | ------ | ↑ serum IgM 100% ↑ serum IgG 70% |
| | | b)$10^6$ Frozen / thawed trophozoites. | b)Jejunal fluid. | ------ | ↑ serum IgA 60% ↑ secretory IgA 50% |
| 5) Nayak *et al*, 1987 | Not Stated | Wells coated with sonicated trophs. | Human milk from infected mothers. | ------ | ↑ secretory IgA in infected mothers. |
| 6) Janoff *et al*, 1988 | WB | Wells coated with $2x10^5$ trophs/ml. Frozen & thawed 3x. | Human sera from AIDS patients and Control sera from immunocompetent patients. | ------ | Normal levels |
| | | | | ------ | ↑ serum IgA, IgG and IgM. |

Abbreviations: Ag. = *Antigen.* 1° = *Primary.* 2° = *Secondary.* S/N = *Supernatant.* Trophs. = *Trophozoites.*

Table 2. A survey of enzyme immunoassays developed by other investigators which used *Giardia intestinalis* trophozoite as antigen (cont.).

| .Reference. | *Giardia* strain. | Method. | Anti-*Giardia* antibody source. | %Sensitivity/ Specificity. | *Giardia*-specific antibody levels. |
|---|---|---|---|---|---|
| 7) Knisley *et al*, 1988 | Portland-1 | Wells coated with sonicated trophs. | Rabbit or goat anti*Giardia* sera. | ------ | ↑ Antibody levels to *Giardia*. |
| 8) Gandhi *et al*, 1989 | Multiple human strains. | Anti-*Giardia* antibody coated . onto beads. | Human sera from patients with giardiasis for more than 2 months. | -/97% | ↑ serum IgM in 33% of cases. |
| 9) Birkhead *et al*, 1989 | WB | Wells coated with 1 µg trophozoite proteins. | Human sera from patients with giardiasis for 3 to 6 weeks. | ------ | ↑ serum IgA |

Abbreviations: Ag. = *Antigen*. 1° = *Primary*. 2° = *Secondary*. S/N = *Supernatant*. Trophs. = *Trophozoites*.

Table 3. Reaction of *Giardia intestinalis* antibodies from laboratory animals with *Giardia* proteins.

| References | *Giardia intestinalis* strain | Antigen recognised MW (kDa) | Localisation | Source of antibody | Technique employed |
|---|---|---|---|---|---|
| 1) Moore *et al*, 1982 | Not stated | High molecular weight fraction | ------ | Rabbit and human anti- sera. | SDS-PAGE EIA |
| 2) Torian *et al*, 1984 | Portland-1 ,WB RS, LT | 170, 155, 53, 55 (Tubulin) *Giardia* cytoskeleton. | ------ | Murine monoclonal to Portland-1 Rabbit anti-*Giardia* Portland-1 sera. | Immunofluorescence Immunoblot, EIA |
| 3) Edson *et al*, 1986 | Portland-1 | 88 | Surface Ag. (trophs) | Murine monoclonal. | Mouse monoclonal antibody. |
| 4) Wenman *et al*, 1986 | WB (ATCC 30957); 4 animal strains; 1 human strain | 62, 52, 38, 31. | 52 & 31 kDa. Surface Ag. of trophs. | Rabbit anti-troph. (WB strain) sera. | Immunoblot |

Abbreviations: Ag. = *Antigen*. 1° = *Primary*. 2° = *Secondary*. S/N = *Supernatant*. Trophs. = *Trophozoites*.

Table 3. Reaction of *Giardia* intestinalis antibodies from laboratory animals with *Giardia* proteins (cont.).

| References | *Giardia intestinalis* strain | Antigen recognised MW (kDa) | Localisation | Source of antibody | Technique employed |
|---|---|---|---|---|---|
| 5) Reiner *et al* 1989 | WB (ATCC 30957) | 21 to 39 kDa polydisperse complex; 66, 78, 92, 103. | Cyst Ag. | Rabbit anti-cyst sera. | Rabbit anti-cyst sera used in immunoblot. |

Abbreviations: Ag. = *Antigen.* 1° = *Primary.* 2° = *Secondary.* S/N = *Supernatant.* Trophs. = *Trophozoites.*

Table 4. Reaction of *Giardia* intestinalis antibodies from human sera with *Giardia* proteins.

| References | *Giardia intestinalis* strain. MW (kDa) | Antigen recognised | Localisation. | Technique employed. |
|---|---|---|---|---|
| 1) Edson *et al*, 1986 | Portland-1 | 88 | Surface Ag. (troph). | Immunoprecipitation test. |
| 2) Taylor and Wenman, 1987 | WB (ATCC 30957) | 31, 27, 28. | Surface Ag. (troph) "Giardin"* | Immunoblot. |
| | | 55 | *Giardia* tubulin of flagella and ventral disc. | Immunoblot. |
| 3) Orlega Pierres *et al*, 1988 | Portland-1 | 82, 63, 55. | Surface Ag. (troph) | Immunoprecipitation test. |

*(*Crossley and Holberton, 1983)*

Abbreviations: Ag. = *Antigen.* 1° = *Primary.* 2° = *Secondary.* S/N = *Supernatant.* Trophs. = *Trophozoites.*

# 2.0 Materials and Methods.

COMPONENTS OF MEDIA , BUFFERS AND REAGENTS ARE LISTED IN THE APPENDICES.

## 2.1 Retrieval of frozen cultures of trophozoites.

i) Axenic Culture Production.

One strain of *Giardia intestinalis* was used for all the experiments described in this thesis: Bris/83/HEPU/106. Source: Duodenal biopsy of an infected symptomatic child (courtesy of Dr. P.F.L. Boreham, Queensland Institute of Medical Research) where 83 is the year of recovery; HEPU is the laboratory where the strain was isolated and cultivated; 106 is the laboratory sample number.

ii) Method.

(a) The trophozoites were retrieved from cultures stored at -80°C by quickly thawing the cultures in a 37°C waterbath and transferring them to sterile Kimax™ tubes (13x100mm borosilicate screw tops).

(b) The Kimax™ tubes were immediately filled with TY1-S-33 growth media and incubated at 37°C for 15 to 25 minutes.

(c) Healthy trophozoites attached to the glass during this time.

(d)The media was discarded and filled with fresh TY1-S-33 and the Kimax™ tubes were allowed to stand for 4 hours to ensure that all the dimethyl sulphoxide (DMSO) had diffused out of the trophozoites into the media.

(e) The tubes were refilled again with TY1-S-33 and all air bubbles were carefully excluded from the tube to minimise exposure of trophozoites to oxygen.

(f) The tubes were incubated at 37°C for five days or until a monolayer of trophozoites was observed.

(g) The culture was then subcultured.

## 2.2 Subculture of *Giardia* trophozoites.

i) Method.

(a) *Giardia* trophozoites at a late log. phase of growth were subcultured by chilling the culture flask (Nunc™) in an ice waterbath for 30 minutes. This detached the trophozoites.

(b) The flask was inverted several times to obtain a homogeneous suspension of trophozoites and one ml. of culture was transferred to a new flask.

(c) The new 50ml flask was filled to the top with TY1-S-33 growth media and all air bubbles were removed.

(d) The flask was incubated at 37°C for four days or until a monolayer of trophozoites was present.

(e) This procedure was repeated to maintain *Giardia intestinalis* in culture.

## 2.3 Harvesting *Giardia* trophozoites.

i) Trophozoites at a late log. phase of growth were harvested as follows:

(a) Flasks of culture were placed into an ice water bath for thirty minutes.

(b) The culture was decanted into 50ml centrifuge tubes (Falcon™) and the trophozoites were pelleted in a centrifuge at 700xG for five minutes.

(c) The supernatant was discarded and the pellet resuspended and washed in phosphate-buffered saline (PBS) and centrifuged at 700xG for five minutes. This step was repeated twice more.

(d) The total number of trophozoites were counted and adjusted to the required concentration.

## 2.4 Procedure for *in vitro* encystation of *Giardia intestinalis* trophozoites.

i) Method.

(a) A culture flask containing *Giardia intestinalis* trophozoites at a late log. phase of growth was decanted and a monolayer of trophozoites was left in the flask

(b) The culture flask was refilled with fresh bile enriched TY1-S-33 encystation media and left to incubate at 37°C for 48 hours.

(c) After 48 hours the contents of the flask, which were now a mixed culture of cysts and trophozoites were decanted into 50ml centrifuge tubes and centrifuged at 700XG for five minutes.

(d) The supernatant was discarded and the pellet was resuspended in sterile distilled water. The distilled water lysed the trophozoites but the cysts remained intact. The cell suspension was stored at 4°C.

## 2.5 Cryopreservation of *Giardia* trophozoites

These cultures served as a backup source for the organism.

i) The cryopreservation procedure was as follows:-

(a) *Giardia* trophozoites were harvested at a late log. phase of growth and the cell suspension was adjusted to 1.2 to $1.5x10^6$ trophozoites per ml with TY1-S-33 growth medium.

(b) 0.25ml of this suspension was pipetted into each Nunc™ Cryotube and 0.25ml of dimethyl sulphoxide (DMSO) was added slowly to each tube..

(c) The tubes were mixed gently and wrapped in tissue-paper (to avoid rapid freezing of the trophozoites) and placed into a polystyrene container.

(d) The whole container was then placed into a -80°C freezer. After 24 hours, the tubes containing the frozen cultures were removed from the container, unwrapped, and stored in a rack at -80°C.

e) For the retrieval of frozen trophozoite cultures, please refer to section 2.1 (ii) of this chapter.

## 2.6 Percoll gradient centrifugation.

i) *In vitro* derived *Giardia intestinalis* cysts were purified as follows:

(a) The mixed suspention of *in vitro* cysts and lysed trophozoites in water (Refer to section 2.4) was pelleted at 1000XG for five minutes in a 50ml conical centrifuge tube (Falcon™).

(b) The supernatant was then discarded and the pellet was resuspended in 15ml of Percoll (Pharmacia™) [specific gravity (sp. gr.) 1.090].

(c) The 15ml of Percoll/cyst-trophozoite suspension was overlaid with 15ml of Percoll of 1.045 sp.gr. followed by 15ml of Percoll of 1.020 sp.gr. To do this the conical centrifuge tube was placed at a slant and the 1.045 sp.gr. and the 1.020 sp.gr. Percoll layers were overlaid dropwise with a pasteur pipette.

d) The Percoll gradients were centrifuged at 1000XG for 30 minutes without braking.

e) Pure cysts were retrieved from the 1.090 sp.gr. and 1.045 sp.gr. interface.

f) This procedure was repeated once more using the sample retrieved from the 1.090 sp.gr. and 1.045 sp.gr. interface to ensure the cyst sample was devoid of lysed trophozoites.

## 2.7 Antisera production: Inoculation of New Zealand white rabbits and Balb/C mice.

i) Preparation of trophozoite membranes as an inoculum.

(a) A 2ml aliquot of harvested trophozoites ($1x10^7$ trophozoites/ml) was placed in a bijou bottle (Please refer to section 2.3).

(b) This was frozen to -20°C and thawed. This procedure was repeated twice more.

(c) A sample of the culture was checked microscopically to ensure that the freezing and thawing had ruptured the cell membranes. Only irregularly shaped, motionless structures were seen with no "normal" trophozoites viewed.

(d) The ruptured cells were then washed by pelleting the cell suspension using an Eppendorf™ Centrifuge 5414S at full speed for five minutes, discarding the supernatant and resuspending the pellet in PBS. This procedure was repeated twice more.

(e) The remaining membranes were resuspended in 2ml of PBS and stored at -20°C.

ii) Preparation of cysts as an inoculum.

(a) *In vitro* cysts were purified by Percoll gradient centrifugation. (Please refer to section 2.6).

(b) The cysts were collected from the 1.090 sp.gr. and 1.045 sp.gr. interface, washed and adjusted to $4x10^7$ cysts per ml in distilled water.

(iii) Immunisation programme for New Zealand white rabbits.

Day 1.- Two rabbits were each immunised with 4x0.25ml of *Giardia* membranes or *Giardia* cysts subcutaneously. (Emulsified in Freunds Complete Adjuvant (Difco™) at a ratio of 3:5). This inoculation was equivalent to $4x10^6$ trophozoites or cysts per rabbit.

Day 14.- Each rabbit was boosted with 0.25ml of *Giardia* membranes or cysts (emulsified in Incomplete Freunds Adjuvant (Difco™) at a ratio of 3:5) subcutaneously. This was equivalent to $4x10^6$ trophozoites or cysts per rabbit.

Day 28.- The procedure was repeated as for Day 14. Three days after the second booster the rabbits were bled from the ear vein and the *Giardia*-specific antibody titre was determined using the indirect immunofluorescence test.

Day 35. The procedure was repeated as for Day 14.

Seven days after the third booster the rabbits were bled by cardiac puncture. Serum was obtained by centrifuging the clotted blood with Serasieve® at 1000XG for ten minutes. The serum was stored at -80°C.

*[Note: This experiment was approved by the Massey University Animal Ethics Committee (88/130 Amended).]*

(iv) Immunisation programme for Balb/C mice.

Day 1- Ten mice were each immunised with 4x0.2ml *Giardia* membranes or *Giardia* cysts (emulsified in Freunds Complete Adjuvant (Difco™) in a ratio of 3:5) subcutaneously. This inoculation was equivalent to $5x10^4$ trophozoites per mouse.

Day 14- The mice were boosted with 0.2ml of *Giardia* membranes or *Giardia* cysts (in PBS) intraperitoneally.

Day 28- The mice were boosted again with 0.2ml of *Giardia* membranes or *Giardia* cysts (in PBS) was given intraperitoneally.

Five days later, the mice were bled and their antisera was obtained by centrifuging the clotted blood with Serasieve® at 1000XG for 10 minutes and stored at -80°C. The immune sera were used in

the indirect immunofluorescence test and enzyme immunoassay.

*[Note: This experiment was approved by the Massey University Animal Ethics Committee (87/61 Amended).]*

## 2.8 Indirect Immunofluorescence Test (IFT) for *Giardia*

i) Method.

(a) Trophozoite suspensions which contain $1x10^5$ trophozoites/ml were centrifuged onto slides in the Shandon™ Cytospin II for five minutes at 8.1XG. These slides were fixed with acetone and used in the IFT.

(b) *Giardia*-specific rabbit antisera* and normal rabbit sera* were diluted 100-fold, 200-fold, 400-fold, 800-fold, 1600-fold, and 3200-fold respectively in PBS.

(c) The slides were placed in a humidified chamber at 37°C and 30ul from each dilution of rabbit sera* was applied to the trophozoite fixed slides.

