

***IN VITRO CULTURE AND ISOENZYME ANALYSIS OF  
GIARDIA LAMBLIA***

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**December 1999**

***IN VITRO* CULTURE AND ISOENZYME ANALYSIS OF  
*GIARDIA LAMBLIA***

by

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(NHD. -MED. TECH)

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE (MEDICAL SCIENCE)

in the

Department of Medical Microbiology  
Faculty of Medicine  
University of Natal  
Durban

1999

*Dedicated to my late parents*

*Nomusa & Sobuthongo*

## ABSTRACT

*Giardia lamblia*, an enteric protozoan parasite, infects a large number of individuals worldwide. In South Africa prevalences ranging between 4 and 63% are documented, however, the impact of giardiasis is underresearched in this country. *Giardia* infections vary from asymptomatic carriage or a self-limiting acute symptomatic illness to chronic, debilitating malabsorption syndrome. The factors responsible for development of symptomatic versus asymptomatic infection are poorly understood. It is believed by some that host factors determine the clinical outcome of infection. On the other hand, the possibility of the existence of pathogenic and non-pathogenic strains (a situation akin to *Entamoeba spp.*) remains to be explored. One requirement for investigation of the potential contribution of strain differences to pathogenicity of infection is establishment of laboratory cultures of different strains isolated from symptomatic and asymptomatic patients. The present study was undertaken to develop and modify existing methods for: (i) establishment of laboratory cultures of *Giardia* trophozoites from excystation of faecal cysts, (ii) long-term maintenance and cryopreservation of the cultures and (iii) preliminary characterisation methodology.

One thousand and twenty-three stool specimens were collected from day care centres, hospital wards and Hlabisa hospital laboratory. A further 6246 were retrieved from the Microbiology Laboratory at King Edward VIII Hospital and screened by direct wet preparation. *Giardia* was detected by light microscopy following formol-ether concentration (127 of 1023 samples) or direct examination of wet preparations (78 of 6246 samples). Cysts were purified from

the positive specimens by sucrose gradient separation. Viability was assessed by a dye-exclusion method (eosin).

Three *in vitro* excystation techniques were employed in an attempt to obtain trophozoites for initiation and establishment of viable cultures thereof. Culture conditions were optimised using two reference strains of *Giardia*, WB & H7 (obtained from the National Institutes of Health, USA). The percentage excystation ranged between 0-42% with all the *in vitro* methods of excystment. Excysted trophozoites remained viable in TYI-S-33 culture medium for periods ranging between 12–72 hours or up to 9 days, and gradually died, hence viable trophozoite cultures could not be established. Some culture initiates (overall 65%) were lost through overwhelming bacterial and/or fungal contaminants.

An animal model was subsequently set up in which C57BL/6 and *Praomys (Mastomys) coucha* mice were used for *in vivo* excystation experiments. 1-3 day old suckling mice were intragastrically injected with  $10^5$ -cysts/ ml in 0,1ml distilled water. Trophozoites were retrieved from the stomachs of infected mice 7-10 days after inoculation and cultivated in TYI-S-33 medium. Six local isolates were axenised using the *in vivo* excystation method. They have been maintained for more than 15 months in culture after stabilates and lysates of confluent growths had been cryopreserved in Liquid Nitrogen. Successful (100%) retrieval of the cryopreserved cultures has been achieved.

Seven isoenzyme electrophoresis systems have been set up and optimised. Reproducible results were obtained in six of the enzymes. Some differences in banding patterns of the enzymes were demonstrated.

## **Declaration**

This study represents original work by the author and has not been submitted in any form to another University. Where assistance was received and use of the work of others was made it was duly acknowledged in the text.

The work described in this dissertation was carried out in the South African Medical Research Council (Durban) under the supervision of Prof. TFHG Jackson and Prof A. W. Sturm as co-supervisor.

Ethical approval was obtained from the Faculty of Medicine Ethics Committee of the University of Natal.

**Parts of this work were presented at the following conferences:**

1. *In Vitro* Culture of *Giardia lamblia*. 1996. Kwitshana ZL, Reddy SG, Sturm AW & Jackson TFHG. Presentation at the 10<sup>th</sup> Annual Congress of the Parasitological Association of Southern Africa. 5-6 September 1996. Waterfront, Cape Town.
2. *In vitro* culture and isoenzyme analysis of *Giardia lamblia*. 1998 Kwitshana ZL, Reddy SG, Sturm A.W & Jackson TFHG. 12<sup>th</sup> Annual Congress of the Parasitological Association of Southern Africa. 9-11 September 1998. Mont-Auxources. Drakensburg.
3. Determination of viability of *Giardia lamblia* cysts: vital staining, *in vitro* and *in vivo* excystation. Kwitshana Z.L.; Reddy S.G.; Sturm A.W. & Jackson T.F.H.G. 14<sup>th</sup> Annual Congress of the Parasitological Association of Southern Africa. 19-23 October 1999. Augrabies Falls. Eastern Cape.

**Publications.**

*In Vitro* Culture of *Giardia lamblia*. (Abstract). 1996. Kwitshana Z.L. Jackson T.F.H.G. Journal of South African Veterinary Association 67(4): 175

*In vitro* culture and isoenzyme analysis of *Giardia lamblia*. 1998. (Abstract) Kwitshana Z.L.; Reddy S.G.; Sturm A.W. & Jackson T.F.H.G. Journal of South African Veterinary Association. 69:6



## ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to the following individuals for their assistance in preparation of this thesis:

- Professor TFHG Jackson, (Supervisor) Regional Executive Director, S.A. Medical Research Council, Durban for supervising and affording me the opportunity to do this work. His constructive criticism, constant encouragement, expert advice, support and guidance are highly appreciated.
- Professor AW Sturm, (Co-supervisor) Head of Department, Microbiology, University of Natal, Medical School, for his expert Microbiology contribution, advice and encouragement.
- Mr. Selvan Reddy for his scientific input, patience, encouragement, support and professional criticism.
- The Superintendent of King Edward Hospital for giving permission to collect stool specimens from patients at the hospital.
- Professor J Coovadia Head of Paediatrics Dept. for allowing stool specimen collection from children's wards.
- Dr. T.E.Nash and Mr. J.T.Conrad of the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIH), Maryland, for their invaluable assistance in the establishment of the animal model and kind supply of *Giardia* reference strains.
- The Medical Research Council and the University of Natal for providing financial support for this project.
- Staff in Medical Microbiology at King Edward as well as Hlabisa hospital Laboratories for their assistance with retrieval of stool specimens.
- Mr Sathyanand Suparsad for his kind assistance with isoenzyme electrophoresis. Celia Anderson, Shirley Epstein, Annalies Gumede & Welcome Mkhasibe for their assistance during stool collections and all the staff at the MRC Amoebiasis Research Unit for their moral support.
- Ms. Charmine Bux & Mr James Wesley-Smith for their assistance with invertoscope and phase contrast photography respectively.
- Albert Hirashen and all the technical staff at the Medical Media Services for their assistance with photography.
- Staff at the MRC's Animal Facility: A. Saikoolal, Rould Cibane & Joseph Shozi for their assistance with animal care.

- Personnel and children at the following day care centres: Othandweni Place of Safety, Kideo, & Amanzimtoti for cooperation and assistance during stool collection.
- To my late parents for gracing me with love and education, and my sisters Thobekile, Qhu, Carol, and brothers Sbongseni and Zama for their support and encouragement.
- Finally, my profound gratitude to my husband Sindile for his enduring patience, encouragement and support; my children Anele and Siyabulela who had to learn to cope on their own at a very tender age.

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## ABBREVIATIONS AND UNITS OF MEASUREMENTS

|          |  |
|----------|--|
| °        | degrees                                    |
| °C       | degrees Centigrade                         |
| DNA      | Deoxyribonucleic acid                      |
| Fig.     | figure                                     |
| GPI      | Glucose Phosphate Isomerase                |
| G6PD     | Glucose-6-phosphate dehydrogenase          |
| GOT      | glutamate oxaloacetate transaminase        |
| g        | gram                                       |
| G        | gravity                                    |
| HK       | Hexokinase                                 |
| KEH      | King Edward VIII Hospital                  |
| LN       | liquid nitrogen                            |
| ME       | Malic enzyme                               |
| mg/ml    | microgram per millilitre                   |
| ml       | microlitres                                |
| mg/ml    | milligram per millilitre                   |
| ml       | millilitre                                 |
| MIC      | minimum inhibitory concentration           |
| min      | minute                                     |
| M        | molar                                      |
| PGM      | Phosphoglucomutase                         |
| PDG      | phosphogluconate dehydrogenase             |
| pi       | post inoculation                           |
| RNA      | Ribonucleic acid                           |
| S.G.     | Specific gravity                           |
| TYI-S-33 | Trypticase yeast iron serum culture medium |
| u/ml     | units per millilitre (activity units)      |
| VSPs     | variable surface proteins                  |
| v/v      | volume per volume                          |
| W/v      | weight per volume                          |

# CHAPTER 1

## PRE-AMBLE

*Giardia* was one of the first protozoans to be described. In 1681 van Leeuwenhoek discovered the trophozoites of this genus. The Dutch microscopist made glass lenses and set them into metal frames which he made into simple microscopes. Among the many specimens he examined with these microscopes was his own diarrhoeic stool. Clifford Dobell, an English scientist translated van Leeuwenhoek's findings of the stool examination thus:

*"My excrement being so thin, I was.... persuaded to examine it....wherein I have sometimes also seen animacules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood globule, but all of the one and the same make; their bodies were somewhat longer than broad and the belly, which was flatlike, furnished with sundry little paws, wherewith they made such a stir in the clear medium and among the globules, that you might e'en fancy you saw a pissabed running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but slow progress"*

(Dobell, 1920).

Willem Lambl (cited in Filice, 1952) redescribed the organisms in greater detail in 1859 and named them *Cercomonas intestinalis*. However, it was later found that this name had been pre-empted by use of the term "*intestinalis*" for another parasite in this genus by Diesing in 1850 (Filice, 1952).

In 1888 Blanchard proposed the genus name *Lambli* in honour of W. Lambl, to describe trophozoites from mammal hosts (Erlandsen & Feely, 1984) while Kunstler (Filice, 1952) had named trophozoites isolated from a tadpole *Giardia*

*agilis* in 1882. In 1914, Alexeieff stated that the *Giardia* from a tadpole described by Kunstler in 1882 and the *Lamblia* from mammals reported by Blanchard in 1888 are members of the same genus (Filice, 1952). However, the organisms became known as *Lamblia intestinalis* and *Giardia lamblia*. Subsequently, the generic name *Giardia* became more popular as it was recognised that the two were synonymous. This name then took precedence and to-date is the definitive name for the organisms in this genus.

The work of these early scientists led to the description of this protozoan parasite that colonises and proliferates in the mucosal surface of the intestine of many mammalian hosts.

### **1.1 Taxonomy and Nomenclature**

*Giardia* belongs to the Phylum Sarcomastigophora, Class Zoomastigophora and Order Diplomonadida. It is regarded as a most primitive eukaryote on the basis of its small subunit ribosomal RNA (Sogin *et al.*, 1989). However, L-arginine transport and metabolic pathways characteristically vary from prokaryotes to eukaryotes. Using these two pathways, Knoder *et al.* (1995), deduced that *Giardia* is in transition between these two Kingdoms.

Early workers based their taxonomic groupings on host specificity (Hegner, 1926b). Filice (1952) proved this to be an inadequate system of classification as more than forty species had been identified. In his report he illustrated that the *Giardia* from some hosts could excyst and set up infections in the intestine of a different species of host. For example, cysts from dogs effected infection in the

guinea-pig intestine; laboratory rats were infected by cysts isolated from human faeces and cysts from man as well as those from the rat infected a domestic fowl. He subsequently characterised constant morphological features such as cell dimensions as well as the number and shape of median bodies, as specific characteristics in addition to host occurrence. He recognised three morphologic species, *G. agilis*, (club-shaped median body) which is infective to amphibians, *G. muris* (two small rounded median bodies) from rodents and *G. duodenalis* (claw hammer shaped median body) from mammals. *G. duodenalis* has a wide host range.

In the ensuing years the taxonomic classification of *Giardia* remained in a state of flux as it does today. Lack of agreement regarding the correct specific name for the mammalian isolates, in particular the *Giardia* of human origin, greatly contributes to the taxonomic confusion. The names *G. lamblia* and *G. intestinalis* have been widely used to describe the human isolates. However, Thompson *et al.* (1990), stated that the soundly structured, reproducible and logical morphometrics scheme proposed by Filice (1952) had found widespread favour and was advocated by many leading authorities in the field. They stated that according to the Rules of Zoological Nomenclature, *duodenalis* has priority over *intestinalis* as a specific name for the vertebrate isolates since there is no justification for using the name *intestinalis*. Other authorities also express the view that the specific name "*duodenalis*" is correct for isolates of human origin; other names ("*intestinalis*" and "*lamblia*") are incorrect on taxonomic grounds. For example, Meyer (1985) concluded of other names for the "*duodenalis*" group (i.e. *G. intestinalis* or *G. lamblia*), that their use for *Giardia* in humans suggests that there is something



unique about *Giardia* in humans, which seemed, on evidence present then, not to be the case. They therefore advocate use of the specific name “*duodenalis*” for taxonomic correctness.

On the other hand, some authorities argue against use of the term *G. duodenalis* to designate a species. Their argument is based on the fact that application of biochemical and nucleic acid techniques has revealed marked genetic diversity among *Giardia* isolates that belong to the *duodenalis* group (morphologically indistinguishable). For example, a report by Erlandsen *et al.* (1990) indicated that a variety of *Giardia* species possess claw hammer shaped median bodies (thus belonging to the *duodenalis* group). However, comparative molecular typing studies such as karyotype analysis, rDNA restriction enzyme pattern (Mahbubani *et al.*, 1992) and rRNA gene base pair sequencing (Weiss *et al.*, 1992) show marked distinction between *G.lambli*a, *G.ardae* and *G.muris* (*lambli*a and *ardae* expectedly belong to the same species (*duodenalis*) and are therefore presumably similar). Therefore, according to Erlandsen (1994), Filice’s description of the two specific morphologic groups, *G.muris* (pair of small rounded median bodies) and *G.agilis* (club-shaped median body) appear to be acceptable. However use of *G.duodenalis* (claw hammer shaped median body) to designate a species is controversial. For these reasons, Erlandsen (1994) suggested that the term *G.duodenalis* should in future be restricted to the description of the morphological type of median body within trophozoites and it should not be used as a species designation because as such it is a misnomer. He argues that although some investigators indicate that *G.duodenalis* is synonymous with *G.lambli*a or *G.intestinalis*, and that this species can infect humans, birds and reptiles, such

speciation is apparently inappropriate.

Use of the name *Giardia lamblia* has for a long time been familiar in diagnostic laboratories. This concurs with the original recommendation made by Dr Stiles in 1915, which reads thus: "If you look upon the form in the rabbit as identical with that in man, *duodenalis* would be the correct specific [trivial] name. If you consider the various forms in man, rabbits, rats, etc, as distinct, then in all probability a new name should be suggested for the form that occurs in man.... I would be inclined to suggest *lamblia* as specific [trivial] name for the form in man ", (Filice, 1952).

Inexplicably, scientists subsequently regarded the *duodenalis* species name to be synonymous with *intestinalis* and *lamblia* (Erlandsen *et al.*, 1990; Hill, 1990; Adam, 1991; Flanagan 1992). Lymbery and Tibayrenc (1994), in their survey of past publications identified seven commonly used species names for this genus, based on host preference and morphology (Table 1.1).

**Table 1.1** Commonly used species names in the genus *Giardia* (Lymbery & Tibayrenc, 1994)

| Species                           | Host preference  | Distinctive morphological features  |
|-----------------------------------|------------------|---|
| <i>G. duodenalis</i>              | Vertebrates      | Median bodies claw hammer shaped  |
| <i>G. agilis</i>                  | Amphibians       | Median bodies club shaped   |
| <i>G. muris</i>                   | Rodents          | Median bodies small, rounded  |
| <i>G. intestinalis/G. lamblia</i> | Humans           | As for <i>G. duodenalis</i>   |
| <i>G. psittaci</i>                | Budgerigar       | Median bodies claw hammer shaped, trophozoites have incomplete ventrolateral flange                 |
| <i>G. ardae</i>                   | Great blue heron | Median bodies small rounded or claw hammer shaped, nuclei tear drop shaped, single caudal flagellum |

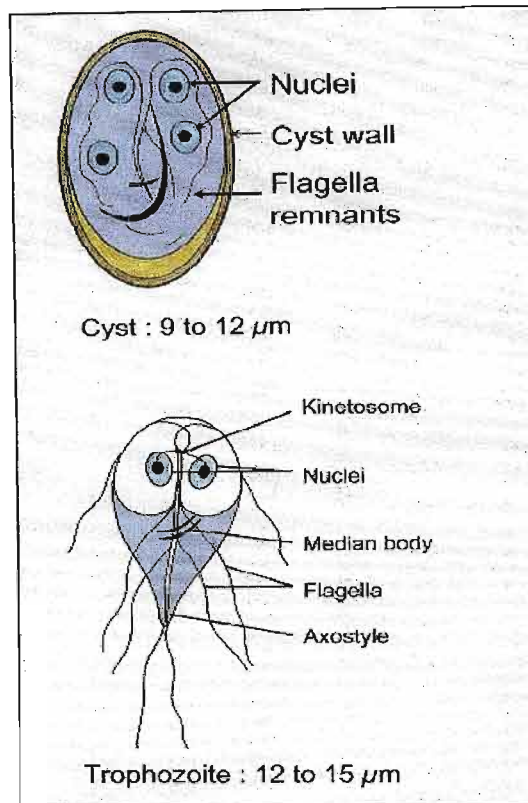
To-date, use of the different specific names for human isolates (viz. *G.lamblia*, *G.duodenalis* and *G.intestinalis*) appears to be based on personal preference. Although Mayrhofer *et al.* (1995) stated that those *Giardia* that infect humans belong to the morphologic group *G.duodenalis* but have been assigned to a separate species *G.intestinalis* (or *G.lamblia*) on the basis of presumed host-specificity, attempts to obtain literature that documents some consensus regarding the specific name for the human isolates were futile.

Lymbery and Tibayrenc (1994) quite correctly stated that the species level systematics have not been resolved. Furthermore, there is increasing evidence that the *Giardia* infecting humans comprise a species complex. For example, Andrews *et al.* (1989) used isoenzyme electrophoresis to characterise 29 Australasian human isolates and 48 clones from these strains. Four distinct types were identified based on the 26 enzyme patterns. Recently, Upcroft *et al.*, (1995) characterised 40 stocks of *Giardia* using biochemical characteristics (karyotype, RFLP and rDNA analyses) over eleven years (1982-1993). During this period at least two major varieties had infected the population of Southeast Queensland. This additional diversity among the human isolates may further complicate the present classification problems currently facing taxonomists. However, it is anticipated that with the advent of advanced molecular typing techniques, the state of flux existing in *Giardia* taxonomy will be resolved.

In the light of a lack of consensus on *Giardia* nomenclature at present, the name *Giardia lamblia* will be used in the current study to describe all isolates obtained from humans.

## 1.2 Life-Cycle and Morphology

*Giardia* has a simple life-cycle with two morphological forms, the trophozoite (vegetative) and a robust cyst (Fig.1.1).



**Fig.1.1** The two morphological forms of *Giardia lamblia*. Cyst (transmissible) and trophozoite (vegetative).

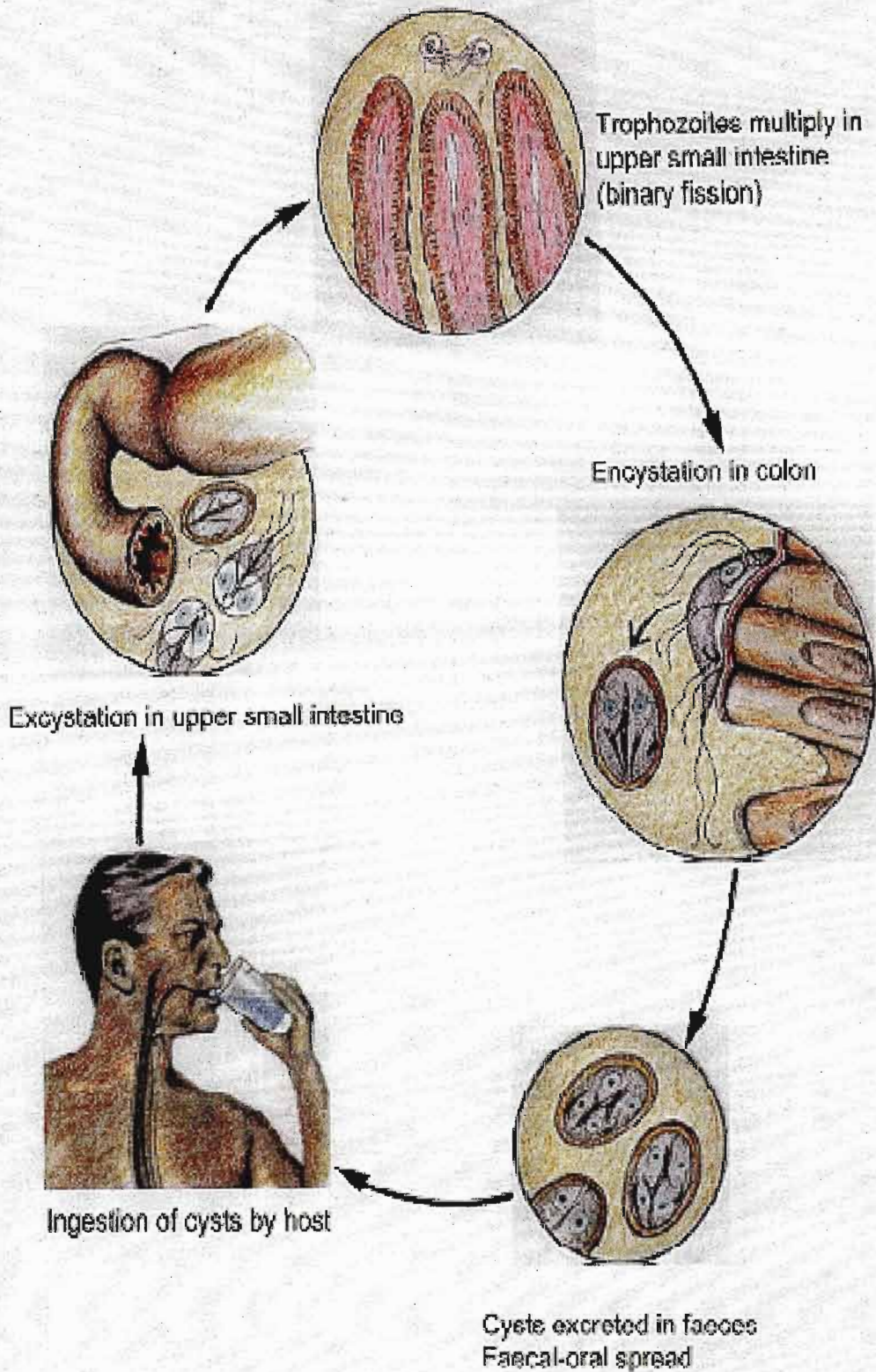
The binucleated pear-shaped trophozoites measure 9-12  $\mu\text{m}$  by 5-15  $\mu\text{m}$  (Hill, 1990). They have a convex dorsal surface and a flat ventral surface with an anterior adhesive disk by which they adhere to the host's mucosa. Four pairs of flagella originate from the kinetosomal mass. This form attaches to and inhabits the upper small intestine of the host. The parasites multiply rapidly by binary fission, although there is evidence that at least some populations may be capable of a form of sexual reproduction (Meloni *et al.*, 1988; Lymbrey & Tibayrenc, 1994).

Differentiation into the cyst stage (8-12µm by 7-10µm) occurs in the relatively drier environment in the lower intestine as trophozoites are flushed downstream by peristalsis. The cyst is the transmissible form of the parasite. In diarrhoeic stools, a shorter time span in the large intestine results in the passage of trophozoites with the faeces. Although they are far more labile than cysts, results from experimental infections suggest that trophozoites may survive passage through the stomach to establish infections in the duodenum (Hegner, 1926a). However they have limited survival outside the host and are therefore an unlikely agent of parasite transmission.

Upon expulsion with the faeces, the cysts can survive for about two months in a suitable environment such as cool water (Jakubowski *et al.*, 1988; Adam, 1991). Ingestion of cysts by the next host through faecal-oral contamination, contaminated water or food results in excystation of cysts in the duodenum, which initiates the next life-cycle (Fig.1.2).

Under a light microscope, cysts are highly refractile oval bodies with fragments of unassembled disk structures and rod-like central axonemes (flagella remnants). Viable cysts can be visually recognised by close adherence of protoplasm to the highly refractile cyst wall. Increased granularity and a large peritrophic space between the cyst wall and parasite indicates loss of viability (Isaac-Renton, 1991).

## LIFE CYCLE OF "GIARDIA LAMBLIA"



**Fig. 1.2.** Life-cycle of *Giardia lamblia*. Ingestion of cysts results in excystation in the duodenum and subsequent colonisation of upper small intestine.

## 1.3 Transmission

### 1.3.1 Human hosts

The cyst is spread through the faecal-oral route. Different mechanisms of transmission have been documented such as direct person to person hand-mouth transfer. This is more common in children (Isaac-Renton *et al.*, 1993) owing to lack of personal hygiene and faecal incontinence. Prevalence is higher in children than in adults in endemic areas (Adam, 1991; Flanagan, 1992). There is suggestive evidence that institutionalised persons and day care attendees are at higher risk of infection, for example Cody and colleagues (1994) isolated cysts from chairs and tables in day care centres. In such settings, transmission rates are expectedly very high, and can subsequently enhance the overall transmission of the parasite. To illustrate this, day care attenders were shown to spread *Giardia* infection to the community in a controlled study by Polis and co-workers (1986). In this study, data suggested that 47% of the children in the centre transmitted giardiasis to at least one household contact. Although humans of all ages are susceptible to infection with *Giardia*, the prevalence is higher in children (Farthing *et al.*, 1986) and infection frequently occurs in immunocompromised individuals (Webster, 1980).

Inadequate sanitation exacerbates dissemination of cysts to the environment.

Surface water supplies are also known to be contaminated with cysts and infected humans replenish this reservoir. Furthermore, wild and domestic animals such as beavers, muskrats, dogs and cats have also been implicated in transmission but their potential as zoonotic reservoirs of giardiasis is still debatable (Erlandsen, 1994). Prolonged occurrence of viable cysts in fresh cool (4°C) water has resulted in *Giardia* being reported as the most common aetiologic agent of epidemic

waterborne diarrhoeal disease in North America and Europe (Craun, 1984; Jephcott *et al.*, 1986). The organisms have been shown to resist the level of chlorination used in many bulk water purification systems. Sole reliance on this method can result in widespread transmission. Filtration and flocculation in addition to chlorination is recommended in order to effectively eliminate *Giardia* cysts from water supplies (Longsdon *et al.*, 1979)

Travel (particularly to endemic areas) has also been documented to facilitate transmission of *Giardia*. For example Brodsky *et al.* (1974) collected data on surveillance of giardiasis in American travellers to the Soviet Union. This data implicated Leningrad as the site of infection and tap water as the probable source.

Flies have been implicated in playing a role in transmission of intestinal protozoa including *Giardia*, as cysts may remain alive in the fly intestine for a considerable period (at least 24hours) and may be deposited in a viable state as early as 40 minutes after the fly has fed on contaminated material (Hegner, 1926b).

Furthermore, it is suggested that since the infective dose is very small (10-25 cysts), it is likely that flies may act as reservoirs and transmit *Giardia* cysts.

However Hall, (1994) noted that it would be difficult to establish whether cysts isolated from flies are viable.

Other modes of transmission have been reported. Food-borne cases of giardiasis have been reported with food-handlers being the most common source of transmission of cysts to freshly prepared food (Adam, 1991) and sexual transmission, particularly in homosexual males also occurs (Schmerin *et al.*, 1978;



Owen, 1984)

### **1.3. 2 The Zoonosis of giardiasis**

The role of wild and domestic animals in the transmission of giardiasis to humans remains unclear. Many animals including dogs, cats, cattle, sheep and wild aquatic mammals have been proposed as both potential reservoirs as well as amplification hosts for spreading human *Giardia* infections (Isaac-Renton, 1991). In the USA beavers have been incriminated as an animal reservoir (Wolfe, 1979; Isaac-Renton *et al.*, 1993). Cross-species infections effected by several investigators in animals such as rats (Filice, 1952), gerbils (Belosevic *et al.*, 1983; Aggarwal & Nash, 1987) and mice (Hill *et al.*, 1983; Byrd *et al.*, 1994) with *Giardia* from humans provide evidence for lack of absolute host-specificity in these organisms. Furthermore, Faubert (1988), proposed that giardiasis is a zoonosis since *Giardia* species isolated from beavers and calves were indistinguishable from isolates of human *G.duodenalis* at the light microscope level and, as with human *G.lambliia* strains the trophozoites could be cultivated *in vitro*. Additionally, a report on the occurrence of giardiasis in backpackers who drank water from areas with no human inhabitants strongly suggested that beavers or other wild animals are reservoirs (Barbour *et al.*, 1976). Thompson *et al.* (1988) reported that all six feline isolates (5 from Australia and 1 from the USA) were genetically identical (as revealed by isoenzyme and DNA analyses). They were also very similar to 20 of 30 human isolates, thereby indicating that cats are a likely reservoir of infection for humans. Meloni *et al.*, (1988) also advocated that felines act as a reservoir of infection to humans; therefore their potential in zoonotic transmission is important.

However, Erlandsen (1994) pointed out that most of the studies implicating animals as potential reservoirs for human infection were flawed and/or biased. During outbreaks, investigators overlooked the potential of birds and muskrats while concentrating on beavers and other aquatic mammals only. Also, contamination by raw sewage (particularly of human origin) was overlooked. Birds were implicated in transmission of giardiasis based solely on the morphological similarity of *Giardia* from Parakeets and Great Blue Herons to *Giardia* found in humans. He further stated that “the evidence for the implication of the beaver or any animal in waterborne outbreaks is very circumstantial”. The fact that waterborne outbreaks of giardiasis are sporadic whilst the aquatic animals are in semi-permanent residence in the water makes their incrimination more questionable. More rigorously controlled studies are still required, hence to-date, the zoonosis of giardiasis is still debatable.

An initial school of thought regarding host-specificity upheld by early scientists was that the *Giardia* of mammals are rigidly host-specific and morphologically distinct (Hegner, 1926b). Filice's breakthrough study (1952) in clarifying the species definition led to the second school which holds that some *Giardia* are capable of infecting more than one vertebrate host species, while others may be host specific (Meyer, 1990) This has been reinforced by several cross-species experiments wherein different animals such as rats, guinea-pigs, domestic fowls (Filice, 1952), mice (Hill *et al.*, 1983; Aggarwal *et al.*, 1983; Mayrhofer *et al.*, 1992, Byrd *et al.*, 1994) and gerbils (Belosevic *et al.*, 1983; Wallis & Wallis, 1986; Aggarwal & Nash, 1988; Visvesvara, 1988) have been successfully infected with *Giardia* of human origin. It would appear therefore that the second school of thought is the view of the majority of the contemporary research community.

#### 1.4 Distribution

The morbidity and misery of giardiasis is experienced world-wide because of the ubiquity of *Giardia*. The global prevalence was reported to be 200 million in 1993 (Crompton & Savioli, 1993). It is the most common protozoan cause of diarrhoea in the United States (Wolfe, 1979; Isaac-Renton, 1991) and is the most frequently reported intestinal parasite of humans in the United Kingdom, (a prevalence of 1,8% of 835 asymptomatic adults screened in a study in Manchester by Kaczmarek & Jones was reported in 1989). In the United States 3,9% of more than 300 000 submitted stool samples (with prevalence values as high as 16% in some areas), were documented (Hill, 1990) and a mean annual rate of infection per annum was reported to be 4,6 per 10 000 population (Flanagan, 1992). In Australia 250 asymptomatic pre-school children in Sydney had a prevalence of infection of 6,8% (Walker *et al.*, 1986) and a rate of 20,2 per 100 000 population was reported (Kaczmarek & Jones, 1989). In Great Britain, an incidence of 0,9 per 10 000 population annually was reported (Flanagan, 1992).

Prevalence varies between <1 and 50% depending on the population being sampled. It is more prevalent in underdeveloped than in developed countries. For example, prevalence estimates vary between 8 and >40% in South America, the Caribbean, the Middle East and South East Asia. In a study in Egypt, 100% of the population was found to be infected over a six month study period (Flanagan, 1992). In contrast, in most developed countries of Western Europe, Australia, New Zealand, and North America, prevalences of between 2 and 7% are more common (Farthing *et al.*, 1986; Flanagan, 1992). However, *Giardia* infections still have an impact on public health problems in these countries.

Evidently *Giardia* differentially infects populations of differing socio-economic backgrounds. It would not be unreasonable to expect rural communities with sparse facilities to have a higher prevalence of giardiasis than the urban population who have proper sanitation, piped and treated water and a relatively higher level of personal hygiene. Contrary to expectations, two independent studies in Bangladesh (Hossain *et al.*, 1983) and Zimbabwe (Mason *et al.*, 1986) found that the prevalence of giardiasis was higher in urban children than in the children in rural areas. It was speculated that this could be related to factors such as high population density in urban areas complicated by overcrowding and poor sanitation of urban slums in developing countries (Rabbani and Islam, 1994).

In rural areas in South Africa, there is often poor sanitation, restricted water supplies and malnutrition, all of which promote *Giardia* infection and transmission. Furthermore, urban areas are overcrowded and have slums with minimal sanitary facilities. Therefore there is a need for diagnostic surveillance of this growth-retarding parasite in this country. Several local studies reflect significant infection levels. For example, Millar and colleagues (1989) performed a survey of parasitic infestation in Cape Town and found that of the 101 children screened, 8 had *Giardia* cysts, and about 46% had multi-parasitosis. More recently, Evans *et al.*, (1998) determined the prevalence of *Giardia* among five communities in the Western Cape by multiple stool assessments. They reported a mean prevalence of 18,1% (with a range of 6-36%) after screening 3976 stools using the formol ether methods.

In Kwa-Zulu Natal, a survey of intestinal parasitic infections in Black school

children by Schutte *et al.* in 1981 revealed *Giardia* prevalence levels ranging between 2.8 and 4.3% within the four regions screened (all in the former northern Zululand). In a recent study undertaken in the Amoebiasis Research Programme laboratory (South African Medical Research Council-Kwa-Zulu Natal), 4% and 13% of 484 and 309 stool samples respectively, from local schools screened for parasites were found to have *Giardia* cysts (Unpublished data). Recently, Jackson *et al.* (1998) reported a 63% prevalence in a cohort of 175 abandoned children in two shelters in Durban (Kwa-Zulu Natal). Furthermore, a prospective study is currently being undertaken by the Amoebiasis Research laboratory involving multiple stool examinations (at least 3 examinations at 3 monthly intervals) of 984 adult subjects recruited from a disadvantaged community in Durban. Preliminary results reveal a prevalence of 4,5% among this population. As most of the reported surveys were based on single stool examinations and all were relying on microscopy (both methods are documented to be insensitive -Ament & Rubin, 1972; Kamath & Murugasu, 1974) these data are an underestimation of the true occurrence of *Giardia* infections locally. However, they provide an estimate of the local prevalence.

### **1.5 Host-parasite Relationship**

*Giardia* organisms were once considered harmless commensals in the human gut (Dobell, 1920; Rendtorff, 1954). Furthermore, Erlandsen and Chase (1974) proposed that the rodent *Giardia* was an indigenous member of the enteric microbiota. After years of debate on the pathogenic potential of this parasite, it became evident that these organisms are associated with a cadre of symptoms producing a disease known as giardiasis. The latter is illustrated by several studies

in which travellers (Brodsky *et al.*, 1974), experimentally infected humans (Nash *et al.*, 1987) and animals (Roberts-Thompson *et al.*, 1976; Aggarwal and Nash, 1987) developed symptoms after exposure. Infections give rise to varying clinical signs and symptoms from asymptomatic cyst passage to acute and chronic diarrhoea associated with villous atrophy, malabsorption and growth impairment in children (Farthing *et al.*, 1986; Hjelt *et al.*, 1992). Consequently, renewed interest has been shown in this genus. It has since been considered an important public health problem and the most frequently identified intestinal protozoan parasite, particularly in the United States (Wolfe, 1979; Smith *et al.*, 1982; Hill, 1990).

Transmission is mainly via the faecal-oral route. For this reason, it is more likely to be endemic in areas that are characterised by poverty, over-crowding, poor personal hygiene, lack of proper sanitation facilities and inadequate purification of water supplies. However, it also has a considerable impact in developed countries such as the United States, Great Britain and Australia as discussed in the preceding section.

Although establishment of axenic laboratory cultures was achieved two decades ago thereby facilitating study of these organisms *in vitro*, many key areas still require clarification such as:

### **1.5.1 Pathogenesis.**

Many theories have been postulated. Early investigators (Erlandsen & Chase, 1974) suggested the possibility that numerous organisms in the small intestine act as a mechanical barrier leading to malabsorption. This was disputed on the basis

of (a) the enormous absorptive capacity of the small bowel (Hill, 1990) and, (b) fewer parasites were isolated in some patients with marked symptoms (Wolfe, 1979). However, Buret (1994) recently stated that the parasite burden contributes to the pathology of giardiasis.

Production of diarrhoea has been ascribed to bacterial overgrowth and bile salt deconjugation, which is common in giardiasis (Tandon *et al.*, 1977). On the contrary, Nash and colleagues (1987) found that bacterial overgrowth was not associated with symptomatic giardiasis in studies of infected human volunteers. Saha and Ghosh (1977) reported superficial invasion of intestinal mucosa in human infection which was associated with steatorrhoea; however, contradictory findings were reported by Owen and co-workers (1979) in their observations of murine giardiasis. They reported that "in animals with intact epithelium, *Giardia* are rare beneath the surface and that penetration probably occurs when trophozoites, randomly moving forward, enter disruptions in the epithelium or cavities left by desquamating cells." A recent finding by Nash and Mowatt (1993) indicated that the variant-specific cysteine-rich surface proteins have a high affinity for certain metals, including zinc. These proteins then can act as a "zinc sink" in which the cations are trapped (on the surface of the trophozoites) and thus rendered unavailable for digestion and absorption functions. They proposed that malabsorption could be caused by zinc deficiency resulting in inhibition of all zinc-dependant enzymes.

Roberts-Thompson (1993) affirmed that the reasons for the development of diarrhoea resulting from infection with *Giardia* species remain unclear. However,

two factors are associated with the development of symptoms, namely the degree of inflammatory changes in the small bowel and damage to microvilli, which lead to a deficiency of brush border enzymes, particularly lactase.

### **1.5.2 Do host factors play a role in increasing the susceptibility of certain hosts to infection?**

Many variables such as age, sex, immune and nutritional status, ABO blood groups, Human Leukocyte Antigen (HLA) type, socio-economic factors and gastric acidity have been implicated in predisposing hosts to giardiasis. The exact nature of the relationship between the disease and these factors has not been conclusively described. Brief discussions on these factors are outlined in 1.7 below.

### **1.5.3 Are immune responses elicited by infection with *Giardia* responsible for parasite clearance and sterilising immunity?**

In some instances these infections are cleared spontaneously. However re-infection of hosts frequently occurs (Farthing *et al.*, 1986; Gilman *et al.*, 1988). It is not clear whether partial protective humoral immunity occurs in giardiasis. Re-infection has been documented in infants and children (Farthing *et al.*, 1986) as well as in adults (Nash *et al.*, 1987; Gilman *et al.*, 1988), thus the issue of immunological maturation with age cannot be used to explain the recurrent infections in some individuals. In experimental human infections, re-infection was confirmed as the volunteers were successfully treated after initial infections and they were successfully re-challenged (Nash *et al.*, 1987). In studies of the *Giardia*-specific immune response in infected human volunteers, serum immunoglobulins



Ig M (100% of patients), IgG (70%), IgA (60%) and intestinal secretory IgA (50%) were documented, but, in these patients the IgA response was not clearly correlated with resolution of infection (Nash *et al.*, 1987). On the other hand, in one clinical study, the protective role of specific secretory IgA was apparently passively acquired by breast-fed infants of *G.lambli*a-infected mothers with high titres of the antibody in their milk (Nayak *et al.*, 1987). The results of the latter study suggest a protective humoral response, whereas in the former study, where re-infection occurred despite the presence of immunoglobulins, such protection was not demonstrated. However, it should be noted that human milk was shown to have a non-immune lethal effect on trophozoites *in vitro* (Gillin, 1987). In the study of Nayak *et al.* (1987) it was not indicated whether this effect was excluded or not.

#### **1.5.4 Are strain differences responsible for variations in the clinical course of infection?**

In 1988, Nash and Aggarwal demonstrated that two distinct isolates of *Giardia* gave rise to different clinical outcomes. In both humans and gerbils, one strain appeared to induce resistance to re-infection on subsequent re-challenge with the homologous organism, whilst infection with the other strain persisted for a longer period and re-challenge resulted in infection. Further, the surface antigens of *Giardia* show a large degree of diversity and undergo surface antigenic variation (Adam *et al.*, 1988; Nash *et al.*, 1988; Nash and Mowatt, 1993). It is not clear what role is played by this antigenic variation in clinical disease. However Nash and colleagues (1991) demonstrated that different isolates expressing varying surface antigens were variably susceptible to intestinal proteases (trypsin and chymotrypsin). Such differences may explain some of the variability in the clinical

features noted in human infections.

#### **1.5.5 Identification of virulence determinants of the organism which would help in new drug and vaccine design.**

IgA1 protease activity (cleaving IgA1 immunoglobulin and haemoglobin) has been described in *Giardia intestinalis* trophozoites (Webster, 1980; Parenti 1989) suggesting that *Giardia* species can survive in the intestine by degrading host IgA. The protease activity may be a non-specific virulence factor that allows the organism to evade host enteric defence mechanisms.

A trypsin-activated, mannose-binding lectin which mediates adherence of the parasite to host epithelia has been demonstrated on the surface of *Giardia* trophozoites (Lev *et al.*, 1986). Whether absence or inhibition of these binding molecules could prevent parasite colonisation and thus interfere with production of clinical illness has yet to be investigated.

*Giardia* has been found to display a large degree of diversity in its surface antigens (Adam *et al.*, 1988; Aggarwalet *al.*, 1989 Nash *et al.*, 1988; 1990). These antigens represent a distinct family of cysteine-rich proteins termed variant-specific surface proteins (VSPs), which cover the entire surface of trophozoites including the flagella (Nash, 1992). The trophozoites can vary their surface antigens and rates of change of VSPs vary markedly among isolates (Nash *et al.*, 1988). These surface antigens appear to play a role in pathogenecity (virulence) of different isolates of *Giardia*:

- The documented differences in resistance to proteases (trypsin and

chymotrypsin) between different isolates expressing different VSPs (Nash *et al.*, 1991) might suggest a possible mechanism for the virulence differences among isolates by enhancing survival in the host intestine.

- Experimental infection of gerbils (Aggarwal & Nash, 1987) and humans (Nash *et al.*, 1987) with two different *G. lamblia* isolates showed a marked difference in pathogenesis between the two isolates. All ten human volunteers inoculated with the GS isolate were infected and 5 developed symptoms, whereas none of 5 volunteers inoculated with the ISR isolate were infected. These two isolates had been shown to express different (Nash *et al.*, 1988). In subsequent studies, human volunteers were inoculated with two cloned GS isolates expressing different VSPs (72 and 200 kDa) (Nash *et al.*, 1990). All 4 inoculated with the GS clone expressing the 72 kDa antigen were infected, whilst only 1 of 13 inoculated with the clone expressing the 200 kDa antigen were infected. These observations also indicate differences in virulence among surface antigen variants from the same isolate.

Further studies are required to find more virulence determinants in these organisms and a possibility of manipulating the VSPs for new drug target sites.

### **1.6 Clinical Manifestations**

In man, the infective dose of *Giardia* was documented to be 10-25 fresh cysts by Rendtorff (1954) when imprisoned human volunteers were given encapsulated *Giardia* cysts. After ingestion of the cysts, a broad clinical spectrum of disease ensues. The most commonly noted feature world-wide is asymptomatic carriage. However, whether these carriers have a transient symptomatic phase, which

passes unnoticed, is debatable. Although the parasite is frequently harboured by apparently symptomless persons who transmit cysts to the environment, this may give rise to symptomatic infections in other individuals.

The clinical course of acute giardiasis is easily recognised in travellers to endemic areas as they present with sudden onset illness during or shortly after travel (Brodsky *et al.*, 1974). The predominant features of acute giardiasis are explosive diarrhoea, abdominal discomfort, nausea, headache and bloating. Symptoms can complicate steatorrhoea and malabsorption in up to 25% of patients (Hjelt *et al.*, 1992) and often cause weight loss (Gerhard, 1989; Heinz, 1988; Meloni *et al.*, 1988; Ukoli, 1984). Frequently, the infection is self-limiting; however, it is estimated that 20 -50% of symptomatic patients will proceed to the chronic stage of the disease. The symptoms can persist for weeks, months or several years. Smith *et al.* (1982), described two and a half years of persistence of symptoms in a patient with giardiasis, while Tandon and colleagues (1977) reported the duration of diarrhoeal symptoms varying from two months to 12 years.

There is increasing evidence that *G.lamblia* infection is very debilitating particularly in younger hosts. Although children apparently tolerate these infections, their physical and mental development is severely retarded. Farthing *et al.* (1986) studied the impact of *Giardia* infection on the growth of infants and children, and found that it had had deleterious effects. The rate of weight gain was significantly lower in the second year of life in *Giardia* infected children, when compared to that of uninfected children. The duration of *Giardia* episodes and their association with diarrhoea appeared to be the most important factors associated with growth disturbance. Similarly, Hjelt *et al.* (1992) illustrated that giardiasis had a negative

impact on the physical growth of 29 children with chronic disease. According to growth charts, the relative heights and weights of the subjects decreased significantly during the course of the disease with severe villus atrophy also being recorded. These workers also demonstrated that the degree of mucosal damage correlated with D-xylose and lactose malabsorption. The younger patients were also more severely affected than the older ones.

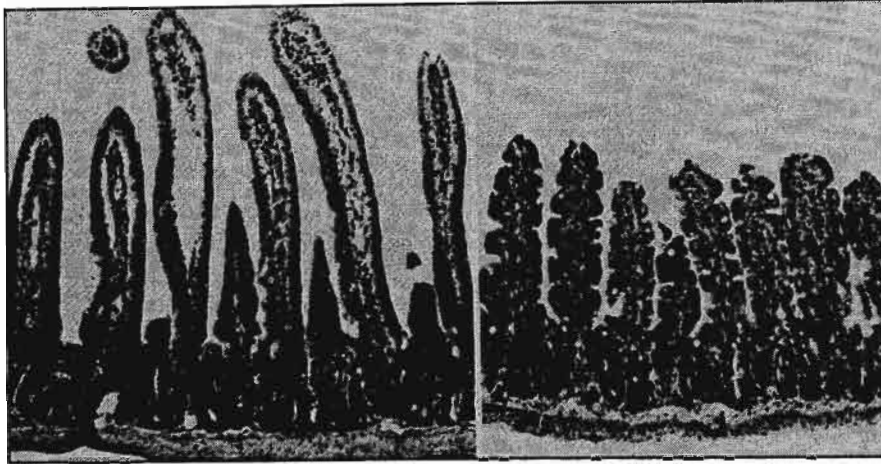
Upadhyay *et al.* (1985), showed that uptake of nutrients by infected, malnourished animals was compromised. A similar phenomenon could be occurring in malnourished children and be attributed mistakenly to the overall effects of deprivation in poverty-stricken areas. Such an association between malnutrition and giardiasis was reported by Gilman *et al.* (1985) where 51% of severely malnourished children living in low socio-economic environments had giardiasis compared to a prevalence of 21% among age-matched control children. A malnourished child has low gastric acidity, depressed intestinal immune functions, retarded enzyme activity, poor intestinal motility and low levels of vitamins, trace elements and minerals. All these factors make the child susceptible to enteric infections including giardiasis (Rabbani and Islam, 1994).

It is suggested that trophozoites can restrict the absorptive area of the gut epithelium, while competing with the host for nutrients, in addition to causing mechanical irritation (Erlandson & Chase, 1974) leading to a malabsorption syndrome (Heinz, 1988). During colonisation, *Giardia* trophozoites were shown to leave imprints of their powerful adhesive disk on the mucosal surface (Owen *et al.*, 1979). This finding supports the theory of mechanical irritation playing a role in

pathogenesis. Malabsorption of electrolytes, solutes and water in the upper small intestine appears to be the primary mechanism of diarrhoea production in giardiasis (Buret, 1994).