(d) The humidified chamber was covered and incubated for 2hrs at 37°C.

(e) The slides were then washed in two changes of PBS for 10 minutes, and once in distilled water for 10 minutes. The slides were then air dried.

(f) The air dried slides were placed into the humidified chamber

at 37°C and 30ul of an 80-fold dilution of fluorescein-conjugated goat-anti-rabbit IgG* (Sigma™) in PBS was applied to each trophozoite fixed slide and incubated for two hours.

(g) The slides were then washed in two changes of PBS for 10 minutes, and once in distilled water for 10 minutes.

(h) The slides were air dried and mounted under the mounting fluid at pH 8.5 (9 parts glycerol: 1 part 0.5M carbonate buffer pH 9.5).

(i) The slides were stored overnight at 4°C in a humidified chamber until examined microscopically under ultra-violet light.

* *Mouse anti-Giardia serum was also tested using the immunofluorecence test. Mouse serum was used in place of rabbit serum at the following serum dilutions: 10-fold, 50-fold, 100-fold, 500-fold, 1000-fold and 5000-fold. A fluorescein conjugated goat anti-mouse antibody was used.*

## 2.9 An Enzyme Immunoassay (EIA) for the detection of *Giardia*-specific antibodies.

i) Method.

(a) One hundred µl of poly-L-lysine (0.01mg/ml)(Sigma™) was applied to polystyrene microtitre wells (Nunc™ Immunomodule). The wells were incubated in a humidified chamber at 37°C for one hour.

(b) The wells were emptied and washed in two changes of PBS-BSA-Tween (125ul/well).

(c) The polystyrene microtitre trays were sensitised with live *Giardia* trophozoites harvested at a late log. phase of growth ($1x10^7$ trophozoites/ml).

(d) A 100ml aliquot of the culture, equivalent to $1x10^6$ trophozoites/well, was used to sensitise these wells. The microtitre trays were placed in a humidified chamber and left for two hours at 37°C.

(e) The wells were emptied and washed in two changes of PBS-BSA-Tween (125ul/well).

(f) The wells were then blocked with PBS-BSA (100µl/ml) at room temperature for sixty minutes.

(g) The wells were emptied and washed in two changes of PBS-

BSA-Tween. After washing, effort was made to ensure that the wells were totally emptied of buffer.

(h) Serum samples which were to be tested were serially diluted in PBS-BSA-Tween.

(i) A 100ul aliquot of each serum dilution was added to each well in duplicate and the wells were incubated at 37°C for two hours.

(j) The wells were emptied and washed in two changes of PBS-BSA-Tween and the fluid was removed by tapping the plates on a paper towel.

(k) A 100ul aliquot of conjugate (alkaline phosphatase labelled anti-immunoglobulin IgG, IgM or IgA (Sigma™) diluted 1000-fold in PBS-BSA-Tween) was added to all the wells and incubated at 37°C for one hour.

(l) The wells were washed in two changes of PBS-BSA-Tween.

(m) A 100ul aliquot of substrate solution (p-nitrophenyl phosphate at 1mg/ml in substrate diluent) was added to the wells and incubated at room temperature for 30 minutes in the dark.

(n) A 50ul aliquot of 3M NaOH was added to the wells to stop the enzyme reaction. The absorbance of each well was read at 410nm using a Dynatech™ Microelisa Minireader MR500.

## 2.10 Protein determination of sample for use in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

i) Protein determination of *Giardia intestinalis* was based on a modified method of Bradford (1976).

The procedure was as follows:-

(a) Bovine Serum Albumin (BSA) (Sigma™) was prepared from 0 to 100ug per 0.1ml in 0.2M NaOH.

(b) The BSA solutions were heated at 100°C for three minutes simulating the sodium dodecyl sulphate (SDS) treatment used in solubilising total *Giardia intestinalis* proteins.

(c) The BSA solutions were cooled at room temperature and 5ml of Coomassie Blue-G reagent (Sigma™) was added to each 0.1ml dilution of the different BSA concentrations.

(d) The tubes were mixed by inversion and 3ml aliquots of the BSA and Coomassie Blue-G reagent mixture were placed into disposable plastic cuvettes and their absorbances were measured at 595nm using a Cecil™ CE599 spectrophotometer.

(e) A standard curve for the estimation of total proteins was derived from these absorbance values. There was a linear relationship between absorbances and protein concentration. The graph was used to determine unknowns.

## 2.11 SDS-PAGE

The SDS-PAGE system used was based on the method of Ionas (1989) which was a modification of the SDS- discontinuous system by Laemmli (1970).

A brief description of the procedure follows:

(i) Materials

(a) Glass plate x 1 (13 x 17 cm)

(b) Glass plate x 1 (13 x 17 cm with a notch 2 cm deep and 10 cm long cut along the 13 cm edge)

(c) Perspex spacers x 2 (16 x 1.5 x 0.15 cm)

(d) Perspex spacer x 1 (13 x 1.5 x 0.15 cm)

(e) Petroleum jelly

(f) " Bulldog " clips

(g) Reservoir gel tank

(h) Perspex stand

(a) A thread of petroleum jelly was piped along the three straight edges of the notched glass plate.

(b) The three perspex spacers were positioned in accordance with the length of the glass edges and pressed onto the petroleum jelly. Care was taken to align the spacers with the edges of the glass. Another thread of petroleum jelly was then piped onto the perspex spacers and the remaining glass plate was pressed into place. The petroleum jelly forms a seal between the glass and the spacers.

(c) The glass mould was set vertically into position on a perspex stand and secured with clips. The notch was positioned on top and facing outwards.

(iii) Preparation of the polyacrylamide running gel

(a) Varying concentrations of acrylamide gels were prepared by mixing the different solutions in the order given below:-

| | *Acrylamide concentrations* | | |
|---|---|---|---|
| | *7.5%* | *10.0%* | *12.5%* |
| *Lower tris buffer* | *5.0 ml* | *5.0 ml* | *5.0 ml* |
| *Running gel solution* | *5.0 ml* | *6.7 ml* | *8.3 ml* |
| *Distilled water* | *10.0 ml* | *8.3 ml* | *6.7 ml* |
| *Ammonium persulphate solution* | *0.1 ml* | *0.1 ml* | *0.1 ml* |
| *N,N,N',N' Tetramethyethylenediamine(TEMED®)* | *0.01ml* | *0.01ml* | *0.01ml* |

(b) The " running " acrylamide mixture was polymerised between two glass plates measuring 13cm by 17cm set apart by perspex spaces.

(c) The running acrylamide mixture was poured into the mould to about 3cm from the edge of the notch and the mixture was overlaid with 5ml of distilled water.

(d) The polymerisation process took forty five to sixty minutes to complete and could be gauged by the appearance of an interface between the gel and the water.

(iv) Preparation of the stacking gel.

(a) The 4.5% stacking gel mixture was prepared by mixing the different solutions in the order given below:-

| | |
|---|---|
| *Upper tris buffer* | *2.5 ml* |
| *Stacking gel solution* | *1.5 ml* |
| *Distilled water* | *6.0 ml* |
| *Ammonium persulphate solution* | *0.05ml* |
| *N,N,N',N' Tetramethylethylenediamine(TEMED®)* | *0.01ml* |

(b) After the running gel mixture has polymerised, the layer of water was removed and replaced by the stacking gel mixture.

(c) The stacking gel mixture was filled to about 0.5cm from the edge of the notch and a perspex comb (with teeth 0.75cm wide, 3 mm apart and 17 mm long) was carefully inserted between the glass plates into the stacking gel mixture. The trapping of air bubbles around the comb was avoided.

(d) The stacking gel mixture took ten to fifteen minutes to polymerise. After polymerisation was completed, the perspex comb was removed and the wells created by the perspex comb were washed in Tris-Glycine Reservoir Buffer.

(e) The glass plate / gel apparatus was then removed from the vertical stand and the bottom spacer was withdrawn.

(f) The upper reservoir edge of the gel electrophoresis apparatus which would be in contact with the gel slab (ie. the notch of the glass plate) was lined with a thread of petroleum jelly. This is to provide a liquid-tight seal between the upper and lower reservoir thus ensuring that the current would flow through the gel.

(g) The glass plate / gel slab was then pressed vertically into position onto the gel electrophoresis apparatus with the notch facing inwards toward the upper reservoir. The whole assembly was secured by clips.

(h) The upper and lower reservoirs were filled with Tris-Glycine Reservoir buffer.

(v) *Giardia intestinalis* protein sample for SDS-PAGE.

(a) *Giardia intestinalis* trophozoites were harvested at a late log. phase of growth and washed twice more in phosphate buffered saline (PBS).

(b) The supernatant was discarded and the pellet was resuspended. A 100ul aliquot of the trophozoite pellet was solubilised with 25ul of SDS-Sample Buffer and 12.5ul of Bromophenol Blue Tracking Dye. The mixture was heated at 100°C for five minutes and cooled at room temperature.

(c) The solubilised sample equivalent to 20, 40, 60, 80, 100, 120, or 140ug of protein (or 300ug of protein for immunoblots) as

determined by the protein assay was applied to the wells cast in the stacking gel and electrophoresed at 15mA until the dye front of the sample had travelled 0.5cm into the running gel.

(d) The protein samples were then electrophoresed at 10mA until the dye front has progressed 10cm into the running gel.

(iv) Staining and destaining of protein bands in the gel.

(a) Immediately after electrophoresis the gel was removed from the apparatus and gently agitated in Coomassie Blue stain overnight.

(b) The gel was then destained in several changes of 10% acetic acid and the process was completed when all the stain had diffused out of the gel and only the protein bands were visibly stained.

(c) The gel was finally washed in distilled water and photographed.

## 2.12 Immunoblotting.

Proteins for use in the immunoblots were separated by SDS-PAGE. The immunoblot procedure was basically that of Ionas (1989) with slight modifications.

### 2.12.1 Setting up of the immunoblot apparatus.

(i) Materials:-

(a) Perspex Gel Holder (Bio-Rad™)
(b) Rectangular tray 4cm deep.
(c) 6 sheets of Whatman™ 3mm chromatography paper (21x16cm).
(d) Two support pads (21x16x1cm).
(e) 0.45um Pure Nitrocellulose paper (Bio-Rad™ or Scheicher and Schuell, Germany) cut to the size of the protein gel.
(f) Trans-blot (Bio-Rad™) gel tank.
(g) Bio-Rad™ 250/2.5 power pack.

(ii) Method of assembly.

(a) The perspex gel holder consisted of two different coloured perspex panels (transparent and grey) joined by a hinge which could be clamped together by a perspex clasp. The holder was left open and the grey panel was placed horizontally into a tray was filled with transfer buffer. The transparent panel was left leaning at a right angle to the grey panel.

(b) A support pad was placed on the grey panel followed by three sheets of Whatman™ 3mm chromatography paper which were all submerged in the transfer buffer. The layers were gently pressed down at the middle and by slowly sliding outwards, any air bubbles trapped between the sheets were eased to the edge and released.

(c) In the mean time, the polyacrylamide gel electrophoresis of the protein samples were completed and a strip of the protein sample from the gel was stained for total protein bands. This step was to ensure proper separation of the protein samples in the SDS-PAGE.

(d) The remaining gel was carefully layered onto the Whatman™ 3mm chromatography paper from step (b). A sheet of 0.45um pure nitrocellulose cut to the size of the polyacrylamide gel was positioned on the gel. (The nitrocellulose sheet was presoaked by capillary action in transfer buffer. This was done by carefully floating the nitrocellulose sheet on the buffer for a few minutes and then submerging the whole sheet.)

(e) Three more sheets of Whatman™ 3mm chromatography paper were placed on top of the nitrocellulose sheet followed by another support pad, excluding all air bubbles in the same manner as in step (b).

(f) The transparent perspex panel was pushed down onto the whole assembly and was securely clamped together; all the time submerged in transfer buffer.

2.12.2 Wet electrophoretic transfer of the immunoblot.

(a) The " Trans-blot " gel tank was filled with transfer buffer and the whole transfer assembly was slotted into position at the middle of the tank with the grey perspex panel facing the cathode end (black electrode) and the transparent panel facing the anode end (red electrode).

(b) The immunoblot was transferred overnight at 30 volts using the Bio-Rad™ 250/2.5 power pack and increased to 60 volts for another two hours the next day.

(c) After transfer was completed the power supply was disconnected and the transfer sandwich disassembled. The nitrocellulose sheet was immersed in Tris-Buffered Saline (TBS) and agitated. The polyacrylamide gel was stained with Coomassie Blue to verify transfer.

2.12.3 Development of Immunoblot.

(a) The nitrocellulose sheet was transferred from the TBS to 100ml of 0.1% Tween Tris Buffered Saline (TTBS)-3% gelatin. The nitrocellulose sheet was blocked at 28°C with agitation for four hours. After this the nitrocellulose sheet was

washed twice for five minutes in TTBS with agitation.

(b) The blocked nitrocellulose sheet was then placed into a plastic bag made to the size of the nitrocellulose sheet along with 5ml of immune serum (diluted 20-fold in TTBS-1% gelatin). Air bubbles caught in the bag were eased out of the bag and the bag was sealed.

(c) The sealed bag was then incubated overnight at 28°C with agitation.

(d) The immunoblot was then washed twice in TTBS and transferred to 100ml of TTBS-1% gelatin containing 33ul of horseradish peroxidase conjugated anti IgG, M, or A which was a 3000-fold dilution of the conjugate.

(e) The blot and conjugate were incubated for an hour at 28°C with agitation, washed twice in TTBS for five minutes each and once in TBS for five minutes with agitation.

(f) While the nitrocellulose sheet was being washed, the horseradish peroxidase colour development solution was prepared. The immunoblot was placed in 100ml of horseradish peroxidase colour development substrate solution and agitated. Protein bands started to appear after about five minutes but the colour reaction was left for up to thirty minutes until the bands were suitably darkened.

(g) The reaction was stopped by rinsing the immunoblot in distilled water.

# 3.0 Results

## 3.1 Enzyme immunoassay

### 3.1.1 Recovery of *Giardia* cysts prepared *in vitro*

*Giardia* cysts prepared *in vitro* are contaminated with trophozoites. This preparation was added to a "stepped" Percoll gradient and centrifuged. Two bands were observed. The upper band was at the interface of specific gravity (sp.gr.) 1.020 and sp.gr. 1.045 and contained both cysts and lysed trophozoites. The lower band at the interface of sp. gr. 1.045 and sp. gr. 1.090 contained only cysts. The percentage of cysts recovered in each fraction is shown in Table 5a.