As pathogenesis depends on successful establishment of infection, adherence has been implicated as an indirect contributing factor. Trypsin-activated lectins have been documented to play a role in parasite adherence to the mucosa (Lev *et al.*, 1986; Adam, 1991; Nash *et al.*, 1991). Inge *et al.*, 1988 showed that the parasite lectins may be required for the selective establishment of *Giardia* infection in the jejunal epithelia rather than colonic cells (a predilection for the host's upper small intestine).

Vast reduction of the villus to crypt ratio (Fig.1.3) has been revealed by transmission electron microscopy in animal giardiasis (Roberts-Thompson *et al.* 1976). Disruption of the brush border in the small intestine, crypt epithelial hyperplasia and mononuclear inflammatory cell infiltration have been documented. The latter is proposed to contribute both to the disease process as well as in parasite eradication (Farthing, 1989; Owen *et al.* 1979). Sometimes the parasites reach the gall bladder and bile ducts and cause jaundice.



**Figure 1.3.** Transmission Electron micrograph of the small intestine in murine giardiasis (right) compared to the normal jejunum of an uninfected control animal (left) (Roberts-Thompson, *et al.*, 1976). (x 160) (Courtesy of A.Mahmoud)

Smith *et al.*, (1982) tested four strains of *Giardia* from humans for toxin production in rabbits and found that these organisms did not produce toxins. To date, no reports showing evidence for toxin production have been published. However, it was suggested that *Giardia* excretory-secretory products may play a role in the pathogenesis of giardiasis, either as a trigger for the diffuse brush border injury, or via direct cytopathogenetic effects during close contacts between host cells and trophozoites, and this needs to be investigated (Buret, 1994). Lysosome-like vacuoles that line the surface of trophozoites have been identified (Feely *et al.*, 1984). These organelles have been shown to release numerous hydrolases, including a thiol-dependant proteinase (Parenti, 1989). A possibility of secretion of the proteinase has been indicated, although there is only limited evidence in support of this. It was further suggested that under selected conditions, the proteinase might be released into the extracellular milieu and play a role in the pathogenesis of diarrhoeal disease (Parenti, 1989).

The association of giardiasis and allergic reactions have been documented.

Balazs and Szatloczky (1978) reported a rare form of *G.lamblia* infection in a patient who presented with a clinical picture of nutritive allergy. An acute hypersensitivity syndrome consisting of urticaria, polyarthropathy and eosinophilia occurring in association with *G.lamblia* was documented by Farthing *et al.* (1983). Perez *et al.* (1994) have also reported the association of the above two clinical conditions. Other extraintestinal manifestations of giardiasis have been documented. For example, a more recent study by Corsi *et al.* (1998) has shown that giardiasis can also cause ocular complications in the form of asymptomatic, non-progressive retinal lesions. These retinal complications were particularly common in younger children with the disease and were detected in both hosts with active and/or past *Giardia* infections. The retinal changes have not been associated with *Giardia* toxin. The authors suggested that they are more than likely caused by immune mechanisms.

Although rare, fatal cases of giardiasis have been documented. For example, Ryder *et al.* (1985) reported eight fatal cases of diarrhoea, one of which was associated with *G.lamblia*.

## **1.7 Risk Factors For Giardiasis**

### **1.7.1 Age and immune status of the individual**

Humans of all ages are susceptible to *Giardia* infections; however, some factors appear to predispose certain individuals within some populations to increased risk of infection. High prevalence levels have been reported in children in many countries; this predisposition usually rapidly declines with increasing age.

Prevalence among adults is usually less than 10% and peak prevalence occurs



among children between the ages of 1 and 5 years (Roberts-Thompson, 1993). Polis *et al.*, (1986) documented prevalences between 21-60% among infants and pre-schoolers in a day care centre. Further, a 21% prevalence in healthy children between 5 and 10-years and up to 51% in malnourished, 1 to 5-year old children was documented by Gilman *et al.* (1985). The results of a prospective, longitudinal study of a cohort of 45 rural Guatemalan children from birth to 3 years showed that the children had an average of 3,6 episodes of *Giardia* in their third year of life. All children had at least one *Giardia* infection by this age (Farthing *et al.* 1986). Prevalence is therefore apparently age-specific. It is speculated that both frequent exposure combined with a low level of personal hygiene among small children plays a predominant role. It is also postulated that malnutrition and chronic diarrhoea (common in children in poorer communities) increases susceptibility (although the true relationship in terms of cause and effect has yet to be established). This view was supported by a study of 31 Gambian children with chronic diarrhoea and malnutrition (Sullivan *et al.*, 1991). Forty-five percent of these children had giardiasis compared with only 12% of 33 healthy age and sex-matched control children recruited from the same geographical location. Similarly, in the study of Bangladeshi malnourished children with chronic diarrhoea aged between 1 and 5 years, a 51% *Giardia* prevalence was documented by Gilman *et al.* (1985). It must be noted though that most of these studies were undertaken in developing countries, therefore the age-specific prevalence must be considered in conjunction with such factors as the socio-economic disposition of the population. For example, in a first world country (Denmark), Hjelt *et al.* (1992) reported a low incidence of 81 per 1 000 000 children aged 0-7 years per annum over a 6-year period.

Some investigators proposed that this age-related prevalence could also be a function of an underdeveloped immune system. This was demonstrated in a *G.lamblia*-suckling-mouse infection study (Hill *et al.*, 1983). In this animal model of *Giardia* infection, all mice, 3, 7 and 14 days old became infected whereas older mice (>14 days old), as well as the mothers of infected suckling mice, which presumably were ingesting large numbers of cysts from their babies' stools, did not become infected. All infected mice cleared the infection 17-21 days after inoculation, and these workers have ascribed this to immunologic maturation, which they reported to occur at one to four weeks of age in mice.

In humans, the possibility of a relationship between the humoral immune response and the development of symptomatic *Giardia* infection was illustrated in a study by Vinayak & Kumkum (1989). They showed that children with chronic symptomatic giardiasis that were resistant to therapy had lower titres of antibody to two *Giardia* antigens than children with acute or asymptomatic infection.

Immunodeficiency has been shown to predispose to *Giardia* infection and appears to be a major contributor to persistence of symptoms as chronic giardiasis occurs in individuals with immunoglobulin deficiency (Webster, 1980; Lo Galbo *et al.*, 1982). Some investigators have stated that patients with hypogammaglobulinaemia are at increased risk of severe giardiasis (Lo Galbo *et al.*, 1982, Adam, 1991). Similarly, Ament & Rubin (1972) reported a strong association between a defective immune system and giardiasis. Seven of 8 patients with hypogammaglobulinaemia and gastrointestinal symptoms were infected with *G.lamblia*. Animal studies have also showed this phenomenon

where immunocompromised mice have presented with the chronic form of giardiasis in comparison with their immunocompetent counterparts (Roberts-Thompson, 1993).

Other conditions suggestive of an association between immune dysfunction and giardiasis are documented. For example, Cruz *et al.*, 1991 and Bassoti *et al.*, 1991) both reported an association of *Giardia* with Whipple's disease in immunodeficient patients.

There is an apparent association of intestinal parasitosis (including giardiasis) and HIV infection. A prevalence of 15,2% among 79 Brazilian, HIV-infected patients was cited by Rabbani & Islam (1994). Connolly *et al.*, (1990) stated that the prevalence of giardiasis among AIDS patients may be underestimated because of incomplete investigation of suspected cases. They showed that extensive microbiological and histological examinations of the intestine revealed 2 cases of giardiasis, diagnoses previously missed among the 33 HIV positive patients.

## **1.7.2 Genetic factors**

### **1.7.2.1 Host factors**

Genetic profile is believed to predispose a host to infection with *Giardia*.

Association between certain ABO blood groups, HLA antigens and giardiasis has been documented (Farthing, 1989). This author suggested that ABO blood groups influence the duration or severity of symptoms because glycoproteins with blood group activity can influence the adhesion of *Giardia*. Patients with giardiasis have been documented to have a higher than expected frequency of blood group A

(Roberts-Thompson, 1993). Furthermore, it has been reported that individuals with blood group A are prone to develop achlorhydria which increases their susceptibility to *Giardia* infection (Paulsen, 1977). In a study in adults in Australia (Roberts-Thompson *et al.*, 1980), patients with prolonged giardiasis had a higher than expected frequency of HLA-A1 and HLA-B12 and a lower than expected frequency of HLA-A3 and HLA-B35. Roberts-Thompson *et al.* (1980) suggested that HLA antigens or linked genes might be associated with a defective immune response to 'host protective' antigens of *Giardia*, however this suggestion still needs to be tested.

#### **1.7.2.2 Parasite factors**

Successful establishment of infection appears to depend on parasite factors as well. For example variations in infectivity in experimental human infection by two distinct *G.lambli*a isolates are documented (Nash *et al.*, 1987). Furthermore, when gerbils were infected with two well characterised *Giardia* isolates, different patterns of infection and degrees of self-cure and varying abilities to induce resistance to homologous and heterologous rechallenge were observed (Aggarwal & Nash, 1987). Other studies demonstrated variation in outcome of infection in gerbils (virulence and infectivity) by *Giardia* organisms isolated from symptomatic and asymptomatic humans (Visvesvara *et al.*, 1988) and in mice (Aggarwal *et al.*, 1983). These studies indicate that intrinsic differences (which may be genetically determined) exist among different isolates of *Giardia*. These differences may confer variations in infectivity of the different isolates.

Clinical isolates of *Giardia* contain mixes of genotypes with a possibility of at least

up to 4 distinct genotypes within each clinical isolate (Andrews *et al.*, 1992). These authors illustrated that *in vivo* and *in vitro* culture methods select different genotypes. Furthermore, it is speculated that in endemic areas where extensive genetic heterogeneity exists within *Giardia* populations, mixed infections with more than one genetic variant are likely to occur (Finn *et al.*, 1994). These authors demonstrated *in vitro* competitive interaction between 2 genetically distinct isolates with different growth rates. In this study, the faster-growing isolate greatly out-competed the slower growing organism. It is likely that such competitive interactions occur *in vivo* during colonisation of hosts. As discussed earlier, *Giardia* genetic variants expressing different surface antigens were shown to be differentially susceptible to the intestinal proteases trypsin and chymotrypsin (Nash *et al.*, 1991). It is therefore more likely that during natural *Giardia* infections, such population dynamics of the parasite play a role in determining the outcome of infections in humans.

### **1.7.3 Travel**

Travel (particularly to endemic areas) has been implicated in increasing the risk of contracting giardiasis, leading to the latter being widely known as travellers' diarrhoea. The water supplies in Leningrad and towns in the Rocky Mountains of North America have been strongly associated with outbreaks of *Giardia* in visitors and local inhabitants. A questionnaire survey of 1 419 travellers to the Soviet Union performed by the Center for Disease Control (CDC) Bethesda, USA, showed that 324 individuals (23%) had acquired giardiasis (Brodsky *et al.*, 1974). Furthermore, in a survey on intestinal parasites by the CDC, the majority of the 670 cases of giardiasis were patients returning from travel abroad (CDC, 1976).

Short-term residents of Colorado were documented to have higher attack rates of giardiasis than did long-term residents (Schultz, 1975). This implies development of acquired immunity among long term residents of communities where *Giardia* is endemic.

#### **1.7.4 Nutritional status and gastric acidity**

There have been reports showing a strong association between malnutrition and giardiasis prevalence. Upadhyay *et al.*, (1985) showed that *Giardia* infected, malnourished animals could not absorb nutrients. Similarly, Gilman *et al.* (1985), reported that 51% of severely malnourished children at a Nutrition Unit in Bangladesh acquired *Giardia* infections.

Giardiasis is more common and lasts longer in patients who are malnourished and therefore immunocompromised. This was demonstrated by Sullivan *et al.* 1991 when they determined the prevalence of giardiasis in 33 Gambian children with chronic diarrhoea malnutrition. Fourteen of the 31 (45%) children with chronic diarrhoea malnutrition syndrome had giardiasis compared with only 4 of 33 (12%) healthy children matched for age and sex.

It is not clear whether giardiasis can contribute to malnutrition or whether malnutrition predisposes to giardiasis (Rabbani & Islam, 1994).

A few findings regarding the role of gastric acid in giardiasis have been published. The acid pH of the stomach was suggested to play a protective role against *Giardia* infection (Giannella *et al.*, 1973), However, the finding that the initial step

in initiation of infection (excystation) is induced by exposure to acid (Bingham & Meyer, 1979) is contradictory. However, hypochlorhydria and achlorhydria have also been documented to predispose to giardiasis (Hass & Bucken, 1967). It has been reported that patients who had had previous gastrectomy had a higher prevalence of giardiasis, probably because of reduced gastric acid production. Although *Giardia* is primarily a duodenal parasite, it may sometimes colonise the gastric mucosa if the stomach pH is low as in achlorhydria patients (Yardley & Bayless, 1967). Feely *et al.*, (1991) illustrated that excystation of *G.muris* cysts can be successfully induced at pH 7,5. This might explain how giardiasis occurs in persons with achlorhydria.

Further studies that will clarify the role of the pH in *Giardia* infections are necessary.

### **1.8 Summary**

Although *Giardia* infections are non-invasive and frequently asymptomatic, they are still a major cause for concern worldwide as they are responsible for growth faltering in infants and children and present large-scale waterborne epidemics of diarrhoea. Moreover, since it is documented that severe forms of the disease are seen in immunocompromised hosts, it is anticipated that the advent of HIV-AIDS will increase the impact of giardiasis.

Owing to the nature of its life cycle, transmission and distribution and its relative resistance to environmental desiccation, it is evident that complete eradication of the parasite will be difficult. Intervention measures will therefore largely depend on

vaccines and chemotherapeutic agents. Control will also depend to a great extent on education of children and adults about hygiene and sanitation. Equally essential is the need to resolve the present taxonomic confusion in order to facilitate effective epidemiological control when accuracy in species and strain definition has been achieved. The extent of interspecies transmission (in order to elucidate the role played by animals in transmission of giardiasis to humans) also requires thorough investigation. These remarks emphasise the crucial need to fully understand the pathogenesis/virulence factors of these organisms.

Finally, several studies have revealed marked genetic diversity among isolates. Some of these studies illustrate that variability exists in many aspects of *Giardia* infection, such as drug susceptibility, infectivity patterns, susceptibility to enzymatic lysis and antigenicity. In view of the fact that many cell-cell interactions occur at the surface (including host-parasite interactions), it remains to be shown whether the surface antigenic variations have any association with virulence determinants during the natural course of infections *in vivo*.

### **1.9 Study objective**

Evidently, giardiasis still remains a poorly understood gastrointestinal infection. Furthermore, *Giardia* is not a fully defined organism at present. The environment, host factors and the parasite cyst interact to propagate giardiasis. A thorough understanding of the organism requires more in-depth knowledge of its biology and biochemistry. Epidemiological intervention to break the life-cycle, preventive control measures and identification of prospective drug target sites and vaccine design would be facilitated by a more comprehensive knowledge of the parasite.



In order to address these and many unanswered questions regarding *Giardia* and giardiasis, both *in vivo* and *in vitro* models need to be established in the laboratory. Characterisation of as many strains as possible and the establishment of cryo-banked stabilates would provide an invaluable research platform.

In South Africa, *Giardia* has been poorly researched and consequently many aspects still require clarification in the local setting.

- It is not known to what extent giardiasis contributes to the morbidity of diarrhoeal disease locally. The possibility that the effects of this parasite are superimposed upon those of other entero-pathogens needs to be investigated.
- The other effects of deprivation such as poverty and malnutrition could be masking the subtle but deleterious effects of giardiasis, particularly in infants and children.
- It is unclear whether different strains of *Giardia* give rise to variation in the extent of disease in South Africa.

The work for this thesis was undertaken to establish a research platform for the characterisation of South African strains of *Giardia*. This was accomplished by:

- I. Initiation of cultures of different human strains of *Giardia* from locally isolated, excysted cysts.
- II. *In vitro* maintenance of cultures.
- III. Establishment of a culture cryo-bank of *Giardia* isolates.
- IV. Preliminary application of isoenzyme electrophoresis to the isolated strains.

## Chapter 2

### SAMPLE COLLECTION AND PREPARATION

#### 2.1 INTRODUCTION

##### 2.1.1 Sourcing And Harvesting of Cysts

In order to fulfil the aims of the present study, there was a need to obtain trophozoites for *in vitro* culture initiation. There are two commonly employed methods of obtaining trophozoites from infected persons. The first derives the parasites by excystation of cysts that are excreted in faeces (Kasprzak and Majewska, 1985; Isaac-Renton *et al.*, 1986; Meloni & Thompson, 1987; Al-Tukhi *et al.*, 1991), while the second method involves the use of a gelatin string-capsule (Enterotest) or intubation to obtain a duodenal aspirate containing the organisms (Meyer, 1976, Gordts *et al.*, 1984; Gordts *et al.*, 1985). *In vitro* cultures of *Giardia* trophozoites have been successfully initiated using both methods of recovery (Gordts *et al.*, 1985; Meloni & Thompson, 1987).

When 4 methods for detecting *Giardia lamblia* (namely examination of stools, duodenal aspirates, intestinal biopsies and impression smears) were evaluated (Kamath & Murugasu, 1974), parasites were detected by 1 or more of these methods in 12 of 21 patients. The study showed that duodenal aspiration was superior to faecal cyst detection. The former could detect the infection in 10 of the 12 *Giardia* infected patients (83% sensitivity), while triplicate stool examinations detected infections in 6 of the 12 (50%) positive samples. (Kamath & Murugasu, 1974). Similarly, Ament & Rubin (1972) reported that *Giardia* was not detected by

multiple stool examinations in 4 of 7 infected patients but was correctly diagnosed by small intestinal biopsy. However as biopsy collection is an invasive procedure and duodenal intubation is unpleasant, collection of faeces became the method of choice for this work, for both identification of infected persons as well as obtaining cysts for experimental work.

Reliable sources of cysts had to be identified during the current study to enable the development of the biochemical methods described in subsequent chapters. Institutionalised persons are documented to be at increased risk of giardiasis (Polis *et al.*, 1986; Cody *et al.*, 1994); furthermore, peak prevalence occurs at ages 2-5 years. Day-care attendees, hospitalised toddlers and children of this age group were therefore targeted as the source population.

### **2.1.2 Detection and isolation of cysts**

It is recognised that direct examination of faecal smears for microscopic detection of protozoan cysts is relatively insensitive. Hence to increase the frequency of detection, concentration techniques are invaluable (Ritchie, 1948). Various methods of concentrating and purifying cysts from faecal debris and bacteria are available. Some of these use sedimentation principles, e.g. the formol-ether technique (Ritchie, 1948) whilst others rely on floatation e.g. Zinc Sulphate (Faust *et al.*, 1938). In 1952 Ritchie *et al.* demonstrated that the formol-ether concentration technique was superior to the Zinc Sulphate floatation method. However, both methods use toxic reagents and the high concentrations of formalin, iodine and ether employed for purification of cysts is unsuitable if they are to be used for immunological, biological or other research purposes (Jyothi *et*

*al.*, 1993). The use of these techniques should therefore be limited to diagnostic detection of cysts and/or ova. Gradient solutions, using buoyant density, have been widely used to purify viable parasite cysts and ova. Roberts-Thompson *et al.*, (1976) developed a method that uses a 1M solution of sucrose to separate *G.muris* cysts. Since then this has been used by many workers to isolate viable cysts that were processed for different experiments (Bingham and Meyer, 1979; Schaefer *et al.*, 1984; Meloni and Thompson, 1987). Percoll (Pharmacia Inc., Sweden) is also widely used for obtaining purified cyst preparations. It causes minimal osmolarity changes, has low viscosity and low toxicity to cells (Sauch, 1984). However, the use of this expensive product for large-scale separations has cost implications. Sucrose, as it is inexpensive and less toxic, was more suited to the current work.

The current chapter deals with specimen collection as well as the assessment of efficacy of various methods in recovering optimum numbers of cysts from faeces. Determination of cyst viability is also covered.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Specimen Collection**

Description of the aims and benefits of the study as well as the rights of the participants to withdraw from the study at any time was discussed with the mothers of the children recruited into the study. In case of the institutionalised children, this was discussed with the caregivers. Following informed consent (Appendix 1), faecal specimens were collected from two groups of subjects.

The first group consisted of patients at King Edward VIII hospital (KEH). It serves Durban and its surrounding areas (mostly the disadvantaged community). This symptomatic group had clinical giardiasis. Collection of samples was accomplished mainly in the hospital's Paediatric wards and also from the Gastroenterology Unit.

Sampling was also accomplished by retrieving all stools routinely submitted to the Microbiology laboratories at King Edward VIII and Hlabisa (Northern Kwa-Zulu Natal) hospitals. These were categorised as symptomatic or asymptomatic according to availability of the following information:

- (i) Clinical history
- (ii) Microbiological culture results
- (iii) Stool consistency (evidence of watery stool and presence of trophozoites)

In cases where any of the above was not available and where concomitant bacterial and/ or other enteropathogens were isolated, classification into symptomatic/asymptomatic was not effected as this would obscure the symptomatology of giardiasis.

The second group consisted of “apparently healthy” children from various institutions: (i) Othandweni Home, (an institution that cares for abandoned children) located 15 km south of Durban. Stools were collected mostly from children aged from six months to ten years (ii) children (2-5 years of age) attending pre-school and day care centres at the following residential areas for the Black African community: (a) Clermont, (Kideo Pre-school), 30km west of Durban; (b) Amanzimtoti (Siyakuthanda Creche) 45 km south of Durban.

### **2.2.2 Sample Preparation for Microscopic Detection**

One thousand and twenty-three faecal specimens collected from the day care centres and Hlabisa laboratory were concentrated by the formol-ether technique to amplify cyst detection.

#### **2.2.2.1 Formol-ether Concentration of faeces for microscopic examination The modified method of Ritchie (1948)**

Approximately 0,35g (pea-size) of stool was placed in a 15ml polypropylene centrifuge tube followed by the addition of 7ml of 10% formalin. The faecal matter was broken up with an applicator stick and the suspension was filtered through two layers of wet gauze into a paper cup. The filtrate was returned to a clean centrifuge tube and 3ml of diethyl ether added. The tube contents were thoroughly mixed by shaking vigorously for 30 seconds prior to centrifugation at 400xg for 5min at 22°C. The supernatant formalin-ether layers were discarded and the deposit was screened microscopically for the presence of *Giardia* forms. After systematically scanning the entire area covered by a coverslip on the slide, all *Giardia* positive samples were semi-quantitated as shown in Table 2.1.

**Table 2.1.** Semi-quantitative assessment of formol-ether concentrated samples.

| Grading | Cysts (x400) |
|---------|--------------|
| Scanty  | <1           |
| 1+      | 1-2          |
| 2+      | 2-50         |
| 3+      | 50 –200      |
| 4+      | >200         |

#### **2.2.2.2 Examination of faecal wet preparations (modified Beemer's Stain)**

During the period of January 1996-December 1997, 6246 stool samples were submitted to the Microbiology laboratory at King Edward Hospital for microbiological investigation. This laboratory routinely analyses all submitted stools for parasitosis by examining direct wet preparations stained with Beemer's and Sheather's stains. These samples were only available for the current study in the afternoon (after routine microbiological analyses had been completed).

Therefore it was only feasible to screen by direct microscopy in order to allow for timely detection of cysts. All these samples (which had been previously screened by a laboratory technologist) were then re-examined for the presence of *Giardia* by repeat stained wet preparations using the modified Beemer's stain (Appendix 2) as follows:

An aliquot of 10  $\mu$ L of Beemer's stain was transferred to a microscope slide with a disposable Pasteur pipette. Approximately 10 $\mu$ g of faeces was thoroughly

emulsified in the stain and a 22 x 40mm coverslip applied. The entire area was systematically examined on the microscope at 100x magnification for the presence of *Giardia* cysts. A higher magnification (400x) was also employed to confirm suspicious objects identified using the 100x objective.