The two fractions were recentrifuged in Percoll gradients. Cysts and lysed trophozoites from the upper band were again found in the upper fraction. Similarly, those from the lower fraction were detected in the lower fraction. (Table 5b).

### 3.1.2 Determination of cyst viability

The cysts obtained from different density regions following Percoll Gradient centrifugation were stained with fluorescein diacetate (claimed to stain living cysts only) and propidium iodide (claimed to stain dead cysts only). The results are shown in Table 6 and Table 7.

## Table 5a. First Percoll gradient centrifugation of *Giardia cysts:* Cyst recovery at specific gravity interfaces

| Sample | Initial cyst count / 10ml | Sp. gr. interface | No. of cysts recovered at interface fraction | % Recovery |
|---|---|---|---|---|
| One | $4.08 \times 10^7$ | (i) 1.020/1.045 | $3.02 \times 10^7$ | 74.0 |
| | | (ii) 1.045/1.090 | $6.60 \times 10^5$ | 1.6 |
| Two | $7.2 \times 10^7$ | (i) 1.020/1.045 | $4.70 \times 10^7$ | 65.0 |
| | | (ii) 1.045/1.090 | $3.20 \times 10^6$ | 4.4 |
| Three | $1.4 \times 10^7$ | (i) 1.020/1.045 | $9.90 \times 10^6$ | 70.7 |
| | | (ii) 1.045/1.090 | $1.30 \times 10^6$ | 9.2 |

---

The above table shows the recovery of *Giardia* cysts prepared *in vitro* from the upper and lower Percoll sp.gr. fractions. The sample from each fraction was recentrifuged in a second Percoll Gradient and the recovery of *Giardia* cysts was recorded in Table 5b.

## Table 5b. Second Percoll gradient centrifugation of *Giardia* cysts: Cyst recovery at specific gravity interfaces.

| Sample Fraction | | Sp .gr .interface | No. of cysts recovered at interface fraction. | % Recovery |
|---|---|---|---|---|
| One | (i)1.020/1.045 sp.gr. | 1.020/1.045 | $2.30 \times 10^7$ | 76.2 |
| | | 1.045/1.090 | $4.30 \times 10^5$ | 1.4 |
| | (ii)1.045/1.090 sp.gr. | 1.020/1.045 | $1.40 \times 10^5$ | 21.2 |
| | | 1.045/1.090 | $4.30 \times 10^5$ | 65.2 |
| Two | (i)1.020/1.045 sp.gr. | 1.020/1.045 | $3.16 \times 10^7$ | 67.3 |
| | | 1.045/1.090 | $2.54 \times 10^6$ | 5.4 |
| | (ii)1.045/1.090 sp.gr. | 1.020/1.045 | $3.52 \times 10^5$ | 11.0 |
| | | 1.045/1.090 | $2.17 \times 10^6$ | 67.7 |
| Three | (i)1.020/1.045 sp.gr. | 1.020/1.045 | $7.17 \times 10^6$ | 72.4 |
| | | 1.045/1.090 | $2.48 \times 10^5$ | 2.5 |
| | (ii)1.045/1.090 sp.gr. | 1.020/1.045 | $2.33 \times 10^5$ | 17.9 |
| | | 1.045/1.090 | $9.26 \times 10^5$ | 71.2 |

This table shows the number of cysts recovered from the upper (1.020/1.045 sp.gr.) and lower (1.045/1.090 sp.gr.) Percoll gradient interfaces after the upper and lower Percoll bands were recentrifuged in a second Percoll gradient.

## Table 6. Viability determination of cysts recovered from the upper Percoll band at the interface of sp.gr. 1.020 and sp.gr. 1.045

| | | NUMBER OF CYSTS COUNTED IN 0.1µl [a] | | |
|---|---|---|---|---|
| Stain | Treatment | Fluorescent cysts | Non-Fluorescent cysts | % Fluorescing |
| Propidium iodide | Untreated | 18 | 60 | 23 |
| | Heat treated[b] | 21 | 52 | 29 |
| Fluoresein diacetate | Untreated | 0 | 85 | 0 |
| | Heat treated | 0 | 81 | 0 |

a *Mean of three count*

b *"Heat treated" indicates Giardia cysts were heated at 100°C for 15 minutes.*

Table 7. Viability determination of cysts recovered from the lower Percoll band at the interface of sp.gr. 1.045 and sp.gr 1.020.

| | | NUMBER OF CYSTS COUNTED IN 0.1µl[a] | | |
|---|---|---|---|---|
| Stain | Treatment | Fluorescent cysts | Non-Fluorescent cysts | % Fluorescing |
| Propidium iodide | Untreated | 36 | 58 | 38 |
| | Heat treated[b] | 25 | 40 | 38 |
| Fluorescein diacetate | Untreated | 9 | 46 | 16 |
| | Heat treated | 0 | 48 | 0 |

a *Mean of three count*

b *"Heat treated" indicates Giardia cysts were heated at 100 °C for 15 minutes.*

This investigation was not primarily concerned with the viability or otherwise of *in vitro Giardia* cysts. We conclude, however, that propidium iodide is not a reliable method of estimating viability since no change in staining occurred after heating.

Fluorescein diacetate stained 16% *in vitro* cysts before heating and none after heating. While the percentage stained is low (16%) this figure probably represents a minimum estimate of viability. We conclude that the lower band contains cysts which better represent *in vivo* derived *Giardia* cysts than do cysts from the upper band. *In vitro* cysts purified by Percoll centrifugation and collected from the lower band were subsequently used in all experimental applications which require *Giardia* cysts in this thesis.

### 3.1.3 Titration of *Giardia* antisera by the Indirect Immunofluorescence Test.

Antibody was prepared in rabbits and mice using *Giardia* trophozoites or cysts as immunising antigen. These antisera were used to develop an EIA test and for immunoblotting to investigate the relative antibody response to different antigens. Preliminary titration of the antisera were undertaken using an indirect immunofluorescence test. The results (Table 8 and Table 9) show that both antisera stained cysts in the immunofluorescence test.

Table 8. Indirect immunofluorescence test of Giardia antisera prepared in rabbits.

| Test antigen | Serum dilution | Anti-cyst sera | Anti-trophozoite sera | Normal Rabbit sera |
|---|---|---|---|---|
| *Giardia* cysts | 100-fold | +[a] | + | -[b] |
| | 200-fold | + | + | - |
| | 400-fold | + | + | - |
| | 800-fold | + | + | - |
| | 1600-fold | - | - | - |
| | 3200-fold | - | - | - |

[a] = *Fluorescent staining detected.*

[b] = *Fluorescent staining not detected.*

Table 9. Indirect immunofluorescence test of *Giardia* antisera prepared in mice.

| Test antigen mouse | Serum dilution | Anti-trophozoite sera | Normal sera |
|---|---|---|---|
| *Giardia* | 10-fold | +[a] | + |
| trophozoites | 50-fold | + | -[b] |
| | 100-fold | + | - |
| | 500-fold | + | - |
| | 1000-fold | + | - |
| | 5000-fold | - | - |

[a] = *Fluorescent staining detected.*

[b] = *Fluorescent staining not detected.*

### 3.1.4 Preliminary development of an EIA

To establish an EIA to detect *Giardia* specific antibodies, different preparations of *Giardia* trophozoite antigen were used to coat polystyrene microtitre wells. EIA tests were repeated three times for each antigen preparation and the results were tabulated (Table 10.).

Table 10. Enzyme Immunoassay with different *Giardia* antigen preparations*.

| Antigen preparation | Assay 1 | Assay 2 | Assay 3 |
|---|---|---|---|
| 1.Frozen and thawed trophozoites | 0.145 | 0.380 | 0.225 |
| 2.Trophozoite membranes | 0.175 | 0.300 | 0.440 |
| 3.Live trophozoites | 0.140 | 0.290 | 0.170 |
| 4.Sonicated trophozoites | 0.210 | 0.170 | 0.140 |

**A 10-fold dilution of Giardia-specific mouse antisera was used in all the assays.*

It can be seen that tests using the four antigen preparations gave inconsistent results. Most investigators use trophozoites as antigen but it was observed in our tests that after the first wash in the EIA procedure, few if any trophozoites remained adsorbed to the microtitre well (Fig. 2). This could account for the low and inconsistent results.

Several methods could be used to attach *Giardia* trophozoites onto the microtitre well. *Giardia* antibodies could be used to capture the *Giardia* trophozoites or the trophozoites could be attached by poly-L-lysine. The latter was investigated. Microtitre wells were treated with poly-L-lysine, a large excess of trophozoites was added to each of the wells, and the time needed for trophozoites to attach is shown in Table 11.

Table 11. Trophozoite adherence to wells coated with poly-L-lysine

| Incubation time (minutes) | % Area with adhered trophozoite* |
|---|---|
| 15 | 40 |
| 30 | 40 |
| 45 | 40 |
| 60 | 40 |
| 90 | 40 |
| 120 | 40 |
| 180 | 40 |

**Note. Area estimated visually using 40x magnification*

After fifteen minutes about forty percent of the microtitre well had trophozoites attached, and remained attached following the initial washing. and that the number of attached trophozoites did not increase with increased time of contact in the well. The trophozoites remained attached throughout subsequent steps in the EIA (Fig. 1).

*Giardia* cysts in turn failed to attach to the microtitre well in the sense that none were observed after the first wash in the EIA procedure. Poly-l-lysine was then used to coat the wells and was found to promote cyst attachment (Fig 3.) which withstood washing *Giardia* cysts attached using poly-l-lysine were used in subsequent EIA tests.

The results of the EIA tests with and without pretreatment of the microtitre wells with poly-l-lysine are shown in Table 12.

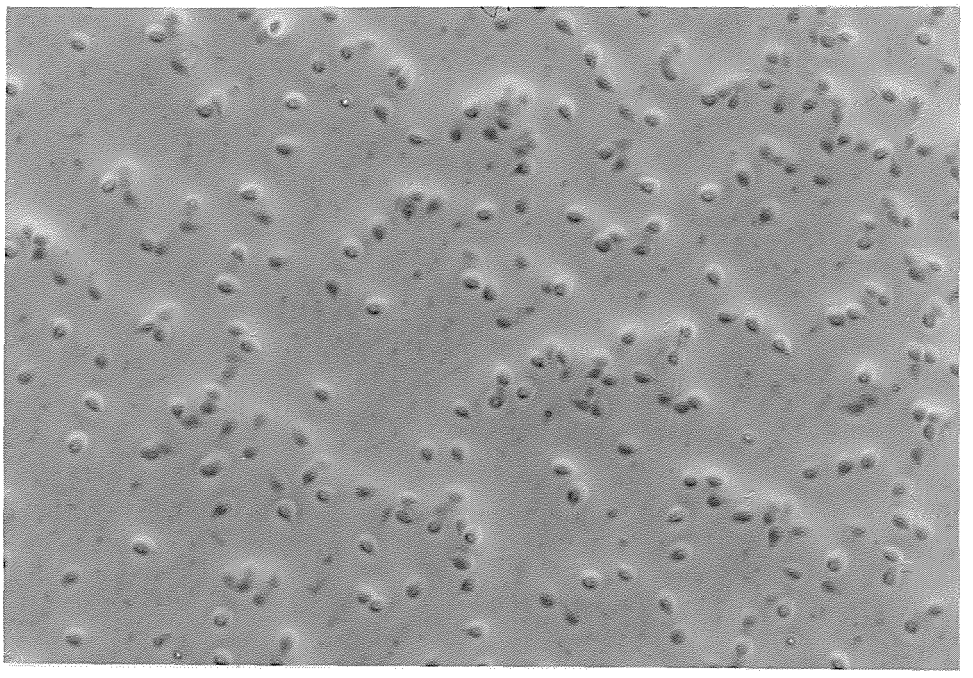
Table 12. Enzyme Immunoassay with and without poly-L-lysine.

| | | Absorbance (410nm) | | |
|---|---|---|---|---|
| Antigen source | Treatment | Assay 1 | Assay 2 | Assay 3 |
| Cysts | i)No poly-L-lysine | 0.120 | 0.190 | 0.250 |
| | ii)Poly-L-lysine | 0.690 | 0.710 | 0.725 |
| Live trophozoites | i)No poly-L-lysine | 0.140 | 0.290 | 0.170 |
| | ii)Poly-L-lysine | 0.760 | 0.790 | 0.750 |

We conclude that attachment of trophozoites and cysts by poly-L-lysine gives more consistent results and increases the sensitivity of the test. This method was subsequently used to assay *Giardia* antibody in human sera.

Figure.1 *Giardia* trophozoites attached by poly-L-lysine to the polystyrene microtitre well.

Figure.2 Microtitre wells not treated with poly-L-lysine. No trophozoites were attached to the well after washing.



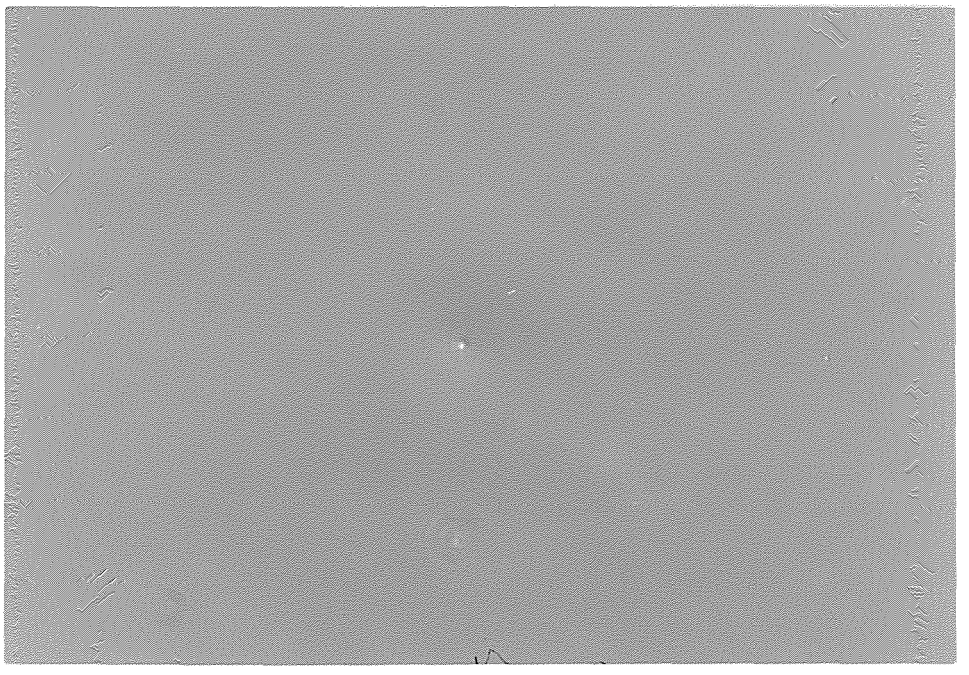
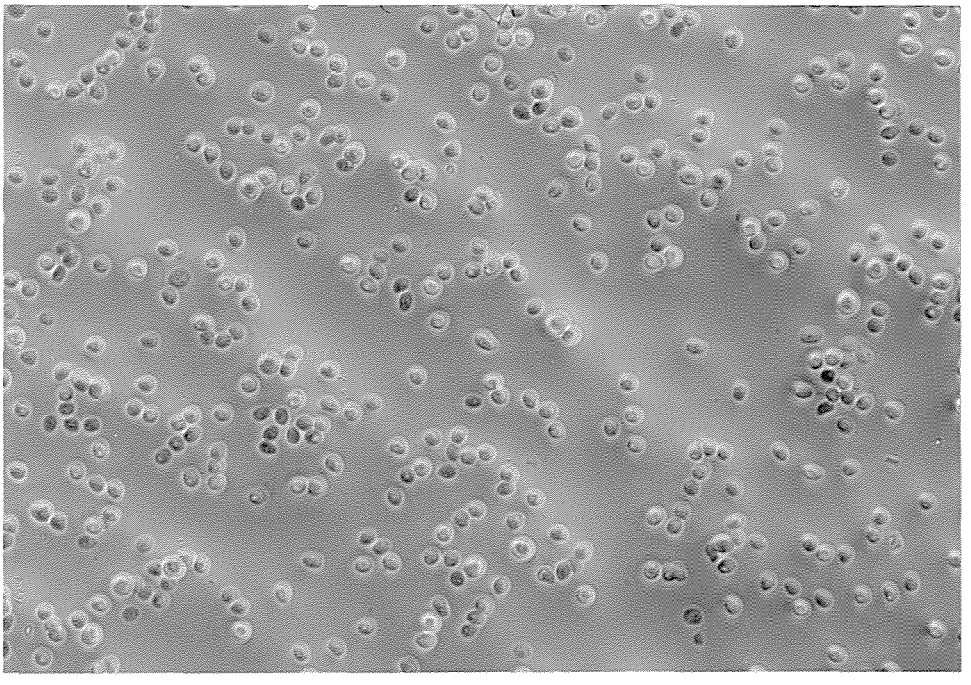


Figure.3 *Giardia* cysts attached by poly-L-lysine to the microtitre well..

*Note: No cysts were observed in wells that were not treated with poly-l-lysine*



### 3.1.5 Detection of different immunoglobulin classes of antibody to *Giardia* in human sera

Using an EIA with *Giardia* trophozoites or cysts attached by poly-L-lysine as antigen, human sera were tested for *Giardia*-specific antibody of the three immunoglobulin classes: IgG, IgM and IgA.

Serum samples were allotted to one of three groups: convalescent patients; patients with current infections and "negative control". Serum samples from patients who had receivec drug therapy for giardiasis were considered as "convalescent" sera. "Current infection" sera were from patients who had not yet received any therapy for giardiasis and "negative control" sera were obtained from volunteers who had no history of giardiasis.

Each serum sample was tested for antibody at three dilutions; 10-fold, 100-fold, and 1000-fold. The results are shown in Tables 13(a) to 14(b) and are also illustrated in histograms (Fig.4 and 5). A 100-fold dilution of serum was found to be the optimum dilution for comparing the *Giardia*-specific antibody levels in the three groups of sera based on the histograms.

The mean absorbances obtained in the EIA are shown in Table 15 and *Giardia*-specific IgM titres of "current" serum samples were slightly elevated as compared to the "convalescent" and "negative control" serum samples. *Giardia*-specific serum IgG titres were

slightly elevated in "convalescent" sera when compared to antibody levels in the other two groups. *Giardia*-specific IgA titres in all three groups were low in the two assays and no significant difference was apparent between the groups. Due to the small sample size, the results for all three immunoglobulin classes showed no statistically significant differences between the groups of patients.

## 3.2 Detection of *Giardia* antibody to individual antigens using immunoblots.

### 3.2.1 Separation of *Giardia* proteins by SDS-PAGE.

To optimise the separation of *Giardia* proteins prior to immunoblotting, different protein concentrations and different polyacrylamide gel concentrations were compared. The results are shown in Figure 6, Figure 7 and Figure 8. The optimum separation corresponded to a concentration of 10% polyacrylamide and a protein load of 100ug per lane. About 30 *Giardia* trophozoite protein bands were detected (Figure 9). Most of these bands fell within the 31 to 200 kilodalton range.

### 3.2.2 Development of immunoblots using rabbit antisera.

To establish the immunoblotting technique, total *Giardia* trophozoite proteins separated by SDS-PAGE were "immunoblotted" with rabbit sera raised against *Giardia* trophozoites and cysts. The results are shown in Figure 9(i) and Figure 9(ii). Rabbit antisera to either

**Table 13(a).EIA of human sera with Giardia trophozoites as test antigen. Group one: "Convalescent" sera.**

| Human serum sample no. | SERUM SAMPLE DILUTIONS* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| 09004 | 0.275 | 0.385 | 0.010 | 0.275 | 0.290 | 0.010 | 0.130 | 0.120 | 0 |
| 09009 | 0.250 | 0.410 | 0 | 0.230 | 0.345 | 0 | 0.170 | 0.145 | 0 |
| 09012 | 0.120 | 0.335 | 0 | 0.220 | 0.275 | 0 | 0.165 | 0.120 | 0 |
| JH(306) | 0.480 | 0.440 | 0.020 | 0.405 | 0.355 | 0.010 | 0.205 | 0.140 | 0 |
| MH(307) | 0.330 | 0.310 | 0 | 0.305 | 0.180 | 0 | 0.145 | 0.060 | 0 |
| PB(305) | 0.260 | 0.400 | 0 | 0.305 | 0.300 | 0 | 0.195 | 0.160 | 0 |
| MR(300) | 0.385 | 0.460 | 0.005 | 0.315 | 0.315 | 0 | 0.155 | 0.115 | 0 |
| CS(202) | 0.305 | 0.290 | 0 | 0.195 | 0.175 | 0 | 0.100 | 0.090 | 0 |
| VO(314) | 0.290 | 0.500 | 0 | 0.285 | 0.395 | 0 | 0.190 | 0.190 | 0 |
| Mean | 0.299 | 0.392 | 0.004 | 0.282 | 0.282 | 0.002 | 0.160 | 0.120 | 0 |

**Absorbance readings at 410nm (Tests were performed in duplicate).*

**Table 13(b).EIA of human sera with *Giardia* trophozoites as test antigen. Group two: "Current infection" sera.**

| Human serum sample no. | SERUM SAMPLE DILUTIONS* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| 09002 | 0.115 | 0.645 | 0.010 | 0.205 | 0.505 | 0.010 | 0.165 | 0.200 | 0 |
| 09003 | 0.190 | 0.260 | 0 | 0.240 | 0.140 | 0 | 0.180 | 0.075 | 0 |
| 09005 | 0.255 | 0.500 | 0.010 | 0.255 | 0.400 | 0 | 0.150 | 0.190 | 0 |
| 09006 | 0.280 | 0.420 | 0.010 | 0.220 | 0.305 | 0 | 0.130 | 0.130 | 0 |
| 09007 | 0.320 | 0.415 | 0 | 0.275 | 0.385 | 0 | 0.135 | 0.135 | 0 |
| 09008 | 0.170 | 0.365 | 0 | 0.190 | 0.285 | 0 | 0.125 | 0.130 | 0 |
| 09010 | 0.240 | 0.390 | 0 | 0.240 | 0.240 | 0 | 0.170 | 0.115 | 0 |
| 09011 | 0.205 | 0.395 | 0 | 0.265 | 0.325 | 0 | 0.190 | 0.205 | 0 |
| Mean | 0.222 | 0.424 | 0.004 | 0.236 | 0.323 | 0.001 | 0.156 | 0.148 | 0 |

**Absorbance readings at 410nm (Tests were performed in duplicate).*

**Table 13(c).EIA of human sera with *Giardia* trophozoites as test antigen. Group three: "Negative control" sera.**

| Human serum sample no. | SERUM SAMPLE DILUTIONS* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| 348 | 0.410 | 0.33 | 0.010 | 0.185 | 0.220 | 0.010 | 0.105 | 0.145 | 0 |
| 349 | 0.140 | 0.325 | 0 | 0.095 | 0.230 | 0 | 0.015 | 0.130 | 0 |
| 350 | 0.130 | 0.400 | 0 | 0.080 | 0.285 | 0 | 0.020 | 0.115 | 0 |
| 351 | 0.165 | 0.275 | 0 | 0.140 | 0.220 | 0 | 0.015 | 0.125 | 0 |
| 352 | 0.190 | 0.320 | 0 | 0.210 | 0.185 | 0 | 0.090 | 0.025 | 0 |
| 353 | 0.220 | 0.345 | 0.005 | 0.105 | 0.210 | 0 | 0.120 | 0.170 | 0 |
| 354 | 0.120 | 0.440 | 0 | 0.080 | 0.340 | 0 | 0.135 | 0.205 | 0 |
| BG | 0.150 | 0.300 | 0 | 0.255 | 0.215 | 0 | 0.185 | 0.115 | 0 |
| JT | 0.145 | 0.275 | 0.010 | 0.225 | 0.205 | 0 | 0.155 | 0.125 | 0 |
| JC | 0.185 | 0.380 | 0.010 | 0.255 | 0.240 | 0.01 | 0.145 | 0.115 | 0 |
| G1 | 0.135 | 0.415 | 0.200 | 0.210 | 0.330 | 0.010 | 0.120 | 0.155 | 0 |
| Mean | 0.181 | 0.364 | 0.012 | 0.167 | 0.244 | 0.003 | 0.100 | 0.130 | 0 |

**Absorbance readings at 410nm (Tests were performed in duplicate).*

Figure.4 Histogram of the absorbance readings in EIA tests for *Giardia* antibody of different immunoglobulin classes in human sera, using *Giardia* trophozoites as antigen.

Figure.4(a) Human sera diluted 10-fold.
Figure.4(b) Human sera diluted 100-fold
Figure.4(c) Human sera diluted 1000-fold.

Figure 4.

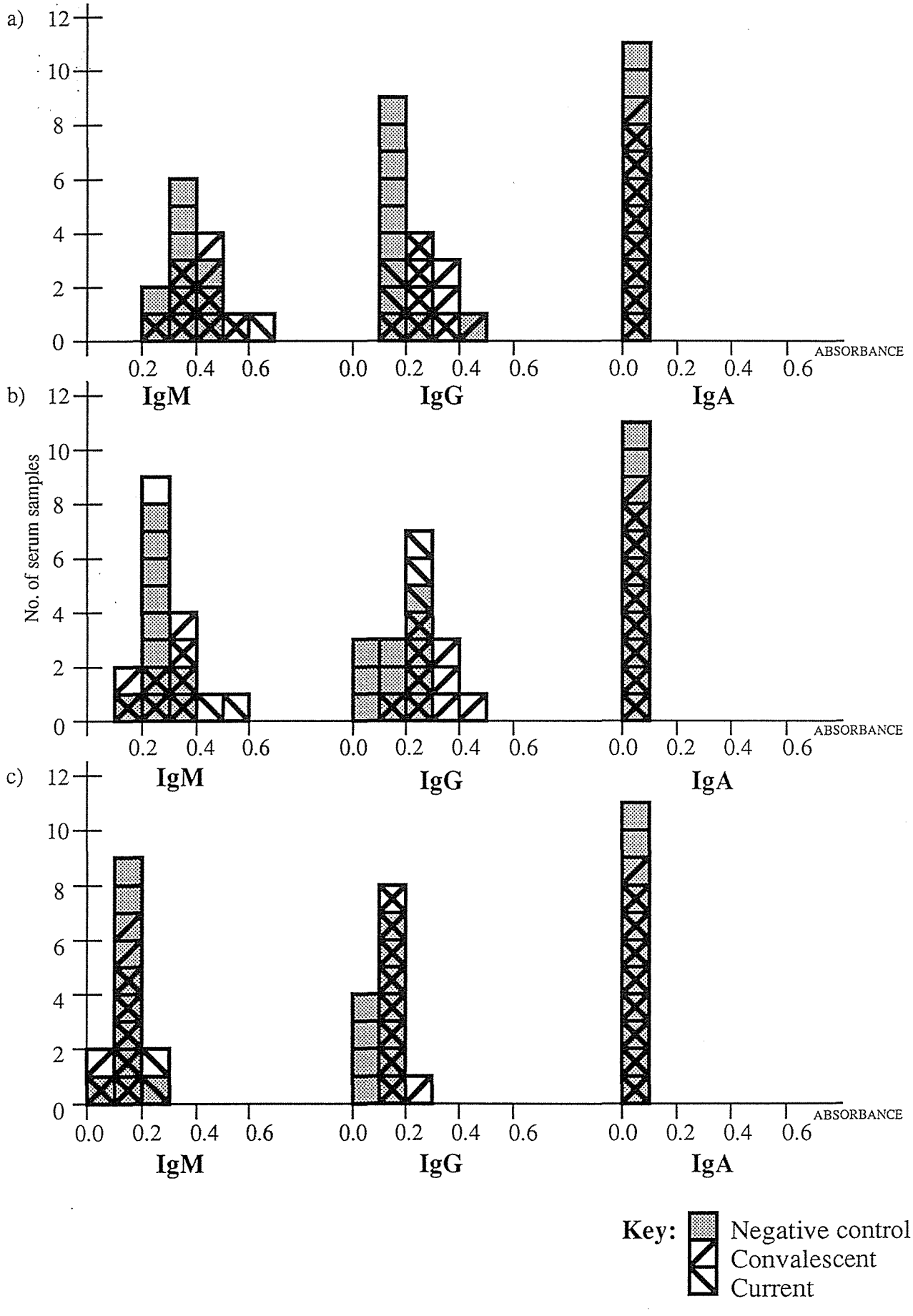

a)
b)
c)
No. of serum samples
ABSORBANCE
IgM
IgG
IgA
Key:
Negative control
Convalescent
Current