### **2.2.3 Purification of Cysts**

It is essential to separate the cysts from the faecal matter as soon as possible to minimise overwhelming fungal and bacterial multiplication, and to prevent undesirable drying effects. If and when separation could not be undertaken immediately, stools were diluted 1:3 in 0,2M Phosphate Buffered Saline (PBS), pH 7,0 and stored at 4°C until purification. Three methods were assessed for optimum recovery of cysts from faecal material as outlined below:

#### **2.2.3.1 The discontinuous gradient method (Al-Tukhi *et al.*, 1991)**

Approximately 0,5g of stool samples (in duplicate) were emulsified in, and vigorously shaken with, 10ml of distilled water and filtered through 2 layers of wet gauze. The filtrate was layered onto a density gradient made with 5ml each of 0,4M and 0,85M sucrose solutions and centrifuged at 400xg for 10min at 4°C in a 50ml polypropylene centrifuge tube. The material at the water/sucrose interface was aspirated, resuspended in cold distilled water and washed by centrifuging twice at 400xg for 5min. Lipid debris was removed by resuspending the cyst pellet in a mixture of 5ml cold distilled water and 7ml ethyl ether, vortexing, and centrifuging at 400xg for 10min at 4°C. The resultant cyst pellet was collected, washed and resuspended in 0,5ml of normal saline. Cysts were enumerated as described in section 2.2.4.



- *In an attempt to optimise this method, the quantity of stool and the volume of distilled water were varied; in addition the use of normal saline for cyst suspension was introduced in order to prolong cyst viability.*
- *The initial supernatant was filtered to further remove faecal debris, and a larger (50ml) centrifuge tube was used for the gradient separation. All samples were processed with the optimised method.*

### **2.2.3.2 The modified Zinc sulphate (ZnSO<sub>4</sub>) floatation concentration method. (Faust et al. 1938)**

Approximately 0,5g of faeces was introduced (in duplicate) into a 15ml polypropylene centrifuge tube using a wooden applicator. Two millilitres of water was added and the contents mixed by vortexing using the applicator as a shaft. The tube was filled with water and applicator removed. The preparation was centrifuged at 600xg for 1min at 22°C and the supernatant decanted. One millilitre of 33% ZnSO<sub>4</sub> was added and the tube tapped with a finger to mix the contents thoroughly. More ZnSO<sub>4</sub> was added to half-fill the tube and the mixture was filtered into a paper cup through 2 layers of wet gauze. The filtrate was returned to the tube. Sufficient ZnSO<sub>4</sub> solution was added to fill the tube to within 3mm of its' top and the sample was centrifuged at 600xg for 1min at 22°C. The uppermost 2mm layer (containing cysts) was collected with a Pasteur pipette into a clean 15 ml centrifuge tube which was filled with distilled water. The preparation was centrifuged at 600xg for 5min at 22°C. This wash step was repeated twice and the pelleted cysts were resuspended in 0,5ml of normal saline. Cysts were counted on a haemocytometer.

*A minor modification of the method was the collection of cysts with a Pasteur pipette from the uppermost layer of the float for subsequent washing, instead of collecting on a coverslip as described in the original method. This was done in order to improve the number of cysts recovered from the float and to preserve the viability.*

### **2.2.3.3 The modified, continuous gradient method (Roberts-Thompson *et al.*, 1976)**

Approximately 0,5g of fresh stools (in duplicates) were broken up in 10ml distilled water in a 50ml specimen jar and gently emulsified using an applicator stick. The slurry was filtered twice through two layers of wet gauze to remove large particles. The filtered suspension was layered onto 5ml of 1M sucrose (S.G.1, 11) in a 50ml polypropylene centrifuge tube and centrifuged at 600xg for 5min at 4°C. The cyst concentrate was removed at the water-sucrose interface with a Pasteur pipette and washed three times by resuspension in 50ml of distilled water followed by centrifugation at 600xg for 10min. The supernatant was aspirated, discarded and the cyst pellet resuspended in 0,5ml normal saline for counting and further processing.

#### ***Modifications involved:***

- ***Removal of large faecal debris by filtration.***
- ***Reducing the gradient centrifugation period to 5min.***
- ***A 50ml centrifuge tube was used into which 5ml of 1M sucrose and 10ml of filtrate was added.***
- ***The gradient centrifugation step was repeated if the specimens were not sufficiently cleaned after the first step.***

### **2.2.3.4 Comparison of the three purification methods**

Thirteen samples containing *Giardia* cysts were purified by the three methods (in duplicate) on different days. After collecting cysts from the respective gradient layers, the resultant deposits were examined to assess cyst losses for each separation method. Following these trials, the modified method of Robert-Thompson *et al.* (1976) using the 1M sucrose gradient was selected for this work with the following modification also being effected:

Preliminary work with five samples having different cyst loads revealed that for stools with mild to moderate (1+ to 2+) cyst loads, a larger volume of stool (approximately 20 to 30g) had to be used. Aliquots of 5g each were filtered through gauze and centrifuged in a 50ml polypropylene centrifuge tube. Cysts were then purified from the pelleted specimens in at least 6 replicates. The replicated cyst concentrates were then pooled in order to obtain an improved yield. For specimens with 3+ to 4+ cyst loads, about 1g was sufficient to produce sufficient numbers of cysts. All subsequent purifications were undertaken with this modification.

#### **2.2.4 Enumeration and sterilisation of Cyst preparations.**

The purified cyst suspension was diluted 1:10 in normal saline. A 10 $\mu$ l aliquot of the dilution was transferred to a Neubauer counting chamber and the cysts were enumerated. Thereafter the following antibiotics were added to the suspensions:

|                |   |                  |
|----------------|---|------------------|
| Penicillin     | (Biowhittaker, Walkersville, MD)        | 10 000u/ml       |
| Streptomycin   | (Biowhittaker, Walkersville, MD)        | 10000 $\mu$ g/ml |
| Amphotericin B | (Biowhittaker, Walkersville, MD)        | 25 $\mu$ g /ml   |
| Amikacin       | (Bristol Myers Squibb, New Jersey, USA) | 20 $\mu$ g/ml    |
| Vancomycin     | (Mast Diagnostics, Merseyside, UK)      | 20 $\mu$ g/ml    |

The cyst suspensions were then aliquoted in 0,1ml volumes in 1ml microfuge tubes. The suspensions were washed twice by centrifugation in sterile distilled water before experiments were initiated. If sufficient numbers were available, an aliquot was processed on the same day of purification and the remainder stored at

4°C for further processing on subsequent days to replicate experiments. At the early stages of this work, cyst numbers that were used ranged between 100 and 10 000 cysts/ml, depending on the number of cysts obtained after purification. Later, cyst concentrations were adjusted to have at least 100 000 cysts/ml in 0,1ml of distilled water and stored at 4°C in microfuge tubes (Nash, personal communication). Where low numbers were obtained, (as determined in 2.2.4) the gradient purification method was repeated and a larger volume of stool was used for replicate purifications and the replicate cyst concentrates were pooled to obtain larger numbers of cysts.

### **2.2.5 Determination of Cyst Viability**

To ascertain whether cysts would be capable of excysting, their viability was assessed by the eosin exclusion method (Bingham *et al*, 1979). Viable cysts inhibit dye penetration while non-viable ones absorb the dye and appear pink internally.

Equal volumes of the washed cyst suspension and 0,0025% aqueous eosin solution were mixed in a 5ml-polycarbonate tube. Ten microlitres of the suspension was transferred to a haemocytometer with a coverslip in position and the preparation incubated in a moist chamber at 22°C for 15 min. Cysts were then examined under a light microscope at 400x magnification for dye uptake and enumerated. Percentage viability was determined by enumerating unstained and stained cysts in the five large squares of the counting chamber and expressing these as percentage viability as follows:

$$\% \text{ Viability} = \text{unstained cysts} / \text{total cysts} \times 100$$

Viability was determined for every batch of cysts prior to any excystment being attempted.

## 2.3 RESULTS

### 2.3.1. Sample Collection and Concentration

One thousand and twenty three stool samples were collected and screened following formol-ether concentration. *Giardia* forms were detected in 127 (12,4%) of these specimens (Table 2.2).

**Table 2.2** A summary of total stool samples screened after formol-ether concentration or direct stained wet preparation.

|                         | FORMOL-<br>ETHER | DIRECTLY STAINED<br>WET PREPARATION | TOTAL |
|-------------------------|------------------|-------------------------------------|-------|
| Sample Screened         | 1023             | 6246                                | 7269  |
| <i>Giardia</i> positive | 127 (12,4%)      | 78 (1,25%)                          | 205   |

One hundred and fifty-three of 205 samples containing *Giardia* could be used in the experimental work. The rest could not be utilised as they contained very scanty and/or morphologically abnormal cysts.

Of the 1023 stools, 591 were collected from the 3 child-care centres and 100 (16,9%) contained *Giardia* cysts/ trophozoites. In one institution (Othandweni), prevalence was as high as 75% and 59% in February and June 1997 respectively.

The overall prevalences varied between 12,5% and 75 % among the institutionalised subjects (Table 2. 3).

**Table 2.3.** Prevalence of *Giardia* in institutionalised subjects from January to December 1997.

| Month     | Hlabisa† |       | Kideo‡ |       | Siyakuthanda<br>‡ |       | KEH Wards |       | Othandweni# |       |
|-----------|----------|-------|--------|-------|-------------------|-------|-----------|-------|-------------|-------|
|           | %        | Total | %      | Total | %                 | Total | %         | Total | %           | Total |
| January   |          | NC    |        | NC    |                   | NC    |           | NC    | 26          | 84    |
| February  |          | NC    |        | NC    | 28,6              | 14    | 23,5      | 17    | 75          | 16    |
| March     |          | NC    |        | NC    |                   | NC    | 10,5      | 19    | NC          |       |
| April     |          | NC    |        | NC    |                   | NC    | 0         | 8     | 0           | 14    |
| May       | 0        | 17    | 28,6   | 30    | 20                | 15    | 4,5       | 22    | 12,5        | 18    |
| June      | 16,7     | 18    |        | NC    |                   | NC    | 0         | 8     | 59          | 83    |
| July      | 12       | 25    |        | NC    |                   | NC    | 0         | 20    | 22,7        | 22    |
| August    | 33,3     | 18    |        | NC    |                   | NC    | 8,3       | 12    | 25,9        | 32    |
| September | 0        | 12    |        | NC    |                   | NC    | 0         | 22    | 36,8        | 49    |
| October   | 3,9      | 52    |        | NC    | 36,4              | 33    | 4,7       | 43    | 36,3        | 69    |
| November  | 2,2      | 46    |        | NC    |                   | NC    | 4,8       | 21    | 20          | 75    |
| December  | 2,8      | 36    |        | NC    |                   | NC    | 0         | 16    | 0           | 70*   |

Total =Total number of stools screened

NC =No collection for the specified period

† =Laboratory

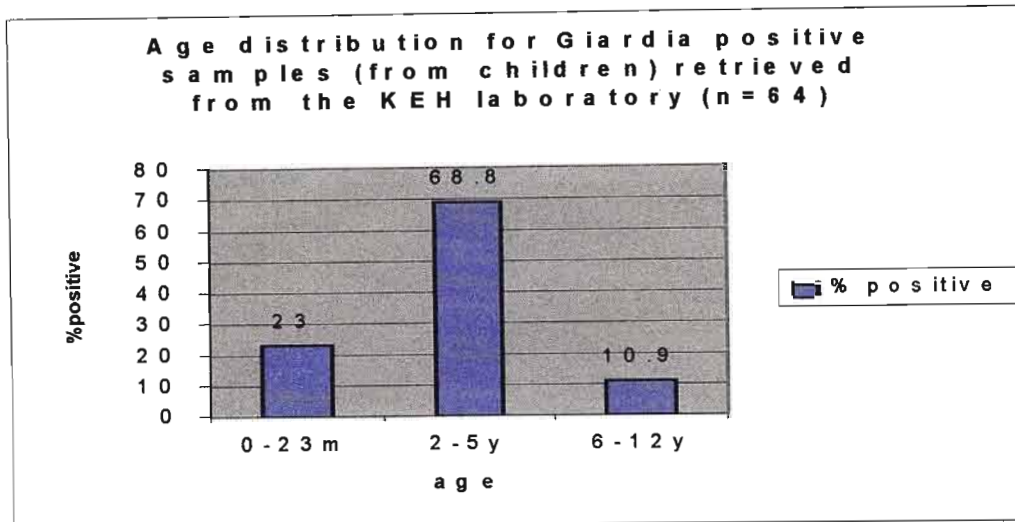
‡ =Day care centers

# =Children's home

\* =Stools were collected two weeks after a mass Mebendazole treatment in the institution.

During the study period, 6246 stool samples were submitted to KEH Microbiology laboratory and *Giardia* was isolated in 78 (1.25%) of these using direct microscopy (Table 2.2). Sixty-four of the 78 samples were obtained from children aged 0-12

years. Figure 2.1 illustrates age distribution for these samples. The majority of the children (68%) were between the ages 2-5 years. Five of the 78 were obtained from adults while no ages were indicated in the hospital records in 9 samples.



**Fig.2.1** Illustrates the percentage age distribution for 64 of 78 patients whose faecal samples (submitted to KEH microbiology laboratory between January 1996 and December 1997) were harbouring *Giardia*. Approximately 69% of the patients were children aged between 2 and 5 years.

The percentage of *Giardia* positive samples ranged between 0,36 (July 1996) and 4,11 (May 1997) as shown in Table 2. 4 and *Giardia* was not detected from samples submitted during the months of May and October 1996. It should be noted that many confounding factors in this sample population do not allow for determination of real prevalence: (i) the submitted samples were obtained from patients who came from different geographical areas because KEH is a referral hospital for many far-outlying areas (ii) the sample population comprised of a mixture of adults and children (iii) direct microscopic screening is known to be less sensitive than the concentration methods, therefore real prevalence would be underestimated.

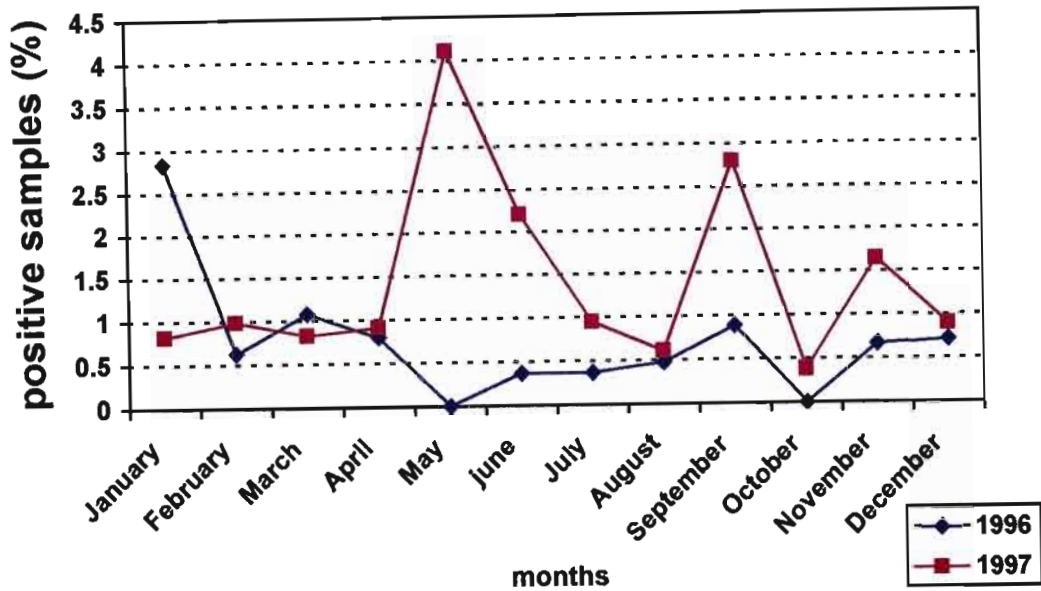


**Table 2. 4.** Monthly summary of stools screened (from the KEH Microbiology lab) and the percentages of *Giardia* positive samples over a two-year period.

| Month     | 1996             |                 | 1997             |                 |
|-----------|------------------|-----------------|------------------|-----------------|
|           | % <i>Giardia</i> | Stools Screened | % <i>Giardia</i> | Stools Screened |
| January   | 2,83             | 460             | 0,82             | 363             |
| February  | 0,62             | 325             | 0,99             | 302             |
| March     | 1,08             | 278             | 0,83             | 240             |
| April     | 0,8              | 251             | 0,91             | 222             |
| May       | 0                | 291             | <b>4,11</b>      | 219             |
| June      | 0,37             | 273             | 2,21             | 226             |
| July      | <b>0,36</b>      | 276             | 0,95             | 211             |
| August    | 0,48             | 210             | 0,61             | 163             |
| September | 0,89             | 226             | 2,8              | 179             |
| October   | 0                | 248             | 0,39             | 258             |
| November  | 0,67             | 298             | 1,65             | 242             |
| December  | 0,72             | 278             | 0,9              | 207             |

Figure 2.2 indicates the monthly pattern of *Giardia* positive samples from the KEH laboratory. There were more positive samples in 1997 than in 1996, however this was not statistically significant ( $p=0.67$ ). This increase was inexplicable because all the technical parameters were constant during the 2-year collection period. Seasonal variation (in terms of rainfall patterns) would be difficult to measure because of the diversity of geographical location of the source patients.

**Pattern of Giardia-positive samples submitted over 2 years**



**Fig.2.2** A graphical illustration of the monthly percentages of *Giardia* positive samples detected among all specimens submitted to the KEH laboratory for routine microbiological analysis from January 1996 to December 1997. The values do not represent prevalences because of sample bias. Detected by direct microscopic screening.

**General Observations:**

- Approximately 25% of the *Giardia* positive stools, particularly from the hospitalised individuals, were found to contain moderate to high numbers of yeasts.
- Many other parasite ova and cysts were detected microscopically from stools that were collected from the day care centers and KEH wards. The predominant ones were the ova of *Ascaris lumbricoides* (overall 62%) and *Trichuris trichuria* (57%) which, more often than not, were found in association with each other. The second most commonly detected were the *Entamoeba*

*spp* (59%). Other parasites less frequently isolated from KEH inpatients included oocysts of *Cryptosporidium* (0,9%) and *Isospora spp* (0,5%). While a predominance of *Ancylostoma duodenalis* (12,2%) was noted in the stools collected from Hlabisa laboratory, *Schistosoma mansoni* (1%) was also detected from these stool samples.

- *Giardia* was commonly found (26%) in association with *Hymenolepis nana*, particularly in the stools collected from the childcare centres. The association of *Giardia* with other parasites for the entire sample was as follows: *Entamoeba spp* 26,8%; *Ascaris* 14,2%; *Trichuris trichuria* 13,1%; *Blastocysts* 3,1%; *Ancylostoma duodenalis* 1,58%; *Isospora*, *Chilomastix* and *Trichomonas hominis* 0,5%.
- Of the 78 stool samples harbouring *Giardia* (obtained from KEH), 13 (16,7%) were sourced from asthmatic children attending the allergy /asthma clinic. Four of the 78 (5,1%) children harbouring *Giardia* presented with eosinophilia. Nine of the 78 (11.5%) were obtained from anaemic patients, predominantly children. *Giardia* cysts were also isolated from a 9-day old premature neonate.
- In the child-care centres, the same individuals were more often found to be infected with *Giardia* over a 2-year period.

### **2.3.2 Cyst Purification**

#### **Comparison of the three methods tested for cyst purification**

Thirteen different stool specimens containing *Giardia* cysts were purified (in duplicate) by each of the three methods. A total cyst count was subsequently performed on each resultant purified sample. This was done on different days. The results of each averaged count are listed in Table 2. 5.

**Table 2.5.** Results of total cyst counts obtained after purifying different stool specimens using the ZnSO<sub>4</sub>; Discontinuous and Continuous (1M) Sucrose gradient separation methods. (Each result represents an average of two counts). Count =N x 10<sup>3</sup> /ml

| Subject No. | ZnSO <sub>4</sub> | Discontinuous sucrose | 1M sucrose |
|-------------|-------------------|-----------------------|------------|
| 1           | 15                | 21                    | 19         |
| 2           | 13                | 17                    | 25         |
| 3           | 12                | 22                    | 18         |
| 4           | 15                | 24                    | 28         |
| 5           | 143               | 104                   | 139        |
| 6           | 83                | 58                    | 62         |
| 7           | 27                | 24                    | 10         |
| 8           | 227               | 183                   | 216        |
| 9           | 21                | 19                    | 15         |
| 10          | 32                | 13                    | 28         |
| 11          | 47                | 69                    | 75         |
| 12          | 71                | 59                    | 67         |
| 13          | 36                | 39                    | 57         |
| *Frequency  | 54%               | 15%                   | 31%        |

\*frequency of maximal cyst detection = total maximum counts observed for each method per total number of samples

Frequencies of maximal cyst detection by the three methods were calculated and were found to be 54%, 15% and 31% for ZnSO<sub>4</sub>, Discontinuous and 1M sucrose methods respectively. It is clear that the sucrose 1M-gradient and ZnSO<sub>4</sub> floatation methods gave better recovery of cysts from stools than the 0,85M and 0,4M gradients technique.

## Observations

- A large proportion of cysts separated using ZnSO<sub>4</sub> floatation appeared granular intracellularly, even though attempts were made to remove the ZnSO<sub>4</sub> solution by washing as soon as possible.
- Fluid and semi-formed stool specimens were much easier to separate and produced cleaner cysts than hard-formed stools, which required repeated gradient separations.
- Upon examination of the deposits obtained after retrieving cysts from the respective gradient material, more cyst losses were observed on the two sucrose gradient techniques compared to that obtained from the ZnSO<sub>4</sub> sediment after cysts were retrieved from the float.

### 2.3.3 Total Cyst Counts

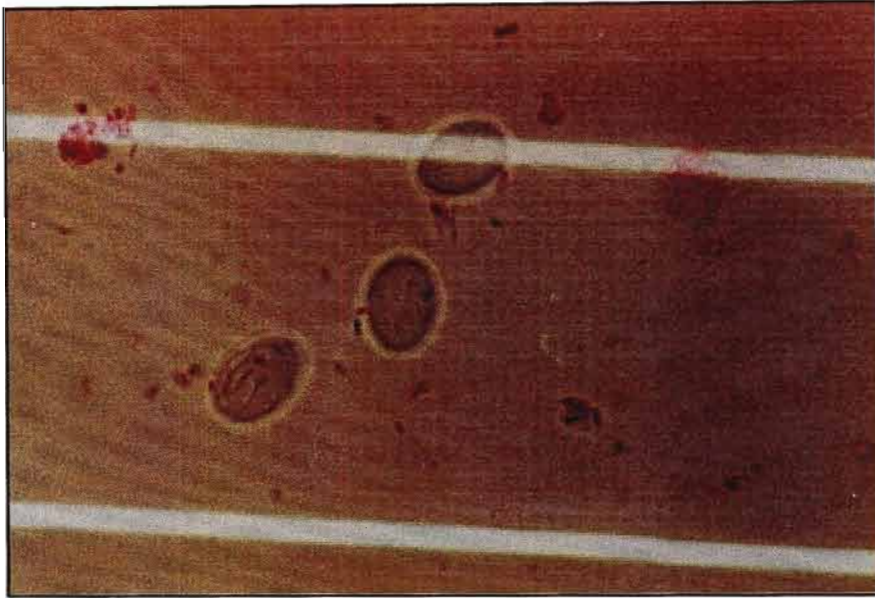
After purification, all cyst suspensions were enumerated on a Neubauer counting chamber and all results appear in Appendices 3-5. Fifty-two samples contained very scanty *Giardia* cysts (detected on the formol-ether concentrated samples). In addition, many of these were non-viable and were therefore considered unsuitable for experimental work.