**Table 14(a).EIA of human sera with *Giardia* cysts as test antigen. Group one: "Convalescent" sera.**

| Human serum sample no. | SERUM SAMPLE DILUTIONS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| 09004 | 0.245 | 0.285 | 0.010 | 0.205 | 0.225 | 0 | 0.080 | 0.150 | 0 |
| 09009 | 0.180 | 0.345 | 0 | 0.165 | 0.285 | 0 | 0.040 | 0.120 | 0 |
| 09012 | 0.060 | 0.220 | 0 | 0.060 | 0.180 | 0.020 | 0.010 | 0.065 | 0 |
| JH | 0.365 | 0.320 | 0 | 0.320 | 0.270 | 0 | 0.210 | 0.140 | 0 |
| MH(307) | 0.320 | 0.120 | 0 | 0.320 | 0.090 | 0 | 0.180 | 0.400 | 0 |
| PB | 0.205 | 0.335 | 0 | 0.180 | 0.240 | 0 | 0.060 | 0.160 | 0 |
| MR(300) | 0.280 | 0.340 | 0 | 0.080 | 0.080 | 0 | 0.010 | 0.015 | 0 |
| CS | 0.215 | 0.230 | 0.020 | 0.160 | 0.145 | 0 | 0.090 | 0.080 | 0 |
| VO | 0.270 | 0.355 | 0 | 0.310 | 0.240 | 0 | 0.090 | 0.120 | 0 |
| Mean | 0.238 | 0.283 | 0.003 | 0.200 | 0.195 | 0.002 | 0.085 | 0.139 | 0 |

**Absorbance readings at 410nm (Tests were performed in duplicate).*

**Table 14(b).EIA of human sera with *Giardia* cyst as test antigen.**
**Group two: "Current infection" sera.**

| Human serum sample no. | SERUM SAMPLE DILUTIONS* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| 09002 | 0.080 | 0.460 | 0 | 0.120 | 0.200 | 0 | 0.060 | 0.100 | 0 |
| 09003 | 0.120 | 0.230 | 0.010 | 0.200 | 0.275 | 0 | 0.140 | 0.050 | 0 |
| 09005 | 0.300 | 0.340 | 0.040 | 0.160 | 0.200 | 0.020 | 0.070 | 0.140 | 0 |
| 09006 | 0.295 | 0.450 | 0 | 0.210 | 0.380 | 0 | 0.110 | 0.205 | 0 |
| 09007 | 0.280 | 0.190 | 0.010 | 0.240 | 0.120 | 0.010 | 0.140 | 0.090 | 0 |
| 09008 | 0.120 | 0.280 | 0 | 0.180 | 0.290 | 0 | 0.110 | 0.145 | 0 |
| 09010 | 0.180 | 0.410 | 0 | 0.210 | 0.385 | 0 | 0.135 | 0.225 | 0 |
| 09011 | 0.215 | 0.335 | 0 | 0.205 | 0.320 | 0 | 0.175 | 0.150 | 0 |
| Mean | 0.199 | 0.337 | 0.008 | 0.191 | 0.271 | 0.004 | 0.118 | 0.138 | 0 |

**Absorbance readings at 410nm (Tests were performed in duplicate).*

**Table 14(c).EIA of human sera with *Giardia* cysts as test antigen.**
**Group three: "Negative control" sera.**

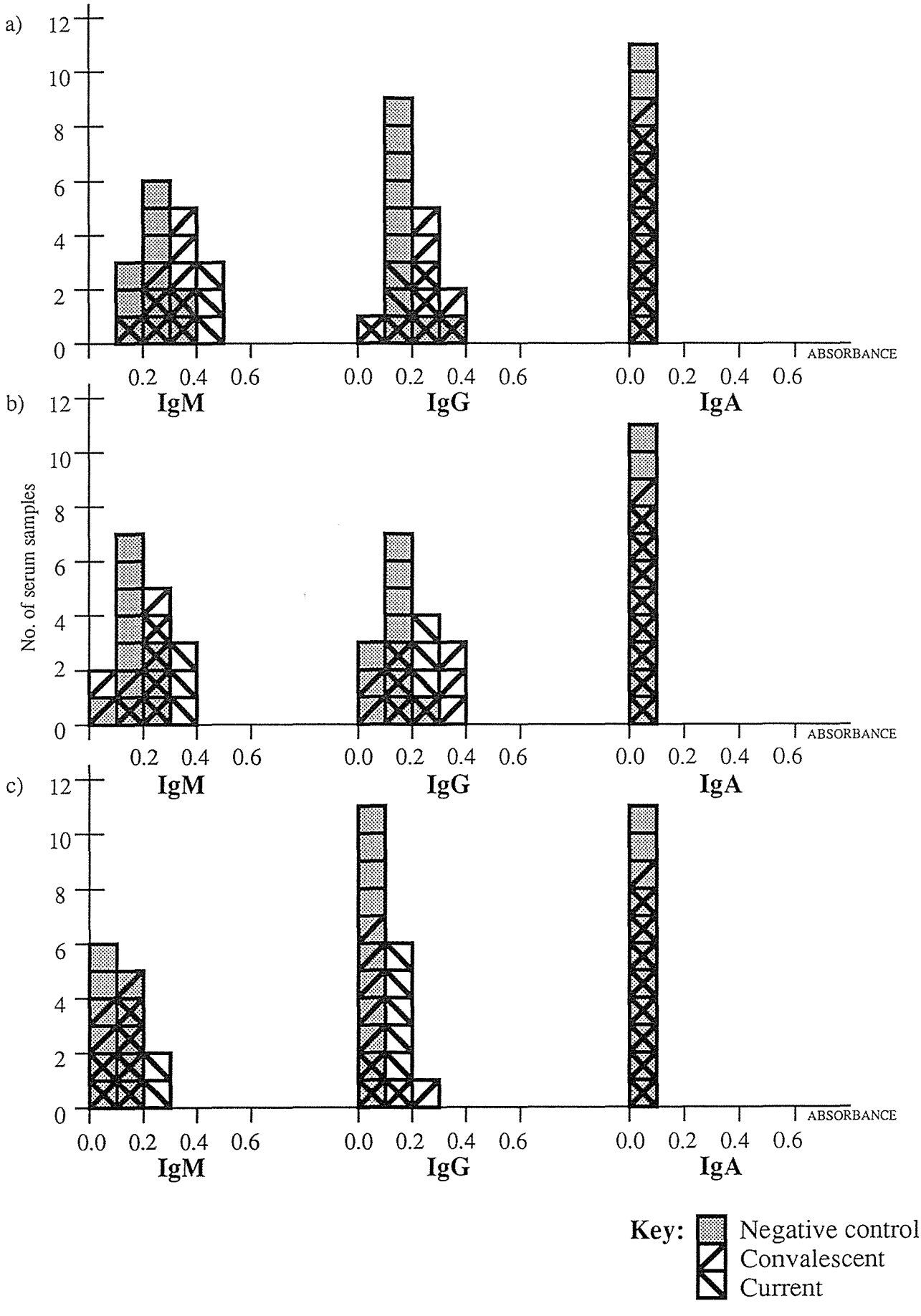
| Human serum sample no. | SERUM SAMPLE DILUTIONS* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| 348 | 0.310 | 0.215 | 0 | 0.205 | 0.135 | 0 | 0.080 | 0.150 | 0 |
| 349 | 0.160 | 0.285 | 0 | 0.140 | 0.185 | 0 | 0.065 | 0.090 | 0 |
| 350 | 0.150 | 0.370 | 0 | 0.095 | 0.280 | 0 | 0.035 | 0.170 | 0 |
| 351 | 0.145 | 0.285 | 0.010 | 0.135 | 0.265 | 0 | 0.070 | 0.125 | 0 |
| 352 | 0.180 | 0.160 | 0.020 | 0.160 | 0.100 | 0.020 | 0.075 | 0.050 | 0 |
| 353 | 0.285 | 0.310 | 0.010 | 0.190 | 0.205 | 0 | 0.080 | 0.125 | 0 |
| 354 | 0.100 | 0.210 | 0 | 0.080 | 0.170 | 0 | 0.020 | 0.065 | 0 |
| BG | 0.170 | 0.205 | 0 | 0.125 | 0.170 | 0 | 0.090 | 0.060 | 0 |
| JT | 0.165 | 0.245 | 0 | 0.090 | 0.155 | 0 | 0.035 | 0.110 | 0 |
| JC | 0.130 | 0.110 | 0 | 0.120 | 0.085 | 0 | 0.070 | 0.025 | 0 |
| GI | 0.190 | 0.185 | 0 | 0.125 | 0.140 | 0 | 0.080 | 0.065 | 0 |
| Mean | 0.180 | 0.235 | 0.004 | 0.133 | 0.172 | 0.002 | 0.064 | 0.094 | 0`1 |

**Absorbance readings at 410nm (Tests were performed in duplicate).*

Figure.5 Histogram of the absorbance readings in EIA tests for *Giardia* antibody of different immunoglobulin classes in human sera, using *Giardia* cysts as antigen.

Figure.5(a) Human sera diluted 10-fold.
Figure.5(b) Human sera diluted 100-fold
Figure.5(c) Human sera diluted 1000-fold.

Figure 5.


a)
b)
c)
No. of serum samples
ABSORBANCE
IgM
IgG
IgA
Key:
Negative control
Convalescent
Current

## Table 15. The mean absorbances obtained in EIA tests for *Giardia*-specific antibody using cysts or trophozoites as antigen.

| EIA with trophozoites as antigen. | SERUM SAMPLE DILUTIONS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| "Convalescent" sera | 0.299 | 0.392 | 0.004 | 0.282 | 0.282 | 0.002 | 0.160 | 0.120 | 0 |
| "Current infection" sera | 0.222 | 0.424 | 0.004 | 0.236 | 0.323 | 0.001 | 0.156 | 0.148 | 0 |
| "Negative control" sera | 0.181 | 0.364 | 0.012 | 0.167 | 0.244 | 0.003 | 0.100 | 0.130 | 0 |

| EIA with cysts as antigen. | SERUM SAMPLE DILUTIONS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10-fold | | | 100-fold | | | 1000-fold | | |
| | IgG | IgM | IgA | IgG | IgM | IgA | IgG | IgM | IgA |
| "Convalescent" sera | 0.238 | 0.283 | 0.003 | 0.200 | 0.195 | 0.002 | 0.085 | 0.139 | 0 |
| "Current infection" sera | 0.199 | 0.337 | 0.008 | 0.191 | 0.271 | 0.004 | 0.118 | 0.138 | 0 |
| "Negative control" sera | 0.180 | 0.235 | 0.004 | 0.133 | 0.172 | 0.002 | 0.064 | 0.094 | 0 |

cysts or trophozoites reacted with most of the trophozoite protein bands. These sera were subsequently used as a positive control when testing human sera. Two faint bands were detected (Figure 9(iii)) using "normal" rabbit sera.

### 3.2.3 Immunoblotting of human sera using *Giardia* proteins

Human serum samples from clinically diagnosed cases of giardiasis were tested in immunoblots to identify *Giardia* proteins which elicited an immune response in patients. Results indicating a positive or negative reaction for IgM, IgG and IgA antibody are shown in Table 16, 17 and 18.

Table 16. Immunoblots with "convalescent" human sera.*

| Human serum sample | Antibody reaction to trophozoite proteins | | |
|---|---|---|---|
| | IgG | IgM | IgA |
| 09004 | +[a] | + | + |
| 09009 | + | -[b] | - |
| 09012 | + | - | + |
| 306 | + | - | - |
| 307 | - | - | - |
| 305 | + | - | - |
| 300 | + | - | - |
| 314 | + | + | + |

[a] = *At least one protein band reacted in the immunoblot.*

[b] = *No protein band reacted in the immunoblot.*

Note. * "convalescent sera" were from patients who had received some form of drug therapy for giardiasis.

# Table 17. Immunoblots with "current sera".*

| Human serum sample | Antibody reaction to trophozoite protein | | |
|---|---|---|---|
| | IgG | IgM | IgA |
| 09002 | -[a] | - | - |
| 09005 | +[b] | + | - |
| 09006 | + | - | - |
| 09007 | + | - | + |
| 09008 | + | - | - |
| 09010 | + | - | + |
| 09011 | + | - | - |

[a] = *At least one protein band reacted in the immunoblot.*

[b] = *No protein band reacted in the immunoblot*

Note * "Current sera" were from patients who had not yet received any therapy for giardiasis.

Thirteen of fifteen (87%) human serum samples from clinically diagnosed cases of giardiasis had IgG antibody which reacted with trophozoite proteins as compared to four of ten (40%) samples from the "negative control" sera group. IgM antibody was detected in three of fifteen (20%) samples from clinically diagnosed cases of giardiasis but was also detected in four of ten (40%) samples of the "negative control" sera group. Five of fifteen samples (33%) from clinically diagnosed giardiasis cases had IgA antibody to trophozoite proteins compared to two of ten (20%) "negative control" serum samples.

IgA antibody in clinically diagnosed giardiasis samples did not react with any particular protein band and the reaction was weak. Two of five samples which had a positive reaction reacted with a 62 kDa protein. A 62 kDa and 66 kDa protein were also reactive in

two "negative control" serum samples.

IgM antibody reaction with trophozoite protein from clinically diagnosed giardiasis samples were also varied. Four of ten negative control serum samples consistently reacted with the 62 kDa protein.

Table 18. Immunoblot with "negative control sera".*

| Human serum sample. | Antibody reaction to trophozoite protein | | |
|---|---|---|---|
| | IgG | IgM | IgA |
| 348 | +[a] | -[b] | - |
| 349 | + | - | - |
| 350 | + | + | + |
| 351 | + | - | - |
| 352 | - | - | - |
| 353 | - | + | - |
| 354 | - | + | - |
| 355 | - | - | + |
| 356 | - | + | - |
| 357 | - | - | - |

[a] = *At least one protein band reacted in the immunoblot.*

[b] = *No protein band reacted in the immunoblot.*

Note. * "negative control sera" were from volunteers who had no history of giardiasis.

Figures 11 to 16 show the protein bands that reacted with antibody in human sera. Antibody in human sera reacted with fewer bands than did the rabbit immune serum. The immune response of humans to *Giardia* infection is clearly complex and varies between

individuals.

*Giardia* proteins which elicited an antibody response in some patyents did not invariably do so in other patients. However, three proteins (200 kDa, 62 kDa, and 42 kDa) elicited an IgG antibody response in many of the clinically diagnosed samples. In contrast, the negative control sera did not react with the 200 kDa protein. However, four of ten "negative control" serum samples reacted with the 62 kDa protein and two reacted with the 42 kDa protein.

Figure.6 Total *Giardia* trophozoite protein electrophoresed in 5% polyacrylamide gel.

The trophozoite protein was electrophoresed at different protein concentrations in each lane.

(From left to right) 10, 20, 40, 60, 80, 100, 120,140, 160, 180, 200, 220, 240, 260 μg of protein per track. Note that the gel front is darkly stained which indicates that it contains many low molecular weight proteins which were not separated at this concentration.

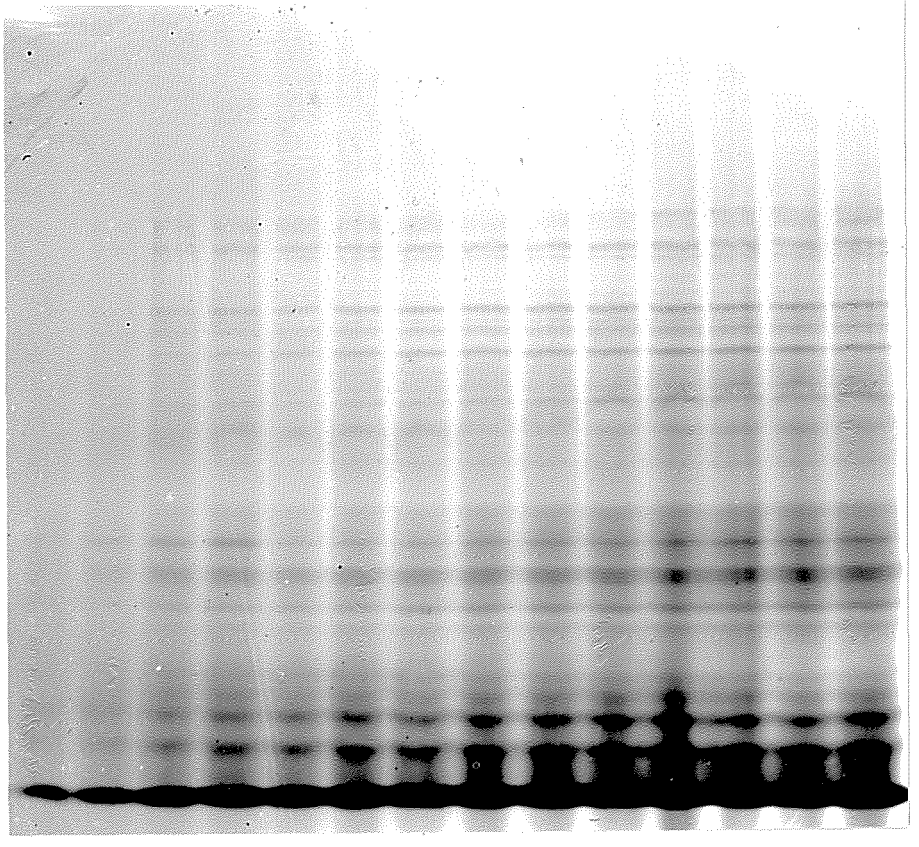


Figure 7 Total *Giardia* trophozoite proteins electrophoresed in 10% polyacrylamide gel.

The total proteins were electrophoresed at different concentrations in each lane.

(From left to right) 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260 μg of protein per track. Note that this gel concentration gave good differentiation of the proteins. The arrow shows the optimal protein concentration which gave the best resolution of the protein bands. About 30 protein bands were separated in the gel.

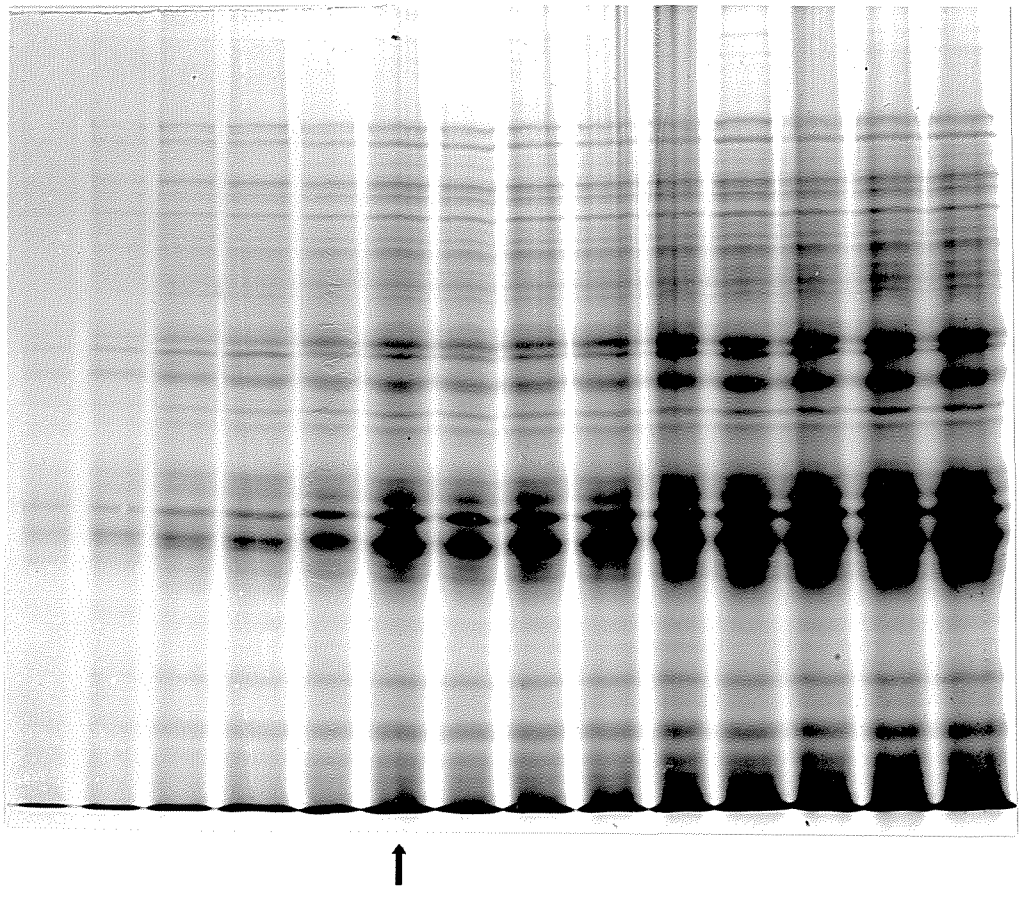


Figure8 Total *Giardia* trophozoite proteins electrophoresed in a 12.5% polyacrylamide gel.

The total proteins were electrophoresed at different protein concentrations in each lane.

(From left to right) 10, 20, 40, 60, 80, 100, 120,140, 160, 180, 200, 220, 240, 260 μg of protein per track.

Note that high molecular weight proteins were not well differentiated.

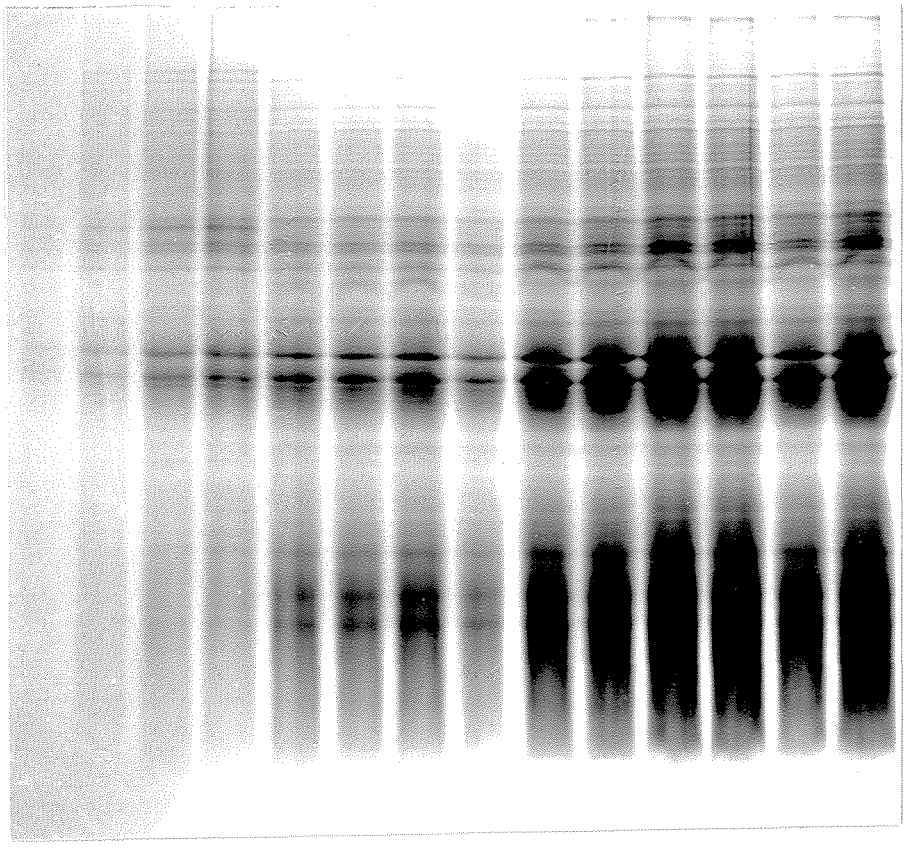


Figure 9 SDS-PAGE of total *Giardia* trophozoite protein in parallel with high and low molecular weight protein markers

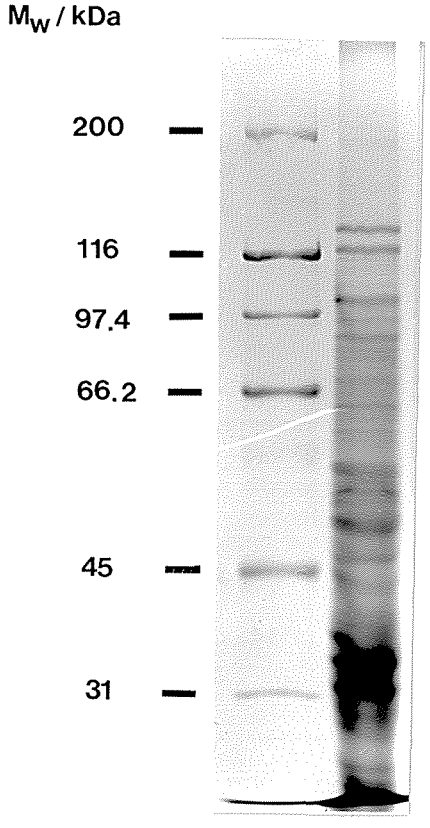
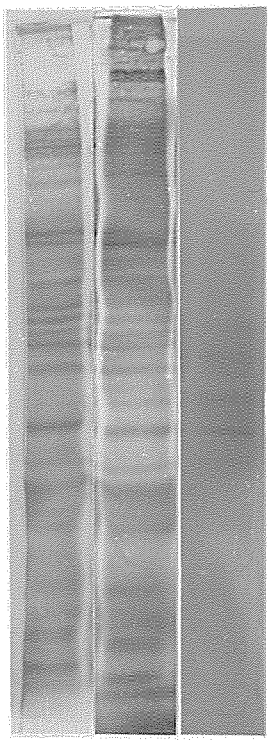

$M_W$ / kDa
200
116
97.4
66.2
45
31

Figure 10 Immunoblotting of total *Giardia* trophozoite proteins with rabbit sera.

Figure.10(i) Rabbit anti-cyst sera.

Figure.10(ii) Rabbit anti-trophozoite sera

Figure.10(iii) Normal rabbit sera.



(i) (ii) (iii)

Figure.11 Immunoblotting of *Giardia* trophozoite proteins with human IgG antibody from clinically diagnosed cases of giardiasis.

Each lane represents the reaction of a different serum sample. (From left to right) 09009; 300; 09007; 09012; 306; 314; 09011; 09010; 305; 09006; 09004; 09005; 09008.

Note the lack of uniformity of the human IgG response to *Giardia* infection.

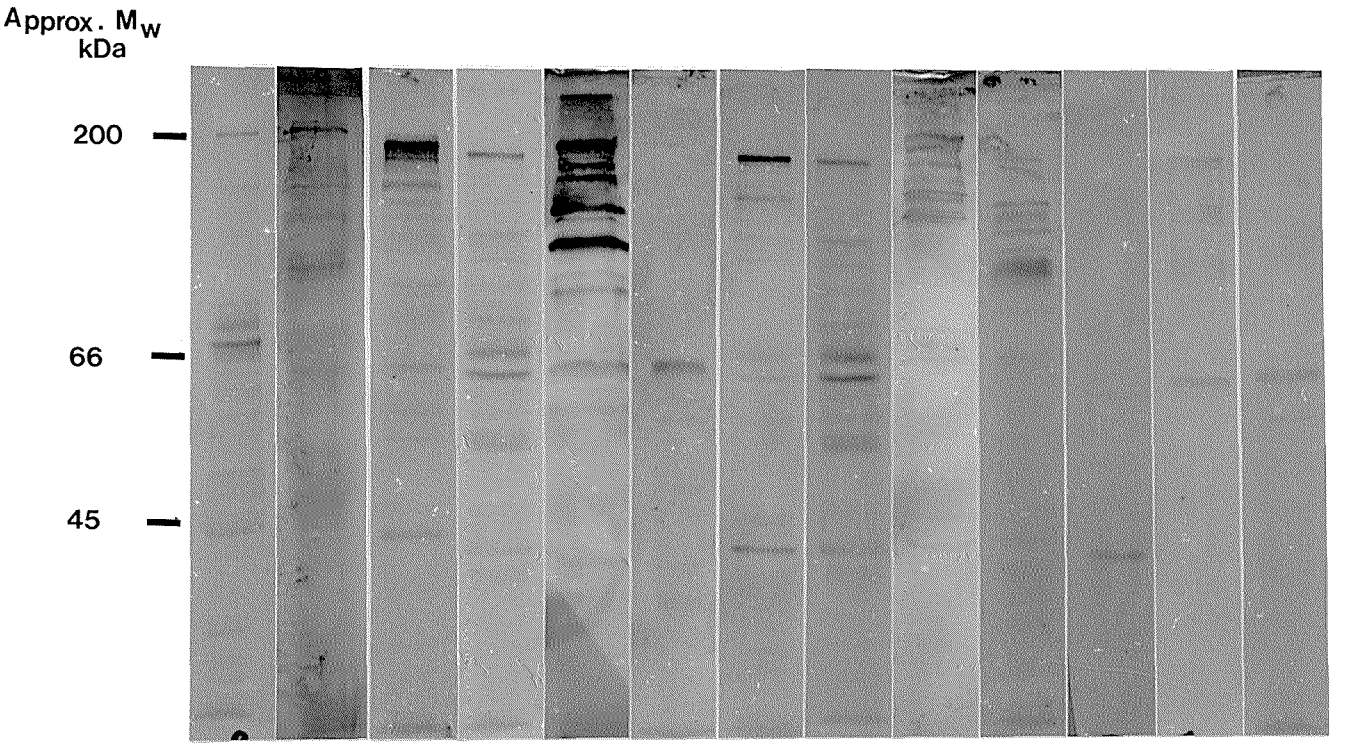

Approx. $M_W$
kDa
200
66
45

Figure.12 Immunoblotting of *Giardia* proteins with human IgG antibody from "negative control" sera.