### 2.3.4 Cyst Viability

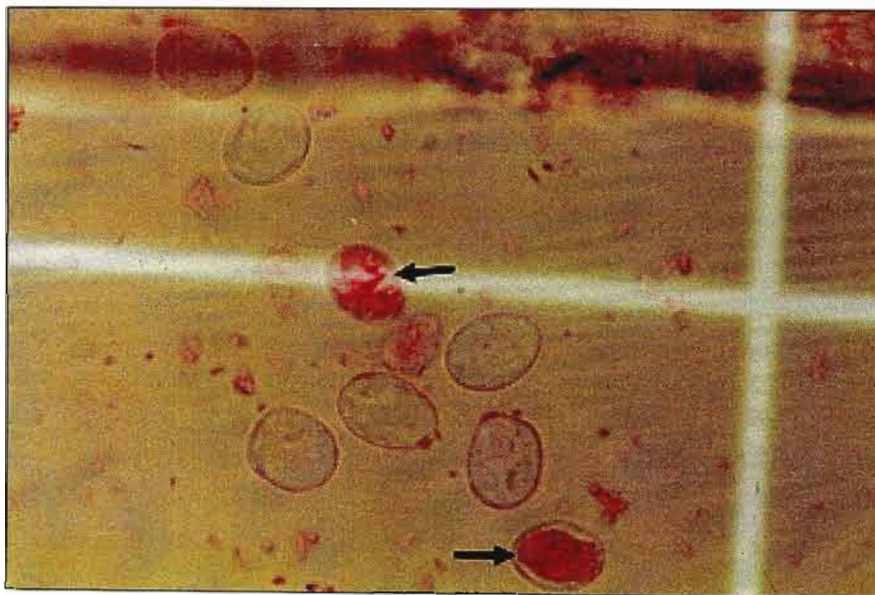
Direct observation of cysts under a light microscope allowed for a qualitative differentiation of viable and non-viable cysts. The former were recognised by their highly refractile cyst wall and clearly defined internal features which are closely adherent to the cyst wall. Similarly, in eosin stained preparations, viable cysts

exhibited these features (Plate 2.1). Non-viable cysts were highly granular, with internal fragments contracted from the less/or non-refractile cyst wall. (Plate 2.3)

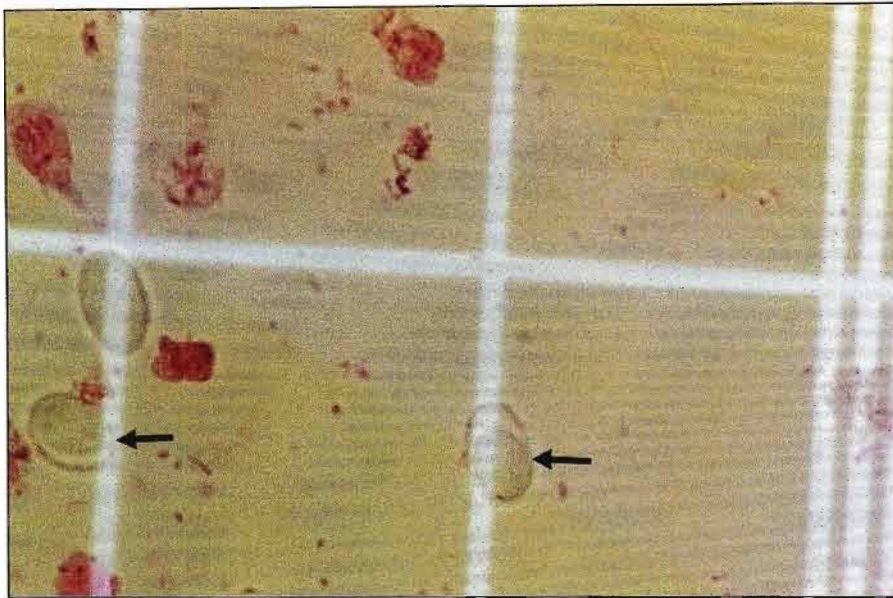
The eosin-exclusion viability assay was performed on all purified cyst preparations to facilitate quantitative assessment of viability and the results are tabulated in Appendices 3- 5. The wall of non-viable cysts allowed the dye to be absorbed and the internal features of the cyst appeared pink (Plate 2.2), whilst viable ones remained pale grey as their walls prevented penetration of the dye (Plate 2.1). However, some cysts failed to absorb the dye despite the fact that their qualitative appearance suggested that they were non-viable. (Plates 2.3 & 2.4)



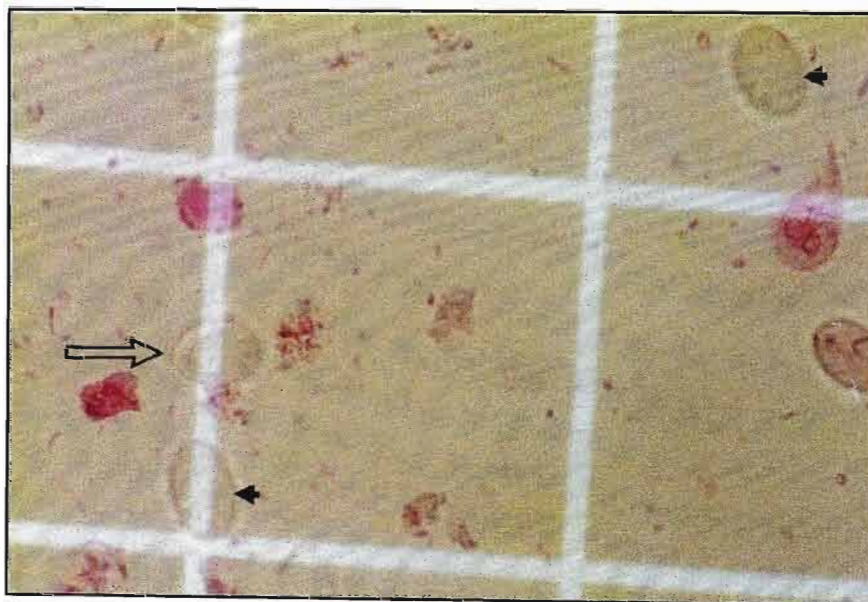
**Plate 2.1.** An eosin-stained preparation. Viable cysts remain unstained and appear pale-grey. (Background consists of bacteria and faecal debris, two non viable trophozoites are also seen). (Magnification 400x)



**Plate 2.2** Eosin stain. Viable cysts as in Plate 2.1 with two non-viable cysts that take up stain (arrows). Background: faecal debris. (400x)



**Plate 2.3** Morphologically non-viable cysts (arrows). The cell contents are partially detached from the cyst wall. The cysts remained unstained. Non-viable trophozoites and a viable cyst are also seen. Faecal debris in the background. (400x)



**Plate 2. 4** A morphologically non-viable (unstained) cyst (arrow outline) and viable cysts (arrowheads). Two stained trophozoites and a stained, nonviable cyst are also shown. (400x)



## 2.4 DISCUSSION

In terms of yield, it is recognised that retrieval of trophozoites from duodenal aspirates is superior to attempting recovery of these stages through excystation of cysts. In this study, however, the organisms were preferentially retrieved from stools for various reasons. The intubation procedure or use of the string test should be performed by a clinician / physician whose presence would then be required during all collection times. Moreover, in random mass collections where infection is not verified, duodenal aspirates would not be practically feasible and thus collection of faecal specimens would be appropriate. Finally, donors, (who in most cases were children and toddlers), might not have consented to repeated intubation which is an unpleasant procedure.

The selected method did, nevertheless, present problems as stool collection and screening were found to be time consuming. Obtaining a steady, reliable source of *Giardia lamblia* cysts proved difficult. In many instances, stools were not readily available from children when required and this resulted in irregular collections. Since *Giardia lamblia* cyst production is intermittent, obtaining a faecal specimen even from an infected individual is no guarantee of cyst recovery. Only a small fraction of all screened stools were found to harbour *Giardia* cysts: 12,4% of 1023 were detected following formol-ether concentration and 1,2% by two examinations of wet preparations. Some, even if positive on screening, were not suitable for use in experiments due to very low cyst numbers and/or abnormal morphology (non-viable).

Seasonal patterns of *Giardia* occurrence have been documented in some

countries with peak infections in late summer/early autumn (Flanagan, 1992; Hall, 1994). In the present study, such a pattern of transmission could not be established from the samples submitted to KEH Microbiology laboratory. This group comprised of a mixture of outpatient and hospitalised subjects coming from different geographical areas. Owing to (1) sample bias (2) the periodic deworming and irregular collection periods from day care centres and institutions, conclusions regarding seasonal trends could not be drawn for this population subset. However, data from this study indicate that the prevalence of *Giardia* in childcare centres ranged between 12,5% and 75% at any one-collection time. Among the faecal specimens submitted to the laboratory and KEH hospitalised patients, the number of positive samples ranged between 0,36 and 23,5%. Predominantly higher infection rates occurred in children aged 2-5 years. This is consistent with the reported peak prevalence at this age interval (Sullivan *et al.* 1991; Hall, 1994). However, the values obtained in this study are most probably an underestimation of the true prevalence of this organism in the local setting for two reasons:

- In most cases, single stool examinations were undertaken. It has been shown that single stool examinations will miss many pathogenic protozoan infections. Hiatt *et al.* (1995), compared the sensitivity of one stool examination with that of three examinations and found that with the additional examinations, the yield increased by 22,7% for *E.histolytica*, 11,3% for *G.lambliia* and 31,1% for *D.fragilis*
- Secondly, the low sensitivity of stool microscopy for *Giardia* results in high rates of false negatives in their detection (Ament & Rubin, 1972; Kamath & Murugasu, 1974).
- All the samples retrieved from the KEH laboratory were screened by direct

examination of wet preparations. This method is documented to be far less sensitive than using a concentration method to enhance detection of cysts (Ritchie, 1948).

Nonetheless, these data reflect a high prevalence of *Giardia* among institutionalised, young subjects. In the one institution where repeated stool collections were made, the same children were consistently found to be excreting cysts at different collection times (time intervals in between collections varied between two weeks to three months). It is not clear whether these were repeated reinfections or persistence of the same infections because at Othandweni Home, regular mass treatments with Mebendazole were undertaken by a clinician attached to the MRC Amoebiasis Research Unit during the study period. (Previous experience with a treatment regimen comprising of Mebendazole and Metronidazole could not eradicate *Giardia* infections at this Home (Jackson TFHG, personal communication). Finally, institutionalised individuals, particularly pre-school children, proved to be a reliable source of *Giardia* cysts. Work requiring a constant supply of fresh cysts, should be based in such a setting.

Early investigators speculated on probable symbiont existence between *Giardia* and yeasts in the small intestine (Karapetyan, 1962; Meyer, 1979). Association of *Giardia* with yeasts was found in 25% of stools from the hospital subjects and was not prominent in stools obtained from the day-care subjects (4,5%). It would appear from this observation that antibiotic-induced disruption of the gut microbial flora (in the hospitalised individuals who in all likelihood were on antimicrobial therapy) resulted in the preponderance of opportunistic yeasts.

Association of *Giardia* with allergy has previously been reported by others, (Farthing *et al.*, 1983; Perez *et al.* 1994). In the present study, a general observation wherein 5 and 17% of the 78 hospital patients harbouring this protozoan presented with eosinophilia and asthma respectively was noted. However, no strict statistical analyses were undertaken because there were no matching controls against which to measure this variable (outside the scope of this dissertation).

In preliminary experiments, the ZnSO<sub>4</sub> floatation and the 1M sucrose step gradient purification methods were shown to yield better retrieval of cysts from stools than the sucrose discontinuous gradient method. Isaac-Renton *et al.* (1986) reported that the ZnSO<sub>4</sub> method generally yielded cleaner samples and produced larger numbers of organisms than the sucrose gradient technique. We found this to be true in the sense that the cysts were indeed cleaner, and the numbers were higher as revealed by the highest frequency. However, cysts separated by the former method appeared morphologically abnormal. This finding is in keeping with results obtained by Truant and colleagues (1981), who reported that the morphology of *Giardia* cysts was better preserved with sedimentation procedures than with the ZnSO<sub>4</sub> floatation technique. However, increased cyst losses were noted when using the sucrose gradient separation method, a finding similar to that of Jyothi *et al.* (1993).

It was essential to determine the viability of purified cysts, as it would impact on the interpretation of all subsequent excystation experiments (described in chapters 3 and 4) in the present work. Isaac Renton (1993) reported that viable cysts are

recognised (on unstained preparations) by, *inter alia*, their high refractility. In the present study, this feature was observed while the cysts were enumerated (section 2.3.3). In our findings, a proportion of morphologically non-viable cysts retained their refractility even though they were non-viable (Plates 2.3 & 2.4). Very old (>2months old) cysts evidently became non-refractile. The eosin-exclusion method was employed in order to quantify the levels of viability. Here viable cysts maintain an intact dye-excluding wall, while dead ones allow dye penetration. On the contrary, some stained preparations revealed cysts that did not allow the eosin to penetrate through the cyst-wall although morphologically they were apparently non-viable. It is likely that the chitin wall of the cysts retained their ability to act as a dye-excluding barrier although the trophozoites inside were non-viable. The eosin exclusion assay consistently suggested high levels of viability. This and the fact that even morphologically nonviable cysts excluded the dye prove that the eosin method is not reliable for determination of viability. Similar findings were reported by other investigators (Bingham *et al.*, 1979; Schaefer, 1990). Contractility and disintegrated morphology were found to be relatively more reliable visual measures of reduced viability than the dye exclusion method. A quantitative value of cyst viability cannot however be derived from the latter. More work is required to develop and evaluate more reliable methods of assessing viability.

## Chapter 3

### **IN VITRO EXCYSTMENT**

#### **3.1 INTRODUCTION**

Research on excystation of *Giardia* began as early as 1925 (Hegner, 1925). In his later report on *in vitro* excystation of human intestinal protozoa, he recognised moisture and a temperature of about 37°C as the only factors necessary for excystation (Hegner, 1927). It was only in 1979 that all conditions responsible for induction of this process were defined by Bingham and Meyer (1979). These authors demonstrated that *Giardia* excystation is pH dependant, being initiated by acidic conditions. The process is only completed successfully if the acid exposure is promptly followed by incubation in an alkaline medium. This process was described as analogous to passage of cysts through an acidic stomach followed by the alkaline environment encountered in the duodenum. They illustrated that the hydrogen ion was responsible for induction of excystation. In this experiment, cysts were successfully excysted after being exposed to synthetic gastric juice containing inorganic salts and pepsin at acid pH.

Most excystation methods subsequently published have been based on these workers' findings, some with slight modifications. Various combinations of salts, bicarbonate and pepsin or trypsin were mainly used in the modified versions (Bingham *et al.*, 1979; Rice & Schaeffer, 1981; Coggins & Schaefer, 1986; Buchel *et al.*, 1987; Hamilton & Jackson, 1990). In recent years, several workers have reported successful excystation of *Giardia* from humans and subsequent

cultivation *in vitro* (Kasprzak & Majewska, 1985; Isaac-Renton *et al.*, 1986; Meloni & Thompson, 1987; Hautus *et al.*, 1988; Al-Tukhi *et al.*, 1991; Feely *et al.*, 1991; Korman *et al.*, 1992).

Bingham *et al.* (1979) investigated the effects of several factors on *Giardia* spp. excystation *in vitro* and noted that temperature, pH, time and incubation medium affect the levels of excystation achieved. They deduced that in general, those conditions most closely approximating the organism's *in vivo* environment induced the highest levels of excystation. Similarly, Schaeffer *et al.* (1984) studied the factors promoting *in vitro* excystation of *Giardia muris* cysts. They reported similar conditions to those noted by Bingham *et al.* (1979) and further deduced that in addition, the presence of CO<sub>2</sub> and positive oxidation-reduction potentials were equally essential. Various combinations of solutions were used to induce excystation, some containing pepsin digest and others with trypsin. They reported that trypsin accentuated excystation as it enhanced the escape of the trophozoites from cysts. There was a significant increase to over 90% excystation when a solution containing trypsin was used in place of a pepsin digest. However, Buchel *et al.* (1987) studied *in vitro* excystation of *G.lambli*a in detail using a scanning electron microscope and indicated that for *G.lambli*a, best excystation is obtained when pepsin is used.

The effector mechanisms of the excystation process are not completely understood, however, many enlightening findings have been reported. Feely *et al.* (1984) described contractile systems that are responsible for excystation. Coggins and Schaefer (1986) proposed the possibility that enzymatic agents are

synthesised internally and then secreted by the vacuoles to digest the cyst wall. Small dense vesicles were seen on the surface of the trophozoites as they emerge from the cyst wall. In 1991, Feely *et al.* induced excystation of *G.muris* cysts in a phosphate-bicarbonate solution. In their experiment, histochemical analysis demonstrated acid phosphatase activity in the lysosome-like peripheral vacuoles in induced cysts. This report tallied with the earlier suggestions of an enzymatic process being responsible for the excystation process. Buchel and co-workers, (1987) employing scanning electron microscopy of the process, observed that flagella play a mechanical role during excystation. It is thus evident that excystation is an active process on the part of the parasite.

One of the aims of the present study was to initiate viable laboratory cultures of *Giardia* from *in vitro* excysted trophozoites. It was therefore necessary to identify a suitable method that would facilitate optimal retrieval of maximum numbers of trophozoites from faeces-derived cysts. Several published methods using different combinations of solutions were then explored in order to identify the ideal technique. This chapter details these methods and the results obtained.



## 3.2 MATERIALS AND METHODS

Three excystment techniques were assessed as detailed below:

### 3.2.1 The Acid Induction method (Al Tukhi *et al.*, 1991).

Cysts purified as described in Chapter 2 (2.2.3.3) were aliquoted in duplicate (where sufficient numbers were available, triplicate aliquots were made). A 0,1ml aliquot of the purified cyst suspension, containing between  $1 \times 10^2$  and  $18,3 \times 10^4$  cysts/ml was suspended in 0,9 ml of 1M Hydrochloric acid (HCl) (Polychem, Durban, SA.) at pH 2 in a microfuge tube. The tubes were incubated upright at 37°C for one hour and the mixture then centrifuged at 600xg for 10 minutes at 22°C. Supernatant acid was gently decanted and the preparation was washed free of acid by resuspending in an alkaline TYI-S-33 medium (Appendix 7) and centrifuging as described above. The pellet was aseptically harvested and inoculated into an 8ml Kimax (Kimble Glass Inc., USA) glass tube containing 7ml of pre-warmed, modified TYI-S-33 medium with bile and antibiotics. The tubes were incubated at 35,5°C at a 5-degree angle (to allow emerging trophozoites, if any, to adhere to glass) for at least 7 days to allow complete excystation. At daily intervals, the tubes were examined on an inverted microscope, and an aliquot was aseptically transferred to a slide and examined for the presence of excysted trophozoites.

*To optimise the excystation conditions, the effects of duration of exposure to acid was assessed by varying this period from 15min to 1hour in 15 minute increments. As no differences in excystation were noted for each of the time periods used, 1hour exposure was thought to be optimal for concurrent reduction of bacteria. The method was modified by: (i) including a wash step after the initial acid incubation (ii) in later experiments, cyst concentrations were adjusted to contain at least  $10^5$  cysts/ml.*

### **3.2.2 The Modified Acid Induction Method of Rice and Schaefer (1981) (Hamilton and Jackson 1990).**

Washed cyst pellets as obtained in Chapter 2 (section 2.2.3.3) containing at least  $10^5$  cysts were transferred to a 5ml polypropylene tube. The tubes were filled with freshly prepared pre-warmed acid induction medium (Appendix 6a) and tightly capped to keep the contents anaerobic. The mixture was incubated at 37 °C for 30min and thereafter centrifuged at 400xg for 1min at 22°C. The acidic supernatant was gently aspirated and discarded. The pellet was resuspended in 5ml excystment medium (HSP-1, Appendix 6b) and centrifuged at 400xg for 1min at 22°C and the supernatant medium was discarded. The resultant sediment was incubated at 37°C in 3ml of prewarmed excystment medium into which 1ml of 0,3M Glutathione (BDH, Poole, England) was added. A liquid paraffin overlay (0,5 ml) was added. After one hour, the tubes were gently centrifuged at 200xg for 2min at 22°C and the pellet transferred to 8ml Kimax glass tubes (in duplicate) containing 7ml TYI-S-33 medium supplemented with serum, bile and antibiotics (Appendix 7). The tubes were then incubated at 35,5°C at a five-degree angle for at least 7 days. Following examination for excysted trophozoites by inverted microscopy, fresh medium was added to the tubes at 48h intervals. If excysted trophozoites were detected, one of the duplicate tubes was retrieved, chilled on ice for 20min and centrifuged at 400xg at 4°C for 5mins. The deposit was resuspended in 0,1ml of sterile TYI medium, diluted 1 in 10 in 1% formalin (to fix motile trophozoites) and transferred to a haemocytometer. Intact cysts and excysted trophozoites were enumerated and the percentage excystation was determined.

#### ***Improvement to methodology***

- *In an attempt to optimise the excystation, Glutathione (BDH, Poole, England) was added to the incubation medium (Rice & Schaefer, 1981) in order to increase the oxidation-reduction potential.*
- *Horse serum (Highveld Biological, Gauteng, SA) was later replaced with fetal calf serum (Sigma, St Louis, USA) for preparation of excystment medium.*
- *All excysted trophozoites were transferred to TYI-S-33 culture medium for in vitro propagation instead of the modified HSP medium.*
- *As difficulty was experienced in accurately enumerating the excysted trophozoites due to their active motility, trophozoites were subsequently fixed in 1% formalin prior to being enumerated.*

### **3.2.3 The modified Acid Pepsin method of Bingham and Meyer (1979)**

One percent Pepsin (Sigma, St. Louis, USA.) was made in 0,15M NaCl solution and the pH adjusted to 2,0 with 10 M HCl (Polychem, Durban, SA). A purified cyst suspension (as described in Chapter 2) containing at least  $1 \times 10^5$  cysts/ml in 0,1ml normal saline was incubated with 0,9ml of 1% Acid Pepsin at 37°C for 30 minutes in a microfuge tube. The tube contents were centrifuged at 200xg for 2min at 22°C. The supernatant was discarded and pelleted cysts aseptically added to Kimax tubes containing TYI-S-33 medium with antibiotics. The culture tubes were incubated at a 5° angle at 35,5°C for up to 7 days. All experiments were performed in duplicate. One hour after incubation, the tubes were examined with an inverted microscope for the presence of excysted trophozoites. Thereafter, one tube was retrieved from the preparation, chilled for 20 minutes and centrifuged at 400xg at 4°C for 5mins. A 10 $\mu$ l aliquot of the pelleted trophozoites (resuspended in 0,1ml of TYI-S-33) was fixed in 0,09ml of 1% formalin and the number of excysted cysts was determined using a haemocytometer under a light microscope.

### **3.3 RESULTS**

Successfully excysted trophozoites are readily identifiable by light microscopy because of their characteristic motility. Where successful excystation was achieved, percentage excystation was determined.

#### **3.3.1 The Al-Tukhi Excystment Method (1991)**

Excystation experiments were undertaken on sixteen samples (5 from symptomatic and 11 from asymptomatic subjects) using the method described in 3.2.1. Mostly, cysts were used while fresh, however, whenever possible a proportion was stored for up to 8 days at 4°C and replicate excystation attempts were undertaken. In 9 samples, duplicated trials were repeated three times on different days while in the remaining 7 of 16 samples, excystation was undertaken in duplicate once only owing to limited cyst numbers. A total of 34 duplicated excystment trials were performed (in 21 trials the modified method was used) and all were unsuccessful in our hands. The total cyst counts and percentage viability ranged from  $1 \times 10^2$  to  $183 \times 10^4$  and 64 to 97% respectively. Varying the period of acid exposure did not alter the results.

#### **3.3.2 Excystation with the modified Acid Induction method (Hamilton & Jackson, 1990)**

Thirty-four faecally derived cyst samples were processed for excystment by the above method. The details on the source of cysts, the total count, viability and excystation results appear in Appendix 3. The excystation process could be observed under a light microscope when at timed intervals, an aliquot of the excystment preparation was transferred to a microscope slide and examined.

- **After 15 minutes incubation:**

Many forms of the cyst were seen at different stages, though the majority were intact cysts. In some, the internal structure was visible; granular, retracted from the cyst wall on one side (cystozoites) (Plate 3.1). Within some of these, there was contractile movement within the *interna*. Some forms appeared as budding cells, showing balloon-like extrusions, which grew larger. There were few partially excysted trophozoites.

- **After 30 minutes incubation:**

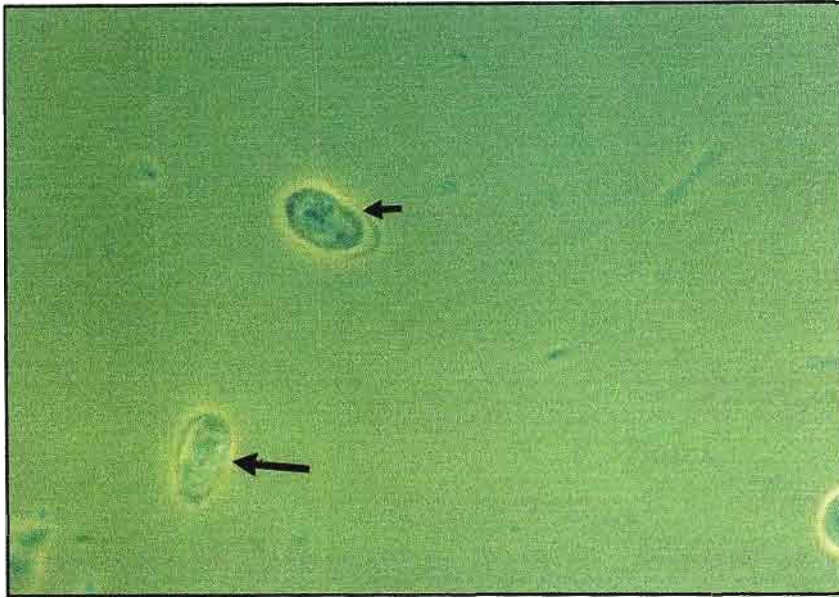
There were more cystozoites with contractile movement within the cyst, and the extrusions in the budding forms grew much larger until a small round trophozoite-like structure appeared (thirty minutes after initiation of excystment). There were also motile forms (atypical in shape) which narrowed at one end as they moved in their typical falling leaf-like motion.

- **After 45 minutes incubation:**

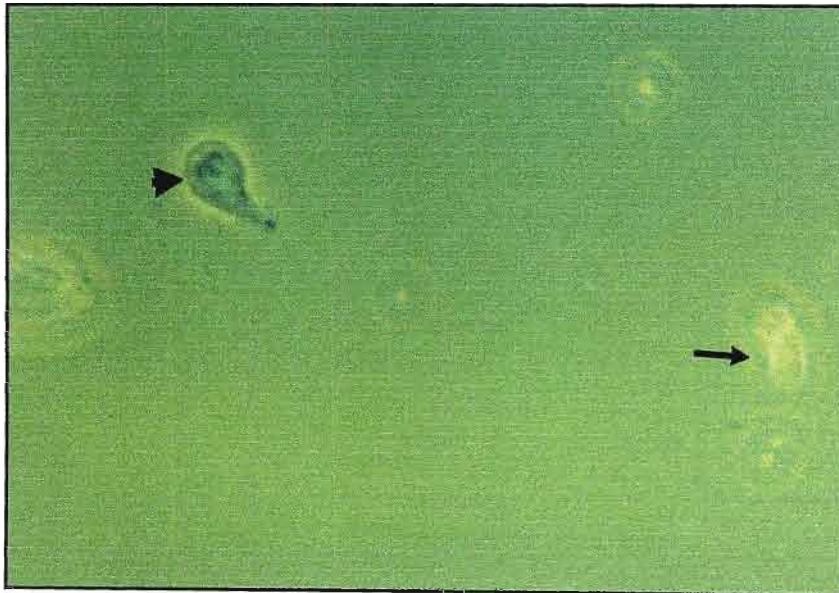
Although there were still some budding cystozoite forms, they were fewer in numbers and appeared larger in size. There were completely excysted trophozoites and morphologically atypical cysts (Plate 3.2. a and b). The completely excysted trophozoites were joined in pairs at the posterior end; they subsequently divided into two trophozoites (Plate 3.3 a and b).

- **60 minutes after incubation:**

Motile excysted trophozoites with their typical appearance and some non-motile, adhering ones were detected. Some cysts were still intact.



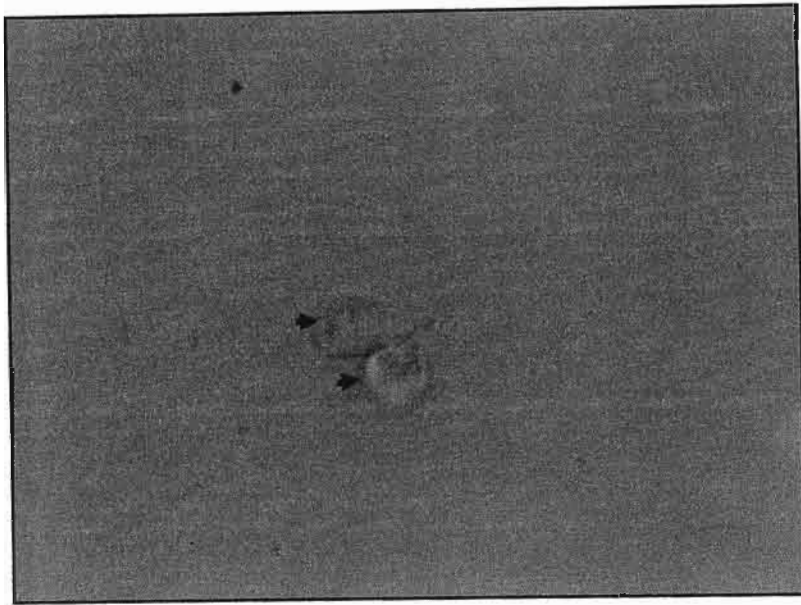
**Plate 3.1** An excysting cyst with the internal contents partially detached (cystozoite) (small arrow) and an induced cyst (wherein contractile movement was observed) (big arrow). An intact cyst is also seen. Background includes faecal debris. (400x).



**Plate 3.2 (a)** A completely excysted trophozoite (arrow-head) and a morphologically atypical cyst in the process of excysting (arrow). (400x)



**Plate 3.2 (b)** A completely excysted trophozoite. (400x)



**Plates 3.3.(a) and (b)** Excysted trophozoite dividing to form daughter trophozoites joined at the posterior end. Bacteria are seen in the background. (400x)



Twenty-four batches of cysts (71% of 34 excystation trials) excysted successfully with this method. The overall percent excystation ranged between 2 and 41% and viability ranged between 30 and 100% by the eosin dye exclusion method.

Addition of Glutathione to the induction medium did not improve the level of excystation (which remained below 40% in all but one of the excysted samples).

The results of successfully excysted samples (expressed in percentages), their viabilities as determined by dye-exclusion and total counts are summarised in Table 3.1. This data was extracted from the full list of all attempted excystations in Appendix 3. The eosin exclusion method showed higher viability levels (with a mean percent viability of 75) whilst the mean excystation value was 8,9%.

In most experiments, low levels of excystation were attained. Only in 3 of the 25 successful excystations were the levels of excystation above 30%, the rest were below 20%.

The method described by Al Tukhi et al. (1991) (3.2.1) was attempted on seven samples that had been successfully excysted previously using the Hamilton & Jackson (1990) method. These cyst samples did not excyst using the former method.

**Table 3.1** Summary data on all successfully excysted samples using the modified Acid Induction method (Hamilton and Jackson, 1990)

| Source | Cyst count (x10 <sup>5</sup> ) /ml | % Viability | % Excystation |
|--------|------------------------------------|-------------|---------------|
| Asympt | 12                                 | 56          | 5             |
| Sympt  | 10                                 | 70          | 10            |
| Sympt  | 79                                 | 44          | *8            |
| Asympt | 11                                 | 82          | 5             |
| Sympt  | 110                                | 27          | *2            |
| Asympt | 18                                 | 75          | 8             |
| Asympt | 10                                 | 88.2        | 10            |
| Asympt | 8                                  | 87.5        | 2             |
| Sympt  | 103                                | 90.9        | *36           |
| Sympt  | 14                                 | 63          | 3             |
| Sympt  | 11                                 | 71          | 12            |
| Asympt | 3                                  | 94          | 5             |
| Asympt | 21                                 | 64          | 33            |
| Asympt | 7                                  | 70          | 10            |
| Asympt | 11                                 | 55          | 2             |
| Asympt | 23                                 | 94          | 20            |
| Asympt | 17                                 | 33          | 2             |
| Asympt | 23                                 | 86          | *41           |
| Asympt | 5                                  | 41          | 7             |
| ?      | 7                                  | 79          | 10            |
| Asympt | 11                                 | 87          | 20            |
| Asympt | 13                                 | 56          | 20            |
| Asympt | 2                                  | 81          | 10            |
| Asympt | 14                                 | 96          | 22            |
| Sympt  | 13                                 | 75.4        | 5             |
| Mean   | 22,24                              | 70,64       | 8,84          |

\*Result represents average of two duplicated attempts.

### 3.3.3 The modified Acid Pepsin excystation method (Bingham & Meyer, 1979)

One hundred and three samples were used for excystation experiments using the modified Acid-pepsin method. Of these, forty-three (42%) excysted successfully. The full details of cyst sources, viability, excystation and culture results appear in Appendices 4 & 5. The individual approximate levels of excystation ranged between 1% and 42% while percentage viability as determined by eosin exclusion ranged between 20% and 100%. With the majority of the excysted samples, low levels of excystation (below 50%) were attained. Table 3.2 summarises the percentage ranges for the two methods. The results obtained with each of the 3 *in vitro* excystation methods are summarised in Table 3.3 and the graphical representation thereof appears in Fig. 3.1

**Table 3.2** Percentage excystation ranges for the Hamilton & Jackson and Acid pepsin methods

| % Excystation Range (both methods) | Number of samples excysted (Percentage) |                     |
|------------------------------------|---|---------------------|
|                                    | Acid Induction* (n=25)                  | Acid Pepsin †(n=43) |
| 1-5%                               | 9 (36%)                                 | 8 (19%)             |
| 6-10%                              | 8 (32%)                                 | 12 (28%)            |
| 11-20%                             | 4 (16%)                                 | 16 (37%)            |
| 21-30%                             | 1(4%)                                   | 5 (12%)             |
| 31-40%                             | 3 (12%)                                 | 2 (5%)              |

\*Hamilton & Jackson, 1990

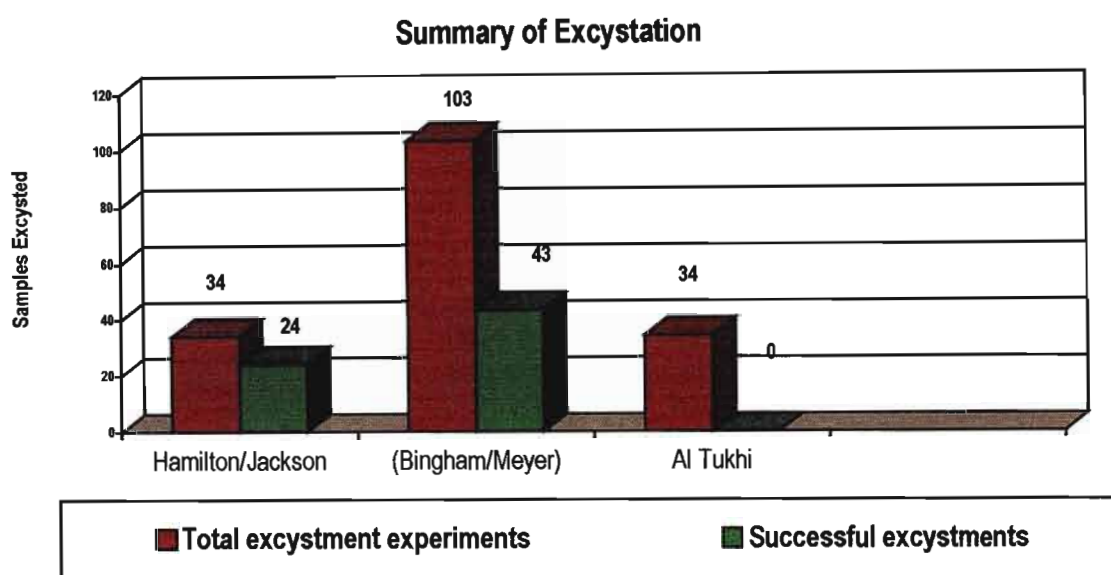
†Bingham & Meyer, 1979

**Table 3.3** Summary results of the 3-excystation methods. At least two excystation attempts were made on each sample.

| Method                | Total No. of attempted excystment experiments | Successful Excystation | % Success |
|-----------------------|---|------------------------|-----------|
| Al Tukhi <i>et al</i> | 34  | 0                      | 0         |
| Acid Induction*       | 34  | 24                     | 71%       |
| Acid Pepsin†          | 103   | 43                     | 42%       |

\*Hamilton & Jackson, 1990

†Bingham & Meyer, 1979



**Fig.3.1** A graphical representation of the summary of *in vitro* excystation using 3 methods. The total number of trials as well as the number of successful attempts are indicated for each method.

It is evident that that the modified Acid Induction method of Hamilton & Jackson resulted in higher levels of successful excystation (overall 71%) than the Acid Pepsin method (overall 42%).

### 3.4 DISCUSSION

Since our source of material for culture initiation was faecally-derived cysts, progress of this work was largely dependent on successful excystation of trophozoites. Several excystment procedures were therefore explored in search of optimal results. It has been acknowledged that none of the available excystment methods work all the time (Nash, 1988). Furthermore, Schaefer (1990) reported large variations in excystation results (2-80%) in his review of available techniques and he concluded that, “*Giardia duodenalis* do not excyst routinely at high levels with available methods”. There are also other investigators of the same opinion (Gordts *et al.*, 1985, Mayrhofer *et al.*, 1992). Similarly, in the current study, varying results were obtained using different excystment methods.

In the classic *in vitro* excystation work of Bingham and Meyer (1979), it was shown that hydrochloric acid alone was capable of inducing excystation and that salts and digestive enzymes did not significantly alter the level of excystation. In our hands, however, 68 replicated attempts at inducing excystation of cysts obtained from both symptomatic and asymptomatic donors by use of HCl only and subsequently incubating in the culture medium (TYI-S-33) as described by Al-Tukhi *et al.* (1991) were unsuccessful. To exclude the possibility of variation in ability of cysts to excyst *in vitro*, the method was repeated (with no success) on seven samples which had been previously excysted with success using the method of Hamilton and Jackson, (1990). Therefore failure to successfully repeat the method described by Al-Tukhi *et al.* (1991) could not be explained in terms of an inherent inability of cysts to excyst *in vitro*.

When salts and trypsin were incorporated into the induction medium (modified

Rice & Schaefer method by Hamilton and Jackson, 1990), a high success rate (71%) was attained in the current work. The method however, involved a post-induction incubation step in modified HSP medium. The excysted trophozoites had to be subsequently transferred to TYI-S-33 medium for propagation and long term *in vitro* maintenance. Later it became apparent that this was not suited for the aim of propagation of viable cultures (as detailed in Chapter 5), therefore the modified method of Hamilton & Jackson (1990) was abandoned.

The acid-pepsin method was better suited for fulfilment of the aims of the present work. Furthermore, in their experiments, Kasprzak and Majewska (1985) showed that although the percentage excystation of *G.intestinalis* and *G.muris* cysts induced by using the TYI-S-33 as the excystment solution is reduced, the resulting yields of trophozoites are greater. The two media (Acid Pepsin induction and TYI-S-33 excystment) were subsequently employed in the current study. Using this method, successful excystation was also achieved, however a comparatively lower success rate was attained.

Excystation is dependent, among other factors upon viability of the cysts. In the current work, viability was assessed by the eosin exclusion method. Although excystation is in itself a measure of viability, the dye exclusion method consistently revealed higher levels of viability than that resulting from any of the excystation experiments (Appendices 3, 4 & 5). The two processes therefore are not comparable. However it must be noted that various factors such as the ability of *in vitro* methods to induce successful excystation must be considered in interpreting these results. On the other hand, the ability of cysts to exclude dye appears to be

an unreliable indicator of viability as shown by some cysts which appeared morphologically non-viable but still retained their ability to exclude dye (Plate 2.3) p59.

It is noteworthy that low levels (<45%) of excystation were obtained with all *in vitro* techniques attempted in the current work and efforts to initiate viable laboratory cultures thereof were unsuccessful (Chapter 5). Since establishment of cultures from excysted trophozoites requires high cyst numbers, the ideal concentration with which to attempt *in vitro* excystation is said to be 100 000 cysts/ml (Nash, 1988). However, in the present study, low levels of excystation were still obtained with all *in vitro* techniques in spite of the high numbers of cysts used for excystation.

Interestingly, in a study for assessing the efficacy of a water treatment agent, Hamilton & Jackson (1990) used *G. muris* cysts *in vitro* excystation to determine the disinfection efficiency. They obtained high levels of excystment (92,2-97,3%). Their experiments were undertaken in the same laboratory where this work was carried out. This finding corresponds with earlier reports that "*G. muris* cysts can be routinely excysted with efficiencies above the 90<sup>th</sup> percentile while *G. duodenalis* excystation efficiencies are erratic and usually much less than 90%" (Schaefer, 1990).

## CHAPTER 4

### *IN VIVO* EXCYSTATION

#### 4.1 INTRODUCTION

Animal models of giardiasis using mice and *G.muris* have been used for various studies since the 1920's. Hegner (1927) reported excystation of cysts from human faeces in laboratory rats. Similar experiments by Filice (1952) illustrated that *Giardia* cysts from humans can excyst and establish infections in the rodent small intestine. Roberts-Thompson *et al.*, (1976) described a novel animal model of murine giardiasis which was later adopted by many workers. They induced reproducible infections by intra-oesophageal inoculation of *G.muris* cysts into Swiss albino mice and investigated the effects of varying doses of inoculated cysts (100, 1000 and 10 000 cysts). Twenty-two of 24 mice receiving inoculations of 100 cysts became infected while all mice receiving 1000 or 10 000 cysts were infected. Maximal cyst output did not differ significantly in the three groups, however the mice inoculated with higher dosages excreted maximum numbers of cysts earlier than those receiving the lower dose. A similar pattern of trophozoite counts was observed; maximum counts occurred on days 5, 7, and 14 post inoculation with 10 000, 1000, and 100 cysts respectively.

Hill and co-workers (1983) pioneered the use of suckling mice for infection with the *Giardia* sourced from humans. They inoculated the CF-1 strain of mice with axenically cultured *G.lambliia* trophozoites to assess the susceptibility of mice to infection. All of the 65 mice that were challenged became infected. Subsequently, Nash *et al.* (1985) first reported the use of neonatal mice as a means of



propagating *Giardia* for subsequent *in vitro* isolation. They axenised five strains using this technique. Recently Mayrhofer and colleagues (1992) also used the suckling mice model for primary isolation of *Giardia* from faecal specimens and for the subsequent growth of the organisms to obtain sufficient material for genetic analysis. Their work revealed that natural infections can be comprised of mixed genotypes. Isolation and purification can lead to selection of distinct genotypes by both *in vivo* and *in vitro* techniques.

Gerbils have also been used as excystation hosts in a study to characterise isolates retrieved from humans and animals in a waterborne outbreak of giardiasis (Isaac-Renton *et al.*, 1993). In another study, gerbils were inoculated with *Giardia lamblia* cysts and infections were effected (Belosevic *et al.*, 1983). In this model it was established that the pattern of cyst release and the number of trophozoites in the intestines of orally and duodenally inoculated gerbils were similar. Visvesvara *et al.* (1988) stated that of all the various animals used as hosts for *G.lamblia*, consistently good results have been obtained only with suckling mice and gerbils. These workers studied the infectivity patterns of *Giardia* cysts obtained from 10 subjects (7 symptomatic and 3 asymptomatic) in gerbils. A strikingly variable infection pattern was reported. One of the ten human isolates (obtained from an asymptomatic person) tested for infectivity in gerbils infected all 12 animals inoculated. Of the nine other isolates inoculated into gerbils, 4 isolates (2 from asymptomatic carriers and 2 from symptomatic patients) failed to establish infections in the animals while cysts from the other five patients produced infections in 11 to 75% of the animals. These results clearly indicated differences between human isolates of *Giardia* in their innate ability to infect gerbils.

The mouse inoculation method using *Giardia* from humans is favoured by renowned *Giardia* authorities like Nash. This method proved to be the most successful for obtaining trophozoites from cysts in his laboratory with a consequent 70% success rate of axenisation of *Giardia* (Nash, 1988).

Although *in vitro* excystation was successfully accomplished (Chapter 3) by various methods, axenic cultures (Chapter 5) could never be established with trophozoites from this source. An alternative method for sourcing trophozoites was needed. Dr T.E. Nash and J. Conrad of the National Institutes for Health (N.I.H) Laboratory for Parasitic Diseases in Bethesda in U.S.A were consulted. They recommended the *in vivo* model and assisted with establishing this method in our laboratory in Durban. The current chapter describes the adaptation of the method to our local environment and the results obtained.

## 4.2 MATERIALS AND METHODS

### 4.2.1. Animal Sourcing and Care

C57BL/6 inbred mice were recommended by Nash and Conrad (personal communication) and these (eight weeks old) were obtained from the Biomedical Resource Centre at the University of Durban Westville. In addition, an indigenous species, readily available in our laboratory, *Praomys (Mastomys) coucha* (outbred) was used. The mice were housed in cages cushioned with sawdust and the temperature maintained at 25°C. A standard pellet diet and fresh water were supplied to the animals *ad lib*. Soiled sawdust was replaced daily.

### 4.2.2 Preliminary Experiments:

To ensure that the animals were well suited for the *in vivo* experiments, some pilot studies were necessary to:

- eliminate any endogenous infections
  - \*establish appropriate cyst dosages with which to effectively inoculate the mice
  - \*establish the appropriate time to retrieve the trophozoites from infected mice
- in order to ensure that maximum numbers of organisms are obtained.

\*Although these have been published previously elsewhere, this type of work was being done for the first time in SA and in our laboratory, furthermore the *Mastomys* strain used here differs from those used by the other investigators.

#### 4.2.2.1 Exclusion of Endogenous *Giardia* Infections

During the acclimatisation period, mouse faecal samples were collected on wetted absorbent cotton-wool pads daily from each of the cages and were screened for the presence of *Giardia* cysts using the formol-ether technique (2.2.2.1) for ten days. The mice were deemed free of parasite infection if all faecal screens were

negative. Metronidazole, 20µg/ml (Flagyl; Rhone-Poulenc, Montreal) was administered orally with a blunted feeding needle for three consecutive days to all the mice. The animals were then mated.

#### **4.2.2.2 Determination of a Reliable Infective Dose of Human *Giardia* Cysts in C57BL/6 Suckling mice.**

Pelleted cysts (prepared as previously described in 2.2.3.3) were diluted to contain different concentrations varying from 100, 1000 to 10 000 cysts/ml in 0,1 ml distilled water. Three different litters of C57BL/6 mice (each consisting of 5, 6 and 9 three-day old neonatal mice born of mothers who were pre-treated with Metronidazole) were intragastrically inoculated with the doses of cysts as follows: Five neonatal mice inoculated with 100-cysts/ ml; nine neonatal mice inoculated with 1000 cysts/ml; and a dose of 10000 cysts/ml injected onto the stomachs of six suckling mice. The mice were sacrificed ten days post inoculation (pi). The entire small intestines were finely chopped with a fine pair of scissors in 1ml of cold TYI-S-33 in Petri dishes. A 0,01ml aliquot was transferred to a glass slide and a 20 x 40mm coverslip was applied. The area of the slide covered by the coverslip was scanned for the presence of trophozoites by light microscopy. The rest of the chopped intestines were transferred to an 8ml Kimax glass tube containing 7ml TYI-S-33 medium and maintained as described in 4.2.3 below.

#### **4.2.2.3 Determination of Peak Trophozoite Growth.**

The optimum period (days after inoculation) during which maximum intestinal trophozoite numbers are obtained had to be determined before the *in vivo* experiments were initiated.

A litter comprising of 7 C57BL/6 mice was inoculated with 1000 cysts/pup by intra-gastric injection. Two to three mice from the litter were sacrificed seven, ten and twelve days post-inoculation. To retrieve at least 80% of trophozoites from their small intestine, a vigorous shaking method (Olveda *et al.*, 1982) was utilised and modified as follows: After killing the mice by cervical dislocation, the entire small intestine (from the gastroduodenal junction) was dissected longitudinally in a Petri dish. The macerated intestines were then transferred to 5 ml of cold, sterile phosphate-buffered saline (PBS) in a sterile 15-ml polypropylene centrifuge tube. The contents of the tube were shaken vigorously for 10 seconds using a vortex mixer. The suspension was thoroughly mixed by sucking in and out with a sterile Pasteur pipette. A 0,001ml aliquot was fixed in 1% formalin, transferred to a counting chamber and the number of trophozoites was counted microscopically. The suspension was then filtered through sterile gauze and the filtrate was centrifuged at 400g at 4°C for 5 mins. The supernatant PBS was decanted aseptically and the pelleted trophozoites were transferred to culture tubes with TYI-S-33 medium containing antibiotics (Appendix 7).