Each lane represents the reaction of a different serumsample. (From left to right) 350; 351; 348; 349.

Note that IgG antibody was detected in a few "negative" control samples but the reaction was with fewer proteins as compared to the serum samples from clinically diagnosed cases of giardiasis.

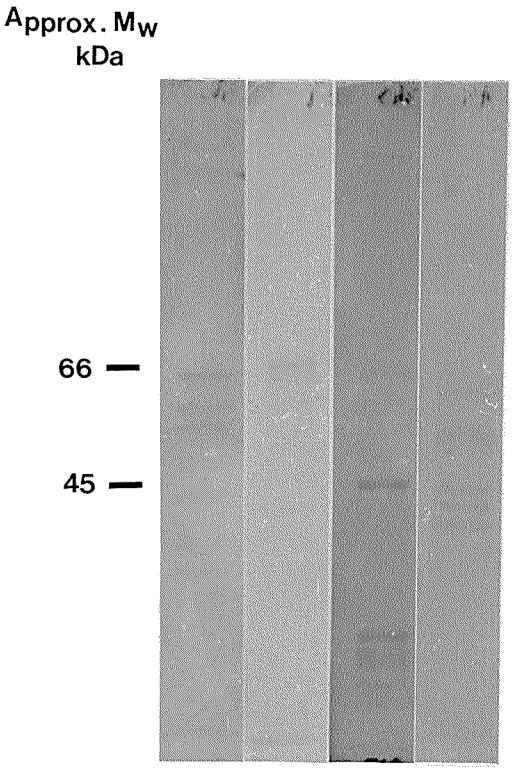

Approx. Mw
kDa
66
45

Figure.13 Immunoblotting of *Giardia* trophozoite proteins with IgA antibody from clinically diagnosed cases of giardiasis.

Each lane represents the reaction of a different serum sample. (From left to right) 09012; 314; 09007; 09010; 09004.

Note that the IgA antibody reacted only with a few bands and the reaction was weak.

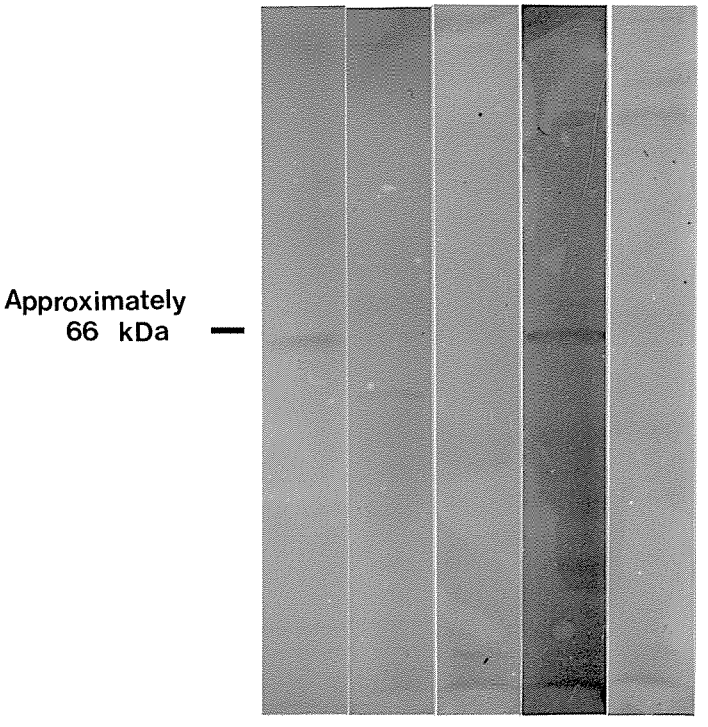

Approximately
66 kDa

Figure.14 Immunoblotting of *Giardia* trophozoite proteins with human IgA antibody from "negative control" sera.

Each lane represents the reaction of a different serum sample. (From left to right) 355; 350.

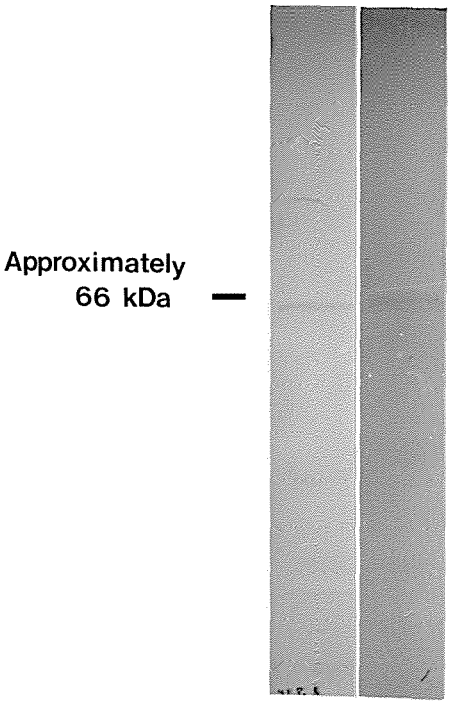

Approximately
66 kDa

Figure.15 Immunoblotting of *Giardia* trophozoite proteins with human IgM antibody from clinically diagnosed cases of giardiasis.

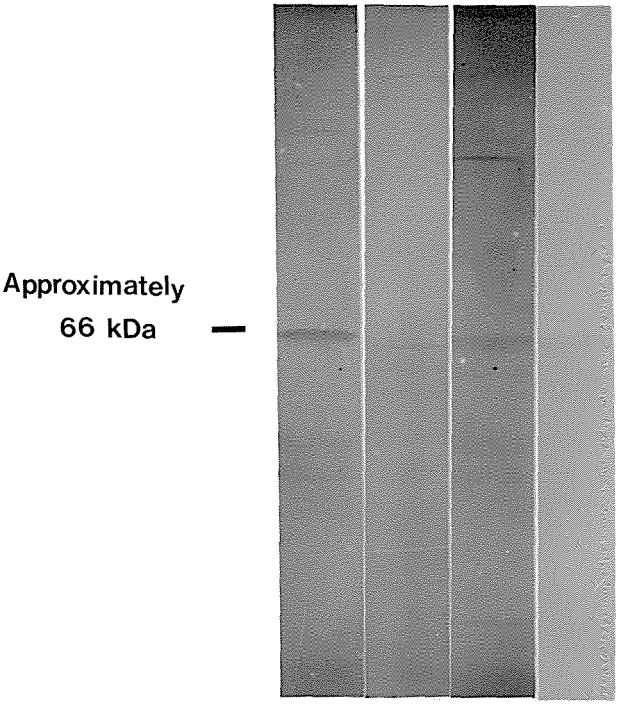
Each lane represents the reaction of a different serum sample. (From left to right) 09005; 314; 09004.

Note that the IgM antibody reacted only with a few protein bands.

Figure.16 Immunoblotting of *Giardia* trophozoite proteins with human IgM antibody from "negative control" sera.

Each lane represents the reaction of a different serum sample. (From left to right) 356; 350; 354; 353.

Note that all four sera reacted with a 62kDa protein but the reaction was weak.


Approximately
66 kDa

# 4. Discussion.

Laboratory diagnosis of infectious diseases usually involves one of two general methods. It can involve the demonstration of the causative organism or associated antigens or immunological methods can be used. In the latter case, the detection of an antibody rise or alternatively a raised level of IgM antibody can give an affirmative diagnosis.

The first of these approaches is used for giardiasis by most diagnostic laboratories. It typically involves a microscopic examination of stools for the presence of *Giardia* trophozoites and/or cysts. The method requires experienced personnel, is labour intensive and has only a sensitivity of twenty to fifty percent (Burke, 1977; Wolfe, 1978; Healy, 1979). This low sensitivity may be due to the fact that *Giardia* cysts are shed intermittently. To increase the rate of detection, examination of three stool samples taken on alternate days is recommended (Burke, 1975; Burke, 1977; Healy, 1979; Wolfe, 1984).

The detection of *Giardia* antigens in stool samples as a diagnostic method has recently been the focus of investigations (Table 1) and a diagnostic test, the "ProSpecT" test detects a *Giardia*-specific antigen (GSA 65). This test is available commercially but it is not yet clear how sensitive and specific it will prove to be.

The present investigation represents a different approach to diagnosis of giardiasis and attempts to develop a serological test for

this purpose. Methods used by other investigators to detect *Giardia*-specific antibodies include the immunoprecipitation test, the indirect immunofluorescence test, the EIA test and immunoblotting (Table 2 and Table 4). These methods have not been entirely successful as far as diagnosis of infection is concerned. However, we decided to investigate the use of the EIA test, since this test is potentially sensitive, accurate, quantitative and relatively easy to perform. It is also able to distinguish between different immunoglobulin classes and can be completed in a few hours.

Initial investigations were concentrated on the most appropriate antigen to use. Frozen and thawed trophozoites, sonicated trophozoites, trophozoite membranes and live trophozoites were compared. Trophozoite membranes and sonicated trophozoites gave inconsistent results and, furthermore, the antibody titres were low (Table 10). Frozen and thawed trophozoites and live trophozoites also gave low antibody titres and inconsistent results although most investigators use these as antigen.

In the course of our work, it was observed that few trophozoites remained attached to the microtitre well following the first wash in the EIA procedure. Another investigator (Smith *et al*, 1981) reported the same observation following the use of live trophozoites but did not suggest a solution to the problem.

The inability of the trophozoites to remain adsorbed to the wells following washing could account for our inadequate result. Consequently, a method was required to ensure that the

trophozoites remained attached. Poly-L-lysine is known to promote cell adsorption to surfaces so we investigated its ability to promote trophozoite attachment. Wells were coated with poly-L-lysine before addition of trophozoites. This caused the trophozoites to remain attached throughout the EIA procedure and consequently this method was used in subsequent tests. This resulted in an increase in the sensitivity and reproducibility of the assay (Table 12).

Since it appeared possible that different antigens predominate on trophozoites and cysts, we also investigated *Giardia* cysts as antigen in an EIA test. *Giardia* cysts like trophozoites also failed to remain adsorbed to the microtitre well in the EIA test so poly-l-lysine was used to coat the wells. This was successful in ensuring that the cysts withstood washing. EIA tests with *Giardia* cysts were relatively sensitive and reproducible (Table.12).

The above preliminary experiments were conducted with hyperimmune sera prepared in mice. Having established a protocol we then used it to detect antibody in human sera.

Human sera was collected from patients who were clinically diagnosed as having giardiasis. These sera were arbitrarily grouped as "convalescent" or "current infection" sera. "Convalescent" sera were from patients who had been diagnosed some time before serum was collected and had already been treated for giardiasis whereas "current infection" sera were from patients who had just then been diagnosed and had not yet been treated for the disease. In addition, "negative control" sera were obtained from volunteers who had no history of giardiasis. However, these

sera probably included some persons with earlier subclinical and thus undiagnosed infections.

*Giardia*-specific antibody was assayed using both trophozoites and cysts as antigen because different antigens may predominate at the two stages and this may affect the immune response. Serological tests using *Giardia* cysts have not been reported by other workers. Since whole cysts and live trophozoites were used in this investigation, the test detected antibody directed against surface antigens. The antibody titres obtained in the EIA tests were similar, which suggests that *Giardia* cysts and trophozoites share many surface antigens.

The IgG antibody titre in "convalescent" sera showed a slight elevation (not statistically significant) as compared to "current infection" sera and "negative control" sera (Table 13(a) to 14(c); Fig 4 and 5; Table 15). Increased IgG antibody has been reported by other investigators (Ridley and Ridley 1975; Radulescu *et al,* 1976; Visveswara *et al*, 1980; Smith *et al,* 1981; Goka *et al,* 1986; Miotti *et al,* 1986; Nash *et al,* 1987; Janoff *et al*, 1988) and the presence of IgG antibody in convalescent sera is not surprising since this antibody is present at a later stage of most infections and tends to persist.

The IgM antibody titres of "current infection" sera were slightly raised as compared to sera from the other two groups. This confirms the observation of other investigators. Goka *et al* (1986) and Gandhi *et al* (1989) detected raised IgM levels in the

sera of giardiasis patients but this only persisted for six weeks. It is interesting to note that the "current infection" sera used in this test were from patients who had been diagnosed with having giardiasis for six weeks or less; so this study is consistent with the earlier observation.

IgA antibody was not detected in any of the human sera used in this investigation. There have been conflicting reports by other investigators (Ridley and Ridley, 1976) on the *Giardia*-specific serum IgA antibody titres in patients but recent investigations by Nash *et al* (1987) Janoff *et al* (1988) and Birkhead *et al* (1989) showed an increase of *Giardia*-specific IgA antibody in the sera of patients.

The role played by serum IgA antibody in giardiasis is still unclear. Since *Giardia* causes a surface infection and is not invasive, it might be expected that IgA antibody production would play a critical role in controlling infection. If so, the IgA antibody must be produced and released locally since the serum titres were low.

A major aim of this investigation was to find if serological diagnosis of *Giardia* is a practical proposition. The slightly elevated IgM antibody titres (Fig 4 and 5; Table 15) detected in the serum of persons currently infected with *Giardia* cannot be used to reliably distinguish a current from a previous infection of *Giardia*. More research in this area is justified and it should be stressed that this project was handicapped by the lack of availability

of an adequate number of human sera particularly from acute giardiasis cases.

A complex organism like *Giardia* inevitably produces many proteins. SDS-PAGE of total *Giardia* proteins (Fig. 8) indicated that the organism has at least thirty proteins and it has probably many more because its total genomic DNA is about $8.0x10^4$ kilobases.

All *Giardia* proteins are in theory, potential antigens for the natural host or following immunisation of any mammalian species with the organism. Thus when rabbits were immunised with trophozoites and sera were used in immunoblots, it was observed that about thirty proteins elicited the production of detectable antibody (Fig. 9).

*Giardia* is not an invasive organism, however, and it might be expected that in a natural infection only a small number of antigens (mainly surface antigens) will elicit an antibody response. This point was investigated by Taylor and Wenman (1987) who used "current infection" human sera to identify the antigens which were most effective in producing an antibody response in humans. They identified four major antigens of molecular weights 27kDa, 28kDa, 31kDa and 55kDa. The 31kDa protein elicited the strongest antibody response and was found to be of the same molecular weight as a *Giardia* protein found in the ventral disc and designated "giardin" by Crossley and Holberton (1985). The 55kDa protein may be *Giardia* tubulin (Torian *et al*, 1984) which is a structural protein of both *Giardia* flagella and the ventral disc.

Our experiments with immunoblotting of human sera were undertaken to confirm these results. Our results indicate, however, that the immune response to *Giardia* infection varies greatly between individuals although the most consistent IgG antibody response was to three proteins: 200 kDa, 62 kDa and 42 kDa. On the assumption that our 62 kDa protein does not represent *Giardia* tubulin (55 kDa), none of our human sera showed a marked immune response to "giardin" or to any of the antigens found by Taylor and Wenman (1987). We therefore conclude that the human immune response to a natural infection of *Giardia* is complex and more work is needed to clarify the major antigens involved in the immune response.

A low percentage of human sera from clinically diagnosed patients had IgA and IgM antibody which reacted to *Giardia* proteins in an immunoblot. IgA and IgM antibody, however, were also detected in "control" sera. In particular, a 62kDa protein reacted both with sera from patients and "control" sera.The presence of antibody in the "negative control" sera is probably due to subclinical *Giardia* infections.

# 5. Appendices.

## Media, buffers and reagents used in this thesis.

### 5.1 Appendix I

General media and reagents.

#### (i) Percoll in 0.1M Phosphate Buffer*.

| | Specific gravity | | |
|---|---|---|---|
| | 1.020 | 1.045 | 1.090 |
| Stock percoll (Pharmacia™) *sp.gr 1.130* | 8.3ml | 29.17ml | 66.7ml |
| 0.1M Phosphate Buffer | 91.7ml | 70.83ml | 33.3ml |

**Based on Schaefer et al, 1986*

#### (ii) 0.1M Phosphate Buffer*. (Specific gravity 1.010)

Solution A: 27.6g $NaH_2PO_4.H_2O$/1000ml solution.

Solution B: 71.7g $Na_2HPO_4.12H_2O$/1000ml solution.

| | To prepare | | | |
|---|---|---|---|---|
| | 50 ml | 100 ml | 200 ml | 500 ml |
| Solution A (ml) | 7 | 14 | 28 | 70 |
| Solution B (ml) | 18 | 36 | 72 | 180 |
| 0.01% Tween 20 in distilled water (ml) | 25 | 50 | 100 | 250 |

**Based on Schaefer et al, 1986*

**(iii) Phosphate Buffered Saline (PBS).**

Dulbecco's PBS is free of calcium and magnesium, since these cations promote cell clumping.

| | |
|---|---|
| NaCl | 8.0g |
| KCl | 0.2g |
| $Na_2HPO_4$ | 1.15g |
| $KH_2PO_4$ | 0.2g |

The components were dissolved sequentially in 800ml of distilled water and the pH was adjusted to 7.3 with 1M HCl or NaOH. The volume was then made up to 1000ml with distilled water and the solution was sterilised at 121°C for fifteen minutes. Store at 4°C.

**(iv) TY1-S-33 Encystation Medium** (Bile enriched)

The bile concentration was increased to 5.0g/litre in place of 0.8g/litre contained in TY1-S-33 growth medium.

The encystation medium was then prepared using the same method as for TY1-S-33 growth medium.

## (v) TY1-S-33 Growth Medium

| | |
|---|---|
| Trypticase Soy Broth (BBL. No. 11768) | 20.0g |
| Yeast Extract Powder (Difco No. 0127-01-7) | 10.0g |
| Glucose | 10.0g |
| NaCl | 2.0g |
| $K_2HPO_4$ | 1.0g |
| $KH_2PO_4$ | 0.6g |
| L-Cysteine Monohydrochloride (Sigma No.C-7880) | 1.5g |
| Ferric Ammonium Citrate (Brown Pearls) | 0.023g |
| L-Ascorbic Acid | 0.2g |
| Bile Bacteriological (Sigma No. B-8381) | 0.8g |
| Benzyl Penicillin | 0.06g |
| Gentamycin Sulphate | 0.05g |
| Vancomycin | 0.02g |
| [a]Bovine Serum | 100.0ml |
| [b]NCTC 135 (Sigma No. N3262) | 100.0ml |
| Distilled water to | 1000.0ml |

The components were dissolved in distilled water to give a final volume of one litre. The medium was adjusted to pH 7.0-7.2 with 5M NaOH and clarified by positive pressure filtration through six pieces of Whatman™ filter paper followed by non-sterile 0.2µm nitrocellulose membrane filter.

A final sterile filtration of the clarified medium was then performed by filtering through a sterile 0.2µm nitrocellulose

membrane filter (sterilised at 121°C for fifteen minutes within the filter unit).

The sterile medium was stored at 4°C until used. (Maximum length of storage - 14 days.)

Notes: [a] Bovine serum was prepared in the laboratory by the centrifugation of blood obtained from the AFFCO Meat Export Works in Feilding.

[b] NCTC 135 (Sigma) The medium was prepared as per manufacturers instructions.

## 5.2 Appendix 2.

Solutions and reagents used in the EIA.

### (i) Phosphate Buffered Saline (PBS)

| | |
|---|---|
| NaCl | 8.0g |
| KCl | 0.2g |
| $Na_2HPO_4$ | 1.15g |
| $KH_2PO_4$ | 0.2g |

The components were dissolved sequentially in 800ml of distilled water and the pH was adjusted to 7.3 with 1M HCl or NaOH.

The volume was then made up to 1000ml with distilled water and the solution was sterilised at 121°C for fifteen minutes. Store at 4°C.

**(ii) Phosphate Buffered Saline - Bovine Serum Albumin (PBS - BSA).**

PBS with 1% w/v Bovine Serum Albumin (Sigma)

| | |
|---|---|
| Bovine Serum Albumin | 10.0g |
| PBS | 1000.0ml |

Preserved with 0.02% $NaN_3$ (0.2g/litre)
Store at 4°C.

**(iii) PBS-BSA-Tween**

PBS with 1% w/v BSA and 0.5% v/v Tween 20

| | |
|---|---|
| Bovine Serum Albumin | 10g |
| Tween 20 | 5g |
| PBS | 1000.0ml |

Preserve with 0.02% $NaN_3$ (0.2g/litre)
Store at 4°C.

### (iv) Carbonate-Bicarbonate Buffer (0.05M)

| | |
|---|---|
| $Na_2CO_3$ (anhydrous) | 1.38g |
| $NaHCO_3$ | 3.11g |
| $NaN_3$ | 0.2 g |

Distilled water was added to a total volume of 1000ml and adjusted to pH 9.5 with 5M NaOH. Store at 4°C.

### (v) Substrate Diluent

9.7% v/v diethylamine (BDH) in distilled water. pH adjusted to 9.8 with concentrated HCl. Preserve with $NaN_3$ to 0.02% final concentration.

### (vi) Substrate Solution

Constitute required volume to a ratio of 1mg p-nitrophenyl phosphate per 1ml of substrate diluent.

## 5.3 Appendix III

Solutions and reagents used in the immunoblotting procedure.

### (i) Horseradish Peroxidase (HRP) Substrate Solution

This solution is composed of two parts and was used immediately after mixing.

Part A:

60mg of 4-chloro-1-naphthol (stored at -20°C) was added to 20ml of cold methanol (AR grade). This solution was dispensed into a dark bottle and stored at -20°C for a few minutes until required.

Part B:

60µl of hydrogen peroxide (30% w/v) was added to 100ml of 1xTBS at room temperature*.

Parts A and B were then mixed immediately to produce the working solution and this was used immediately.

*Note: If the working solution (part A plus part B) is stored at 0-4°C, the substrate will precipitate out of the solution and the sensitivity of the working solution may be substantially decreased. At the time the solutions were mixed (part A and part B), the substrate (Part A) was at -20°C and the 1xTBS-hydrogen peroxide solution was at room temperature, which maximises their activity

and prolongs their useful life.

### (ii) Transfer Buffer

| | |
|---|---|
| Tris (Hydroxymethyl) Aminomethane | 12.12g |
| Glycine | 57.60g |
| Methanol (Analytical Grade) | 800.0ml |
| Distilled water to | 4000.0ml |

Note: This solution does not require adjustment to pH 8.3 and the addition of HCl for minor pH corrections should be avoided as it will cause an ion imbalance

### (iii) Tris Buffered Saline (1xTBS)

| | |
|---|---|
| Tris (Hydroxymethyl) Aminomethane | 4.84g |
| NaCl | 58.48g |
| 12N HCl adjust to | pH 7.5 |
| Distilled water to | 2000.0ml |

Stored at room temperature.

### (iv) Tween-20 Tris Buffered Saline (1xTTBS)

| | |
|---|---|
| Tris (Hydroxymethyl) Aminomethane | 4.84g |
| NaCl | 58.48g |
| 12N HCl adjust to | pH 7.5 |
| Tween-20 (polyoxethylene sorbitan monolaurate) | 1.0ml |
| Distilled water to | 2000.0ml |

Stored at room temperature.

### (v) 1xTTBS-1% Gelatine (Antibody Buffer)

| | |
|---|---|
| Tris (hydroxymethyl) aminomethane | 0.48g |
| NaCl | 5.84g |
| 12N HCl adjust to | pH 7.5 |
| Tween-20 | 0.10ml |
| Gelatin (EIA purity reagent, Bio-Rad™) | 2.00g |
| Distilled water to | 200.ml |

This solution was made immediately prior to use. The gelatin was dissolved in pre-warmed (37°C) 1xTTBS and cooled to 28°C before use.

### (vi) 1xTTBS-3% Gelatin (Blocking Solution)

| | |
|---|---|
| Tris (hydroxymethyl) aminomethane | 0.24g |
| NaCl | 2.92g |
| 12N HCl adjust to | pH 7.5 |
| Gelatin (EIA purity reagent, Bio-Rad™) | 3.0 g |
| Tween-20 | 0.05ml |
| Distilled water to | 100.0 ml |

This solution was made immediately prior to use. The gelatin was dissolved in pre-warmed (37°C) 1xTTBS and cooled to 28°C before use.

## 5.4 Appendix IV

Solutions and reagents used in the SDS-PAGE.

### (i) Ammonium Persulphate Solution

| | |
|---|---|
| Ammonium Persulphate | 0.1g |
| Distilled water | 1.0ml |

This solution was made fresh before use.

**(ii) Bovine Serum Albumin**

| | |
|---|---|
| Bovine Serum Albumin | 10.0mg |
| 0.2M NaOH to | 10.0ml |

Made fresh with every batch of Coomassie Blue Reagent (G-250).

**(iii) Bromophenol Blue Tracking Dye** (x10)

| | |
|---|---|
| Bromophenol Blue | 0.05g |
| Glycerol | 40.0ml |
| Distilled water to | 50.0ml |

The tracking dye was diluted 10-fold in the SDS sample buffer/protein sample mixture prior to boiling.

**(iv) Coomassie Blue Reagent** (For protein determination)

| | |
|---|---|
| Coomassie Blue G-250 | 0.1g |
| 95% Ethanol | 50.0ml |
| 85% (w/v) Phosphoric Acid | 100.0ml |
| Distilled water to | 1000.0ml |

Coomassie Blue G-250 was dissolved in a mixture of ethanol and phosphoric acid and diluted to 1000ml with distilled water. The dye reagent was filtered through two layers of Whatman™ No.1

filter paper and stored at room temperature in a dark bottle.

**(v) Coomassie Blue Stain.** (For SDS-PAGE protein gel)

| | |
|---|---|
| Coomassie Brilliant Blue R-250 | 0.4g |
| Isopropanol | 250.0ml |
| Glacial Acetic Acid | 100.0ml |
| Distilled water to | 1000.0ml |

The stain was filtered through two layers of Whatman™ No.1 filter paper before being stored at room temperature.

**(vi) Lower Tris Buffer** (pH 8.8)

| | |
|---|---|
| Trizma Base (Sigma) | 18.17g |
| 10% SDS Solution in distilled water | 4.0ml |
| 12N HCl to | pH 8.8 |
| Distilled water to | 100.0ml |

Trizma Base was added to 70ml of distilled water and the reaction adjusted to the required pH with 12N HCl. The 10% SDS solution was then added and the volume was made up to 100ml. The buffer was filtered through two layers of Whatman™ No.1 filter paper and stored at 4°C.

**(vii) Running Gel Solution.**

| | |
|---|---|
| Acrylamide | 30.0g |
| Methylene-bis-Acrylamide | 0.5g |
| Distilled water to | 100.0ml |

Acrylamide was dissolved in 70ml of distilled water and allowed to return to room temperature. Methylene-bis-Acrylamide was dissolved in the acrylamide solution and the volume was made to 100ml. The solution was filtered through a double layer of Whatman™ No.1 filter paper and stored at 4°C for no more than six weeks.

**(viii) SDS-Sample Buffer** (x4)

| | |
|---|---|
| ß-mercaptoethanol | 10.0ml |
| SDS | 6.0g |
| Upper Tris Buffer | 25.0ml |
| Distilled water to | 100.0ml |

This solution was diluted 4-fold when added to the protein sample.

**(ix) Stacking Gel Solution**

| | |
|---|---|
| Acrylamide | 30.0g |
| Methylene-bis-Acylamide | 1.6g |
| Distilled water to | 100.0ml |

Acrylamide was dissolved in 70ml of distilled water and allowed to return to room temperature. Methylene-bis-Acrylamide was dissolved in the acrylamide solution and the volume was made to 100ml. The solution was filtered through a double layer of Whatman™ No.1 filter paper and stored at 4°C for no more than six weeks.

**(x) Tris-Glycine Reservoir Buffer** (pH 8.3)

| | |
|---|---|
| Trizma Base (Sigma) | 6.07g |
| Glycine | 28.8g |
| SDS | 2.0g |
| Distilled water to | 2000.0ml |

This solution was stored at room temperature.

**(xi) Upper Tris Buffer** (pH 6.8)

| | |
|---|---|
| Trizma Base (Sigma) | 6.06g |
| 10% Solution of SDS in distilled water | 4.0ml |
| 12N HCl to | pH 6.8 |
| Distilled water to | 100.0ml |

Trizma Base was added to 70ml of distilled water and the reaction adjusted to the required pH with 12N HCl. The SDS was then added and the volume was made up to 100ml. The buffer was filtered through two layers of Whatman™ No.1 filter paper and stored at 4°C.

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