In a separate experiment, to determine the duration of infection in the mice One litter (9 pups) of *Mastomys* was inoculated orally with a feeding needle with 1000 cysts/ml/mouse. Another litter of the C57BL/6 (7 pups) was inoculated similarly with 1000 cysts/ml/mouse. Cyst excretion was monitored on days 6, 7, 8, 10, 15, 20, 24 and 42 days by retrieving stools from the cages. The mice were sacrificed at intervals on days 8, 12, 24 and 42 days post inoculation.

### **4.2.3 *In vivo* Excystation**

This method was based on that of Dr TE Nash & JT Conrad of the N.I.H (Bethesda, Maryland) (personal communication) and modified for local experimentation.

Suspensions containing 1000 cysts/ml in 0,1ml aliquots of distilled water were injected into each of the stomachs of 1-2 day old suckling mice in a litter (a litter consisted of at least 4 up to 9 pups). A total of 53 litters were inoculated. Depending on the availability of neonatal mice and the number of cysts in a sample, the inoculations were replicated either within the same strain of mice or between the two strains. Seven to ten days after inoculation, the infected mice were sacrificed by cervical dislocation. The proximal 8-cm of small intestine was removed and macerated with scissors in 1ml of cold TYI-S-33 medium in a Petri dish. The mixture was transferred to a sterile 9` 0 x 10mm Kimax glass tube filled to 80% capacity with the TYI-S-33 culture medium containing antibiotics and its cap was immediately screwed on. Inoculated tubes were chilled for 30 minutes to detach trophozoites from the macerated intestines and then incubated at 35,5° C for 1 hour at a five-degree angle to allow the trophozoites to adhere to glass. Medium containing intestine fragments was filtered through a double layer of gauze to eliminate the toxic intestines. The filtrate, which contained the unattached trophozoites, was transferred to a sterile 8ml Kimax tube and the latter filled to 80% capacity with fresh TYI-S-33 medium with antibiotics. All the tubes were incubated at 35,5°C for 1 hour and supernatant medium gently decanted and replaced with an equal volume of fresh TYI-S-33 with antibiotics. All tubes were then incubated for 1 hour and the medium was changed one more time. The tubes were examined on an inverted microscope

for attached trophozoites and maintained at 35,5°C for at least seven days even if no trophozoites were detected on the first day.

***Improvement on methodology***

- ***A concentration of 1000 cysts/ml was found to be effective in establishing infections in mice (preliminary experiments). Medium containing the chopped intestine was filtered through a double layer of sterile gauze into a sterile 50ml polypropylene tube and later used for propagation of trophozoites, and not discarded as in the original method.***

**Note:**

Aliquots of all batches of cysts inoculated into mice were concurrently subjected to attempted excystation *in vitro* by the modified acid pepsin method described in 3.2.3. Where cysts numbers were sufficient, (and depending on the availability of neonatal mice when cysts were isolated) repeated inoculations of the same mouse strains and different mice strains were attempted using the same cyst batches which had been aliquoted and maintained at 4°C.

## 4.3 RESULTS

### 4.3.1 Preliminary Experiments

#### 4.3.1.1 Endogenous infections

None of the mice were found to be excreting *Giardia* cysts when their stools were screened for 10 days. The mice were nevertheless treated with Metronidazole to eliminate any occult infections that might have been present.

#### 4.3.1.2 Determination of a reliable infective dose of human-derived cysts in mice.

Three different concentrations of cysts 100, 1000 and 10 000 inoculated into mice produced infections in 60, 100 and 83% of the mice respectively (Table 4.1).

Consequently, standardised inoculations of 1000 cysts/ml were used for the subsequent experiments.

**Table 4.1.** Results of infection of three different litters inoculated with 100, 1000 or 10000 cysts /ml/mouse ten days post inoculation

| Dosage (cysts/ml/pup) | Mice infected (Total inoculated) |
|-----------------------|----------------------------------|
| 100                   | 3 (5)                            |
| 1000                  | 9 (9)                            |
| 10000                 | 5 (6)                            |

#### 4.3.1.3 Optimal period for maximal numbers of trophozoites

Suckling mice inoculated with 1000 cysts/ml/mouse and sacrificed for quantification of trophozoite production on days 7, 10 and 12 pi were found to harbour a greater number of trophozoites at 10 and 12 days in comparison to 7



days pi. However, accurate quantification was difficult. The results are listed in Table 4.2.

**Table 4.2.** Summary of average numbers of trophozoites detected in the small intestine of infected mice 7-12 days after inoculation.

| Days (pi) | Mean number of trophozoites ( $\times 10^6$ ) | Range ( $\times 10^6$ ) |
|-----------|---|-------------------------|
| 7         | 4   | 3-5                     |
| 10        | 10.5  | 8-13                    |
| 12        | 9.5   | 9-11                    |

pi = post inoculation

#### 4.3.1.4 Duration of infections

Cyst excretion (stool microscopy) and trophozoite recovery (sacrificed mice intestines) were monitored on different days from the 2 mice strains inoculated as described in 4.2.2.3 above. Tables 4.3(a) and (b) list the results.

**Table 4.3(a)** Cyst excretion in *Mastomys* and *C57BL/6* mice 6-42 days after inoculation with *G.lambli*a cysts.

| Cyst Excretion.<br>Days pi |                 |                |
|----------------------------|-----------------|----------------|
|                            | <i>Mastomys</i> | <i>C57BL/6</i> |
| 6                          | -               | -              |
| 7                          | -               | -              |
| 8                          | +               | -              |
| 10                         | +               | +              |
| 15                         | +               | +              |
| 20                         | -               | -              |
| 24                         | -               | -              |
| 42                         | -               | -              |

+ Cysts detected  
- no cysts detected

**Table 4.3(b)** Trophozoite recovery from intestines of *Mastomys* and C57BL/6 mice sacrificed 8-42 days after inoculation with *Giardia lamblia* cysts.

| Trophozoite detection.<br>Days pi |                 |         |
|-----------------------------------|-----------------|---------|
|                                   | <i>Mastomys</i> | C57BL/6 |
| 8                                 | +               | +       |
| 12                                | +               | +       |
| 24                                | +               | -       |
| 42                                | -               | -       |

+ trophozoites detected  
 - no trophozoites detected

#### 4.3.2 *In Vivo* Excystation Results

Successful *in vivo* excystation was demonstrated by the presence of trophozoites in the small intestine of inoculated mice. Inoculation with at least 1000 *Giardia* cysts/ml/mouse in distilled water was attempted with fifty-three litters of *Mastomys* and C57BL/6 mice. The results of these *in vivo* excystation experiments are summarised in Table 4.4 and the full details of cyst sources, counts as well as their *in vitro* (acid pepsin) excystation results are listed in full in Appendix 5.

**Table 4.4.** Summary of the inoculations attempted in *Mastomys* and C57BL/6 mice with resultant findings.

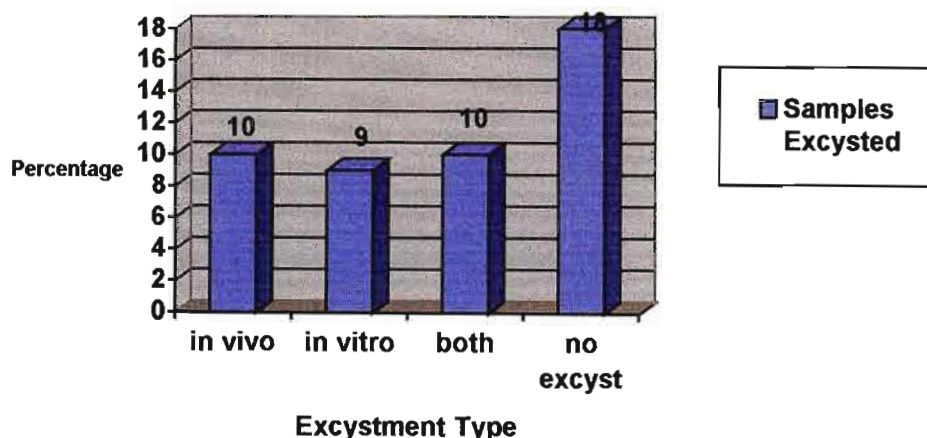
| Mouse Strain    | No. of litters inoculated | Successful excystation/(% resisted infection) |
|-----------------|---------------------------|---|
| C57BL/6         | 39(-1)*                   | 15/(61%)                                      |
| <i>Mastomys</i> | 14(-4)*                   | 5/(50%)                                       |
| <b>Total</b>    | <b>†53</b>                | <b>20</b>                                     |

\* Lost through cannibalism after inoculation. This was more common in *Mastomys* (29%) compared with the C57BL/6 (3%). Approximately 61% of C57BL/6 resisted infection while 50% of the *Mastomys* were not infected.

†Of the 53 inoculation trials, 47 were interpretable, as 5 litters were lost through cannibalism and 1 litter was heavily infested with *Hexamita muris* (synonym. *Spirotrichia muris*, a common rodent parasite). Twenty of the 47 inoculated litters (43%) became infected while 26 (55%) failed to harbour trophozoites (22 in C57BL/6 and 4 in *Mastomys*), despite replicate attempts in 12 batches (Appendix 5). In two instances, *Mastomys* mice were co-infected with numerous flagellates (*Hexamita muris*) and the *Giardia* trophozoites subsequently died *in vitro*. In one litter the numbers of the contaminating organisms were so overwhelming that it was not possible to confirm the presence or absence of the *Giardia* trophozoites.

In addition to the mouse inoculations, cyst samples were concurrently processed for *in vitro* excystation. A comparison between the two excystation methods was subsequently undertaken. Different outcomes were observed for each of them. It was found that in some instances cysts would excyst by the one method but not

the other while in others excystment occurred with both methods. A proportion of the samples could not be excysted with either method. The summary of comparative results of excystation using both *in vitro* (acid pepsin) and *in vivo* (mouse inoculation) methods is presented in Fig.4.1 below.



**Fig.4.1** Summarises the outcome of 47 attempted excystations using animal inoculation (*in vivo*) and acid pepsin (*in vitro*) methods. A total of 53 excystation attempts were made but only 47 were interpretable. Of those, 10% excysted in vitro only and 9% excysted in vitro only. Ten percent excysted by both methods while 18% did not excyst at all.

#### 4.4 Observations

##### Infectivity patterns to mice

Variations in infection patterns of the 2 mice strains were observed:

- The *Mastomys* appeared to be more susceptible to infection than the C57BL/6 mice. Firstly, repeated inoculations were required to effect infections in the C57BL/6 mice (personal observations) whereas *Mastomys* were often infected on the first attempt. Secondly, 23 of 38 (61%) inoculated C57BL/6 litters could

not be infected in comparison with 5 of 10 (50%) *Mastomys* litters. (Appendix 5 & Table 4.4). Thirdly, repeated attempts to inoculate different litters of C57BL/6 mice with the same isolate of *Giardia* failed to effect infections in 12 instances. Finally, three separate repeated inoculations of the 2 mouse strains consistently produced infections in *Mastomys* but not the C57BL/6 mice.

Different batches of cysts varied in their ability to infect mice:

- In some instances, both *Mastomys* and C57BL/6 mice were found to be equally susceptible. Four of the six axenised *Giardia* strains (described in Chapter 5 and arbitrarily designated SA 18, 24, 29 and 305) established infections in both mouse strains. Attempts were not made to infect both strains with the other two isolates SA6 and SA7. On the other hand, repeated inoculations of both mouse strains failed to establish infections with 9 batches of cysts (from symptomatic patients).

## 4.5 DISCUSSION

In the current work, two mice strains have been successfully infected with *Giardia* cysts of human origin. These results strongly suggest that the parasite displays host-preference rather than strict host-specificity and this confirms the findings of earlier workers (Filice, 1952; Hill *et al.*, 1983; Mayrhofer *et al.*, 1992, Mayrhofer & Andrews, 1994) who reported cross-species infections with *Giardia* organisms. Furthermore, in the current study, mice were infected with *Giardia* of human origin; this may have some implications in terms of mice acting as reservoir hosts. However, in the current study the infections were of short term duration in neonatal mice therefore further research is still required to clarify the role played by adult mice in transmission of *Giardia* to humans and vice versa. As *Mastomys* is an indigenous species there could be serious health implications in the South African context if these were indeed acting as reservoir animals.

Animal infectivity was compared with excystation *in vitro* with the same sets of cysts. Of the 53 excystation trials by both animal inoculation and *in vitro* methods, varying results were obtained. Although some could be excysted by both methods, a proportion of the isolated cysts could not excyst *in vitro* but produced infections in mice while some failed to produce animal infections whilst they excysted *in vitro*. The fact that some cysts could excyst in one model but not the other suggests that the two methods provide different environments for factors that initiate the excystation process. Alternately, it is possible that some stimulatory factors within the cysts are favoured by one of the two excystment conditions but not the other. These excystment methods therefore provide a marker that implies strain differences in these morphologically identical *Giardia* of human origin.

A proportionately higher percentage of apparently viable cysts could not be excysted by either of the two methods. Possibly, the factors responsible for excystment were suboptimal or depleted, or the cysts were non-viable. These results indicate wide variations in behaviour of *Giardia* in different conditions. It is however noteworthy that there may be important inconsistencies in both the mouse inoculation method and *in vitro* excystment, therefore the results should be interpreted with caution. For example, it is likely that numerous host factors determine its susceptibility to successful infection by a parasite. Likewise, parasite factors also play a role in determining its ability to establish an infection in the host.

Judging from the inconsistent pattern of excystation in the two models that were set up in the present study, it is evident that successful *in vitro* excystation does not necessarily imply animal infectivity and vice versa. However, using *G. muris* cysts for comparison of animal infectivity and excystation, Hoff *et al.* (1985) indicated that *in vitro* excystation is an adequate indicator of *G. muris* cyst infectivity. This report does not correlate with our experience. This disparity of results may be explained by reports that *G. duodenalis* cysts require greater stimulation to excyst *in vitro* than those of *G. muris* (Schaefer, 1990). Therefore, for *G. lamblia*, as demonstrated in the present work, it would not be appropriate to infer negative animal infectivity from a negative *in vitro* excystation result.

Andrews *et al.* (1989) provided electrophoretic evidence that the *Giardia* of human origin is a species complex. Also, it has been shown that infections can be composed of mixed genotypes and that isolation and purification techniques can be selective (Mayrhofer *et al.*, 1992). Our results may be an indirect reflection of

these findings, i.e. different genotypes responding differently to the different excystment conditions. Mayrhofer *et al.* (1992) suggested that some strains of *G. intestinalis* do not grow in suckling mice. Differences in the ability of cysts obtained from infected humans to infect neonatal mice were also reported by Nash & Aggarwal (1988). Similarly, Visvesvara and colleagues (1988), using gerbils as hosts, concluded that only certain strains of *Giardia* from humans could infect gerbils in their study. In the present work, twelve repeated inoculations of *Giardia* cysts into litters of the two mice strains failed to effect infections. This observation suggests that cysts vary in their intrinsic ability to cause infection in mice.

Between-mouse strain variations in infectivity patterns were also observed in the current study. For example, occasionally, when the same *Giardia* organisms that failed to produce infections in the C57BL/6 mice were injected to the *Mastomys*, infection was established. The *Praomys (Mastomys) caucha* (known to have a defective immune system) appeared to be more readily infectable when compared with the C57BL/6 mouse strain (observations). Because the C57BL/6 mice are inbred and were maintained as such in our facility (therefore genetically identical), a consistent pattern of infection would be expected from this strain. However, some litters completely resisted infection whilst others became infected. Further unexpected were within-litter variations in which some mice within a litter became infected while others resisted infection. During inoculations, meticulous effort was taken to maintain consistency in technique, therefore it would be unlikely that this variation resulted from technical discrepancy. Possibly, factors such as the gastric pH of the individual mice at the time of inoculation may have been responsible for the irregular infection patterns.



These findings strongly suggest that host factors also play an important role in determining successful establishment of infection. Furthermore, evidence suggesting that host dynamic factors play a role in establishment of and type of infection is documented. For example, Tsuchiya (1931) showed that the diet of the host affects the size of *Giardia* cysts excreted while a study by Leitch *et al.* (1989) illustrated that dietary fibre reduces the rate of intestinal infection by *G.lambli*a in gerbils.

Use of surrogate animals has proved to be superior to use of *in vitro* excystation techniques (Chapter 3) in the current study. This excystation technique offers added value for the aims of the study, as a larger number of trophozoites is available for *in vitro* culture, which in turn increases the likelihood of axenisation. Furthermore, the contaminating bacterial /fungal flora encountered with the *in vitro* excystation methods are minimised or eliminated in the animal excystation method.

Schaefer (1990) reviewed all available excystation techniques and concluded that: (1) great variability is to be expected with the currently developed procedures for *Giardia duodenalis* (2) The optimal conditions for repeatable, maximal excystation of *G. duodenalis* cysts are not yet known (3) *G.duodenalis* cysts do not excyst routinely at high levels. Furthermore, it is also documented that none of the methods of excystation work all of the time for all strains of the parasite (Nash, 1988). Our excystation results agree with these conclusions as the present study illustrated that some *Giardia* isolates can readily infect animals but fail to excyst *in vitro* and *vice versa*. *Giardia* seems to display inherent variation in it's ability to

establish infection; interestingly this was demonstrated in human experimental infections by Nash *et al.* (1987). It is not established whether this variation occurs during the natural course of *Giardia* infections.

## CHAPTER 5

### IN VITRO CULTURE OF *GIARDIA* TROPHOZOITES

#### 5.1 INTRODUCTION

Of all the common intestinal protozoa, organisms of the genus *Giardia* have reportedly been among the most difficult to establish in culture (Meyer, 1979). To date, of the three recognised morphologic types of *Giardia* namely *G. agilis*, *G. muris* and *G. duodenalis* (Filice, 1952), only the latter has been successfully cultured *in vitro*. However, *G. duodenalis* strains of canine origin were documented to be refractory to culture (Meloni and Thompson, 1987).

Reports on *in vitro* cultivation of these organisms appeared in the early twentieth century when Chatterjee (1927) first reported maintaining *Giardia* trophozoites alive *in vitro* for up to 5 weeks. Karapetyan then reported in 1960 that he had cultured *Giardia intestinalis* xenically with chick fibroblasts and *Candida guilliermondi* for seven months. The *Giardia* trophozoites continued to grow even after the chick fibroblasts had died. In 1962, Karapetyan successfully cultured *G. duodenalis* isolated from a rabbit monoxenically with *Saccharomyces cerevisiae* without fibroblasts. However, his attempts to axenise *G. duodenalis* were unsuccessful. His complex culture medium included serum, chick embryo extract, chick amniotic fluid or a tryptic digest of meat with Hank's or Earle's solutions. The medium had to be replaced daily.

In 1970, Meyer first axenised *G. duodenalis* from the rabbit, chinchilla and cat. His method entailed use of a U tube, wherein the *Giardia* trophozoites were inoculated

together with yeasts on the right arm of the tube. The motile trophozoites migrated across its base and were isolated, free of yeast, on the left arm of the tube.

In 1976, Meyer developed a culture medium (Hanks, Serum, Phytone-1) (HSP-1) for axenic cultivation of human isolates. One of the major components of Meyer's medium was human serum (15-20%). Visvesvara (1980) highlighted the limitations of using this product as batches of human serum varied in their ability to support good growth of *G.lamblia*; some even inhibited growth and caused cultures to be lost. He also recognised that *G.lamblia* grown in media containing human serum (HSP-1) were unsuitable for use as antigens in serological tests because of non-specific staining reactions. To circumvent this, he gradually adapted the human *Giardia* to grow in Diamond's Trypticase-Panmede-Serum (TPS-1) medium, which is supplemented with bovine or rabbit sera.

A new medium, Trypticase-Yeast-Iron-Serum-33 (TYI-S-33) was developed for axenic culture of *Entamoeba spp* by Diamond *et al.* (1978) to replace the TPS-1 medium, which was at that time the most widely used for these organisms. One of the essential ingredients of TPS-1 medium was Panmede (Paines and Byrne, Ltd., Greenford, Middlesex, England), a papain digest of ox liver. In time it became increasingly difficult to obtain batches which supported good growth, hence TYI-S-33, which is based on Trypticase, a casein digest (BBL, Cockeysville, USA) and yeast, was developed.

Keister (1983) modified TYI-S-33 for cultivation of *Giardia* organisms by adding bile and increasing the concentration of L-cysteine to the original formulation of

Diamond *et al.*'s (1978) medium. He successfully cultured four strains, two of which had been grown and maintained in TPS-1. This was a significant contribution to *G.lamblia* cultivation methodologies as this modified version of TYI-S-33 is currently the most widely used medium for axenic cultivation of *Giardia*. This strategy obviates the need to resort to monoxenic culture first, thus eliminating all problems related with associated organisms.

Although no reports on a clearly defined medium for *Giardia* are presently available, scientists have identified some of the essential ingredients of TYI-S-33 (the medium which supports luxuriant growth of this parasite). Gillin and Diamond (1981a) evaluated the responses of growth of *Entamoeba histolytica* and *G.lamblia* to reducing agents. They found that the requirement for L-cysteine was very specific for growth of *Giardia*, and could not be substituted by other reducing agents. They also discovered that attachment in complex growth media, growth and survival in culture required cysteine (Gillin and Diamond, 1981a, b). In 1994, Lujan and Nash demonstrated that L-cysteine is essential for growth of *Giardia* trophozoites *in vitro* (as it protects the trophozoites against the lethal effects of Oxygen) and that its uptake depends on the presence of serum: thus serum is one of the key components for successful culture. Lujan *et al.* (1994) also showed that bovine serum Cohn fraction Cf-IV-1 enhances the uptake of L-cysteine by trophozoites. In addition to its ability to enhance cysteine uptake, serum also provides growth factors and nutrients. Lipids were reported by Adam (1991) to be necessary for growth.

Keister (1983) showed that the vitamin-Tween mixture added to TYI-S-33 by

Diamond *et al.* (1978) does not enhance the growth of *G.lamblia* in bile-supplemented medium. Bile, therefore, among other factors apparently obviates the need for vitamin supplementation. *Giardia* are unable to synthesise their own membrane phospholipids, thus the mechanism by which bile promotes parasite growth is related to the requirement of pre-formed phospholipid which is abundant in bile and whose uptake is facilitated by presence of conjugated bile salts (Farthing, 1989).

Certain factors can affect *in vitro* *Giardia* cultures.

- Although many antibiotics such as the penicillins, gentamycin, streptomycin, clindamycin and amphotericin B do not affect the growth of *Giardia* trophozoites *in vitro* (Jakubowski *et al.*, 1988), some however can impede its propagation and growth, e.g. Amikacin, an aminoglycoside, can be toxic to the trophozoites at high concentrations.
- Bacterial and viral endosymbionts have been found in *Giardia* of human and animal origin (Feely *et al.*, 1990). The presence of bacteria in trophozoite cultures was reported to cause the rate of *Giardia* multiplication to decrease markedly (Meyer, 1976) while viral endosymbionts in *Giardia* trophozoites are associated with decreased adherence and growth rate (Adam, 1991).
- Low cysteine concentration, high oxygen tension and low temperature cause detachment of trophozoites from the culture vessel.

Although it is difficult to culture *Giardia lamblia*, many workers from various parts of the world have successfully axenised these organisms: Portland (Meyer, 1976); Atlanta (Visvesvara, 1980); Maryland (Gillin & Diamond, 1981a,b; Keister, 1983); Australia (Phillips *et al.*, 1984); Belgium (Gordts *et al.*, 1985); Poland (Kasprzak &

Majewska, 1985) United States (Nash *et al.*, 1985); Western Australia (Meloni & Thompson, 1987; Meloni *et al.*, 1988); Mexico (Cedillo-Rivera *et al.*, 1989); Saudi Arabia (Al Tukhi *et al.*, 1991); Israel (Korman *et al.*, 1992). No reports of similar work have been documented from South Africa. This chapter describes procedures to initiate, expand and maintain viable cultures of *Giardia* trophozoites derived from excystation of locally isolated cysts. Since the trophozoites used for initiation of the cultures were derived from *in vitro* (Chapter 3) and *in vivo* (Chapter 4) excystation experiments, reference to these chapters will be made constantly in the current chapter.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Culture Medium**

TYI-S-33 was utilised for initiation and maintenance of *Giardia* cultures.

Essentially it was prepared as described by Diamond *et al.* (1978) and modified by Keister in 1983. The main ingredients are Biosate (a pancreatic digest of casein and yeast extract), bile, serum, salts, glucose and iron. Details of its preparation appear in Appendix 7.

### **5.2.2 Optimisation of Culture System**

Since biological products vary in their ability to support growth of cells *in vitro* (Diamond *et al.*, 1978), several trials using different brands of reagents were necessary to identify suitable lots of the different products.

Two strains of *Giardia*, (WB, isolated from a patient infected in Afghanistan; and the H7 clone of the GS isolate obtained from a scientist from the National Institutes of Health (NIH) who had camped in Alaska) were obtained from Dr Nash at the NIH, USA. These have been well established as axenic cultures and hence were used as control reference strains to assess good growth-supporting reagents.

#### **5.2.1.1 Sera**

Several batches of bovine sera (lots: CN 1872; CN1399 and CN2290) from Highveld Biological, Gauteng, (SA); fetal bovine serum (lots: 35-603-240 -1419; 35-603-240-1410 & 34-511-248-75) from Delta Bioproducts, Gauteng, (SA); fetal bovine serum from Sigma St. Louis USA, lots 25H4602, 46H4648 & 65H4664



were evaluated.

#### **5.2.1.2 Biosate**

Biosate from BBL- Becton Dickinson and Company, USA, lots 1000L 7DESR and 1000E9DGNP were evaluated.

#### **5.2.1.3 Antibiotics**

Because of expected widespread penicillin/streptomycin resistance among local bacterial strains, various antimicrobial agents (such as Gentamycin, Vancomycin, and low dose Amikacin) were used over and above the penicillin/streptomycin originally incorporated into the culture medium.

While the optimisation experiments were undertaken, an aerophilic laboratory contaminant, *Xanthomonas maltophilia* (identified by biochemical reactions in the Api 20E system (BioMerieux) was detected on some culture tubes of the WB reference strain (the trophozoites continued to grow and multiply in the presence of these microbes). A disk-diffusion susceptibility assay revealed that the organism was resistant to the antibiotics incorporated in the medium, including Amikacin but sensitive to Ciprofloxacin. Subsequently, this antibiotic was required to eliminate the bacterial contaminants without inhibiting the growth of *Giardia* trophozoites. An assay to determine the Ciprofloxacin concentration tolerated by trophozoites was therefore performed, as literature citing use of this quinolone on *Giardia* cultures was not found.

### **Assay for Susceptibility of Giardia to Ciprofloxacin**

A 100µg/ml Ciprofloxacin (Bayer) stock solution was prepared (from a 2 mg/ml intravenous fluid concentrate) in sterile TYI-S-33 medium. Various dilutions of the stock solution were prepared to give final concentrations of 5; 2,5; 1,25; 0,625 and 0,3125 µg/ml in 7ml of TYI-S-33 in the culture tubes. The two reference strains (WB and H7) were retrieved from liquid nitrogen (as described in Chapter 6) and re-cultured in TYI-S-33 medium. Following 48 hours of incubation, the cultures were ice- chilled for 15min, centrifuged at 400xg for 10min at 4°C and the supernatant medium decanted. The pelleted cells were resuspended in 1ml of cold sterile medium and counted using a Nuebauer counting chamber. Aliquots of 0,1ml containing 1000 cells/ml of each isolate were distributed to 6 Kimax glass tubes containing the TYI-S-33 culture medium. Each of the twelve cultures was incubated with each of the concentrations of Ciprofloxacin. Two tubes (one of each strain) incubated without the antibiotic served as control tubes. The tubes were incubated and maintained at 35,5°C at a 5° angle. Growth rate and attachment to glass were monitored in the twelve tubes for 20 and 48 hours by modifying the adhesion assay (Meyer, 1976; Farbey *et al.* 1995) as follows: After 20 and 48 hours incubation, all tubes were observed for adherent cells under a 40x microscope field in the entire inner surface of the culture tubes. A number (growth rank) was assigned to each culture tube (including control tubes). This number represented the average number of adherent trophozoites present per 40x-microscope field in the lower inner surface of a culture tube using the guide outlined in Table 5.1.

**Table 5.1** Outlines a relative semi-quantitative assessment of growth (ranking) of *Giardia* trophozoites in culture which could be used to monitor growth rate over time by counting adherent trophozoites on the lower inner surface of a culture tube under a 40x microscope field. (Adapted from Meyer, 1976).

| Growth Rank | Equivalent average number of trophozoites /40x field |
|-------------|--|
| 1           | 1-5  |
| 2           | 5-50   |
| 3           | 50-250   |
| 4           | 250-500  |

The assay was repeated with higher concentrations of the drug ranging from 10-50 µg /ml of Ciprofloxacin.

Data obtained from the Bayer electronic publication (1997) indicated a minimum inhibitory concentration (MIC) of 4µg /ml for *X. maltophilia*. This concentration of Ciprofloxacin was subsequently incorporated into the contaminated cultures until no bacterial growth was detected on subcultures.

## 5.2.2 Propagation of Trophozoites *In Vitro*.

### 5.2.2.1 Culture of *in vitro* excysted trophozoites

Cultures were initiated with 67 trophozoite batches derived from *in vitro* excystation (Chapter 3). These were aseptically transferred to sterile glass tubes containing TYI-S-33 medium with antibiotics. The tubes were incubated at 35,5°C at a 5° angle to facilitate adherence of excysted trophozoites. After 48-72 hours, old medium was decanted (without dislodging the trophozoites from the tube walls) and replaced with fresh TYI-S-33. Culture tubes were maintained at 35,5°C

for up to seven days even when no trophozoites were detected by microscopy. To monitor bacterial and fungal contamination, subcultures of medium, aseptically aspirated from the tubes, were inoculated onto aerobic and anaerobic blood plates; the latter were incubated in a Gas-Pak MD 21030 (BBL Microbiological Systems; Becton Dickinson & Co; Cockeysville, Maryland, USA) anaerobic jar. The plates were incubated at 37°C for 24 (aerobic) and 48hrs (anaerobic).

#### **5.2.2.2 Establishment of cultures from suckling mice**

Twenty batches of trophozoites (a batch consisted of 4-9 tubes) obtained from mice intestines as described in Section 4.2.3 were incubated in TYI-S-33 medium with antibiotics at 35,5°C at a 5° angle. Cultures were examined daily using an inverted microscope at 100x and 400x magnification. At 48 or 72hour intervals, old culture medium containing non-adherent and dead trophozoites was carefully decanted (without dislodging the attached trophozoites) and 7ml of fresh medium was added. Aseptic conditions were maintained at all times. When cultures formed a confluent adherent monolayer, antibiotics were gradually withdrawn from the medium. Cultures were deemed axenic when they continued to multiply in antibiotic-free medium in the absence of other organisms. Under aseptic conditions, subcultures of TYI-S-33 medium from the culture tubes were streaked onto aerobic and anaerobic blood agar plates to assess for the presence of bacterial /fungal contaminants.

#### **5.2.2.3 Routine Maintenance of cultures**

For routine maintenance and expansion of the cultures, trophozoites that grew well and formed a dense monolayer on the surface of the tube walls were

dislodged by chilling the culture tubes in an ice bath for 20 minutes. Thereafter the tube contents were mixed by gentle inversion and centrifuged at 400xg for 5min at 4°C. Old supernatant medium was gently decanted and pelleted trophozoites resuspended in 1ml of fresh TYI-S-33. A 1:10 dilution of trophozoites was prepared in 1% formalin in a clean tube to fix and enumerate the trophozoites. Aliquots of 0,1ml of the remaining trophozoite suspensions containing  $1 \times 10^4$  cells /ml were distributed to two or more tubes containing 7ml TYI-S-33 medium. The expanded cultures were maintained as above until confluent monolayers were obtained for cryopreservation (Chapter 6) and preparation of lysates for isoenzyme electrophoresis (Chapter 7).

## **5.3 RESULTS**

### **5.3.1 Optimisation Experiments**

Using the two reference strains, good viable growth was feasible with fetal bovine serum from Sigma St. Louis USA, lots 25H4602, 46H4648 & 65H4664; and Biosate from BBL- Becton Dickinson and Company, USA, lot 1000E9DGNP. None of the sera from Highveld Biological and Delta Bioproducts respectively, could support good growth and caused loss of cultures.

The Ciprofloxacin assays showed that concentrations between 0,3125-50µg/ml were well tolerated by the trophozoites. The rate of growth and adhesion of trophozoites to glass was comparable to that of the control tubes (antibiotic-free) 20 and 48 hrs after incubation at all these antibiotic concentrations (Table 5.2).

Culture tubes containing the WB strain showed evidence of cell division after 8 hours incubation. By day 3, a confluent monolayer was formed in all tubes.

The H7 strains showed signs of division after 12 hours incubation in the bottom-most part of the tube. A confluent adherent monolayer was detected 5 days after incubation. A similar pattern of results was observed when the experiment was repeated with the high concentrations (5-50µg/ml) of Ciprofloxacin.

**Table 5.2.** Sensitivity to Ciprofloxacin of *Giardia* reference strains WB and H7: Summary of growth rank of the culture tubes containing different Ciprofloxacin concentrations after 20 and 48 hours incubation.

| Ciprofloxacin Concentration (ug/ml) | Growth Rank (at 40x microscope field) |    |       |    |
|-------------------------------------|---------------------------------------|----|-------|----|
|                                     | 20HRS                                 |    | 48HRS |    |
|                                     | WB                                    | H7 | WB    | H7 |
| 5                                   | 2                                     | 1  | 3     | 2  |
| 2,5                                 | 2                                     | 1  | 3     | 2  |
| 1,25                                | 2                                     | 1  | 4     | 2  |
| 0,625                               | 2                                     | 1  | 3     | 2  |
| 0,3125                              | 2                                     | 1  | 3     | 2  |
| 0 (control)                         | 2                                     | 1  | 3     | 2  |

### 5.3.2 Cultivation of *In Vitro* Excysted Trophozoites.

Table 5.3 summarises the results of all culture initiates derived from *in vitro* excystations.

The behaviour of *in vitro* excysted trophozoites was not consistent in culture.

Some survived for up to six hours, rounded up and died. Others survived for 12, 24 or 72 hours or as long as 9 days in culture but gradually died. The majority of these never adhered to the culture tube surface but swam around vigorously and later became sluggish. A few attached to the glass but remained as single cells at

the bottom of the culture tubes and never showed signs of division. Most of these gradually began to die until, by day 7, no living trophozoites were seen in the culture tubes. In 36% of them (Table 5.3), no evidence of contaminants (aerobic and anaerobic) could be demonstrated.

Sixty-four percent of the cultures were overwhelmed by bacterial contaminants and died, despite the incorporation of antibiotics in the culture media (Table 5.3). Some contaminating organisms were identified. Most frequently gas-forming anaerobes (foul smelling gas evident in the TYI-S-33 culture tubes and growth only occurred on the anaerobic blood plate) contaminated the cultures. Occasionally, yeasts and *Pseudomonas spp* were identified as the contaminating organisms. In some cases *Klebsiella* and *Aerobacter spp* were isolated.

No viable cultures were established using the trophozoites obtained from *in vitro* excystation.



**Table 5.3.** Summary results of *in vitro* culture initiates for trophozoites obtained from excystation using different methods.

| Excystation Method | Successful Excystation | Culture Results        |                    |
|--------------------|------------------------|------------------------|--------------------|
|                    |                        | Contamination detected | No contamination   |
| Hamilton & Jackson | 24/34                  | 16/24                  | 8/24               |
| Acid Pepsin        | 43/103                 | 27/43                  | 16/43              |
| <b>TOTAL</b>       | <b>67/137 (49%)</b>    | <b>43/67 (64%)</b>     | <b>24/67 (36%)</b> |

### 5.3.3 *In vitro* culture of *in vivo*-derived trophozoites

Fifty-three mice litters were inoculated with cysts for *in vivo* excystment and subsequent trophozoite cultivation *in vitro*. Trophozoites were harvested from 20 of those and inoculated into TYI-S-33 medium (the results are summarised in Table 5.4). Of the 20 attempted isolations, 14 failed to establish viable cultures. Eight of the 14 isolates died even though they were not contaminated; they survived from 12 hours to seven days in culture, but eventually rounded up and died. Six were overwhelmed by contaminants. Six isolates were successfully adapted to culture and subsequently, the first axenic South African strains were established *in vitro*. They have been maintained in culture for over 15 months. Stabilates and lysates of the axenised strains were cryopreserved for future use. They have been arbitrarily assigned numbers corresponding with the sample number, preceded by the prefix SA (for South Africa) viz SA6, SA7, SA18,

SA24, SA29 and SA305.

**Table 5.4.** Culture results using *in vivo* excysted trophozoites as inoculum. Thirty percent of the culture initiates were lost through contamination, while 40% failed to establish cultures.

| ANIMAL INOCULATION             | CULTURE RESULTS |                               |                            |
|--------------------------------|-----------------|-------------------------------|----------------------------|
| Excystation/Total inoculations | Contaminated    | No contamination but died out | *No contamination-survived |
| 20/53 (38%)                    | 6/20 (30%)      | 8/20(40%)                     | 6/20 (30%)                 |

\*6 isolates axenised (30% success).

## 5.4 Observations

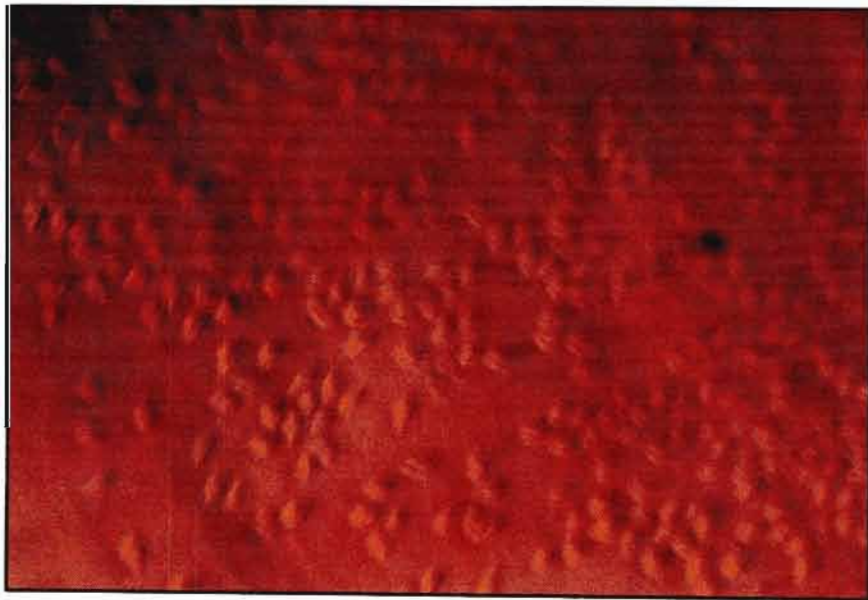
### Behaviour of trophozoites *in vitro*

For fresh isolates, during the initial period of culture, the trophozoites adhered to the bottom of the culture tube mostly as individual cells without signs of division (Plate 5.1). This inert period varied with different isolates (from 10 to 15 days) and could be as long as twenty days in some instances after which they showed signs of division (Plate 5.2). However, as the organisms became adapted to the culture environment, this period became shorter. When the trophozoites are continuously passaged they can be seen dividing by day three after subculture. A confluent monolayer of cells was seen by day 18 in some isolates (Plates 5.3 and 5.4) However one isolate took 32 days to start dividing; the concentration of fetal bovine serum was increased from 10 to 15% on day 24 and confluent growth was reached after 55 days. Interestingly, some trophozoites continually swam around, never adhering to the surface of the tube.

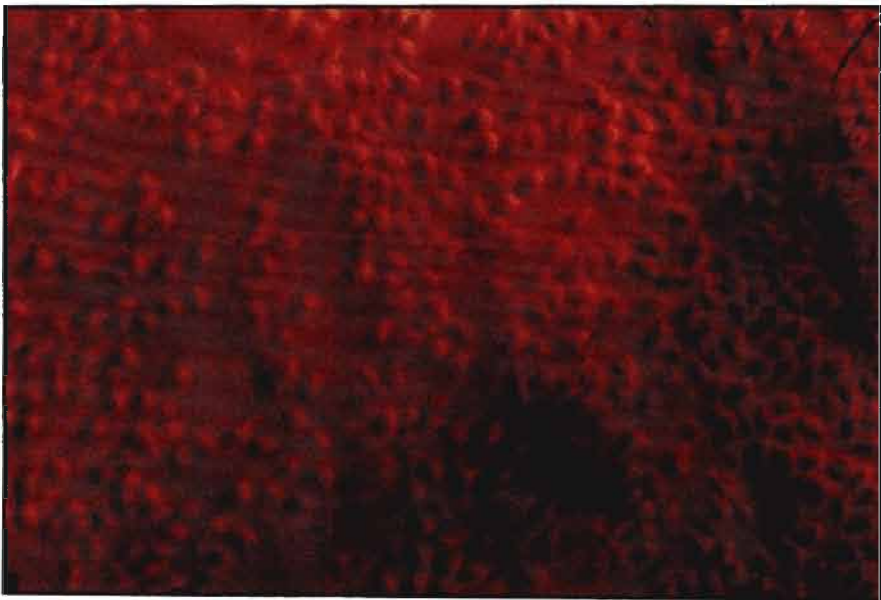
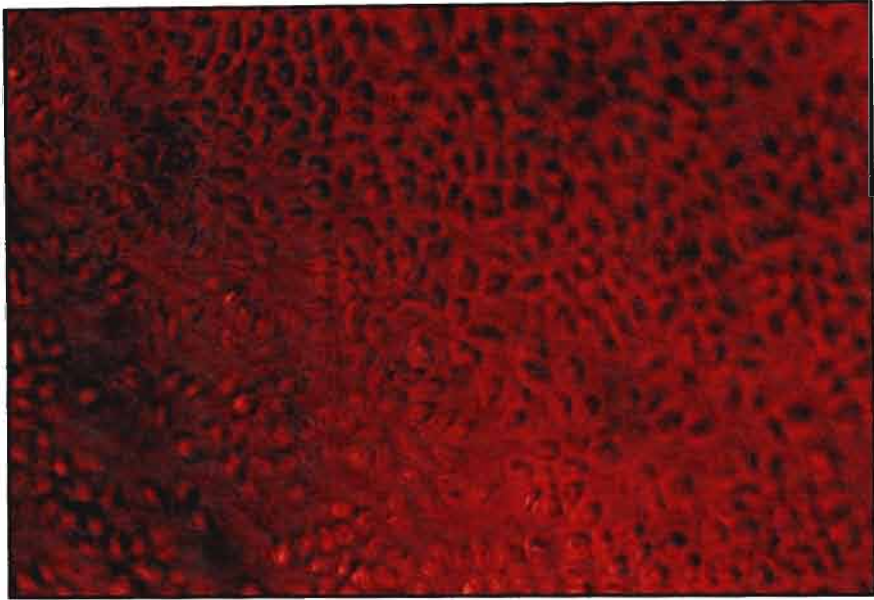
Once established in culture for a long time, the trophozoites became highly resilient. Some trophozoites survived despite long periods without medium replacement. Two tubes of the reference strain (WB) were kept in the 35,5°C incubator for 10 and 45 days respectively without changing the medium and later took to culture. The local strains have also survived up to 96 hours without medium changes. In these cases, although many were rounded up and detached from the glass surface, plenty were still adhering and they continued to grow well after replenishment with fresh medium.



**Plate 5.1** Trophozoites of *G. lamblia* that had been isolated in culture for 8 days. All exist as individual cells (400x).



**Plate 5.2.** Trophozoites after 15 days in culture. Signs of division were noted (400x).



**Plates 5.3 and 5.4** Confluent growth of *G. lamblia* trophozoites in Culture. Magnification x400.

## 5.5 DISCUSSION

In the present work, cultures were initiated from excystation of faecally derived cysts, therefore parts of this discussion consider the results in accordance with the *in vitro* and *in vivo* culture findings.

Initiation of viable long-term cultures from *in vitro* excystation proved to be impossible. Excystment and trophozoite survival were the two major factors that governed successful attempts at *in vitro* cultivation of the organisms. Although successful excystment was achieved in some cases, the individual levels of excystation were low, and if successfully excysted, trophozoites survived for several hours and subsequently died. Therefore, only short-term cultures were established. While some authors reported successful establishment of viable cultures from *in vitro* excystation of cysts (Bingham & Meyer, 1979; Isaac-Renton *et al.*, 1986; Meloni & Thompson, 1987; Meloni *et al.*, 1988; Cedillo-Rivera *et al.*, 1989; Al-Tukhi *et al.*, 1991), others, as in the present study experienced difficulty in establishing cultures from *in vitro* excysted trophozoites (Isaac-Renton *et al.*, 1986; Kaur *et al.*, 1986).

In the current study, apart from the scanty numbers of trophozoites that resulted from low levels of excystation, contaminating faecal microorganisms played a negative role in the failed attempts to establish cultures from *in vitro* excysted cysts of faecal origin. Although a variety of antibiotics were used, a large proportion of cultures was overwhelmed by faecal contaminants. This might be a reflection of the wide distribution of resistant organisms in the local population from which cysts were obtained. The combined effect of these factors made it

difficult to produce viable cultures.

Use of surrogate animals as excystation hosts proved to be superior to *in vitro* excystment. Viable cultures of *Giardia* have been established using this model. Subsequently six local strains have been axenised for the first time in South Africa. The greater numbers of trophozoites available for culture initiation as well as the reduction of contaminating faecal organisms are considered to be key factors in the efficacy of this method.

In the present study only a limited number of *in vivo* excysted trophozoites resulted in the establishment of a successful culture. Therefore the establishment of viable cultures of *Giardia* through mice inoculations can be considered to be dependant on many complex factors such as: *Giardia* isolate variations, mouse strain variations, selection pressures placed by both *in vivo* and *in vitro* conditions and the fact that *Giardia* is fastidious with no defined medium being available for these organisms. Little is known about the growth dynamics of populations of *Giardia*. Furthermore, previous studies reflect that multiple attempts are required to successfully initiate viable cultures of a single isolate. For example, Kasprzak & Majewska (1985) reported that up to 107 consecutive attempts were required to isolate strains (from faecal cysts or duodenal aspirates) *in vitro*. Owing to the limited numbers of cysts available for simultaneous *in vitro* and *in vivo* experiments, few faecal samples allowed for repeated attempts in the current work.

While working with *Giardia* it became clear that establishment of these organisms

in culture is complex, time consuming and requires patience. In the culture system, different batches of excysted trophozoites showed marked variation. Some organisms swam around in the medium for several hours or up to 7 days in some cases. These never adhered to the glass surface. Other trophozoites adhered within minutes or hours to the glass inner surface. They remained attached to the bottom-most inner surface of the tube as single cells for different periods. This was assumed to be an adaptation period that was then followed by cell division and multiplication. Some isolates took 3-4 weeks to produce an adherent, confluent monolayer of cells while one isolate was successfully harvested after approximately two months. Some were easily axenised while others grow axenically with difficulty or not at all. Differences in growth rates between the different strains including the two reference isolates were also observed. This phenomenon strongly suggests there are differences in growth characteristics among the different strains.

The observation that only those trophozoites that adhered and adapted to the culture conditions produced established cultures indicate that selection occurred. The non-adherent trophozoites eventually died or were lost when old medium was decanted and replaced with fresh medium. Upcroft *et al.* (1994) noted that not all *in vitro* cultivated samples will generate viable laboratory cultures and that it has been shown through DNA fingerprinting that what finally establishes as a viable laboratory culture is effectively a clone, but it is not known at what stage this occurs. Mayrhofer *et al.* (1994) and Andrews *et al.* (1992) suggested that adherence is a potential point at which selection can occur. Furthermore, it is equally possible that differences in adherence capability can lead to the



dominance of certain genotypes under *in vitro* conditions.

The differences in behavioural characteristics of *Giardia* isolates that have been highlighted above suggest that not all members of this group have identical growth requirements. Because of this, it is likely that only isolates that are able to adapt to the conditions of the methods of *in vitro* propagation can be cultured. If this is the case, it is possible that several different media will eventually be required to propagate the range of species (and strains) within the genus. It is clear that presently, culture methods inadvertently select for certain genotypes.

## CHAPTER 6

### CRYOPRESERVATION OF TROPHOZOITES

#### 6.1 INTRODUCTION

Diamond *et al.* (1961) reported the first successful attempt at preservation of protozoa in liquid nitrogen. The first attempt to culture *Giardia* from cryopreserved material was made by Meyer & Chadd (1967). Subsequently, *Giardia* cultures have been cryo-preserved and retrieved with success by other workers (Warhurst & Wright, 1979; Lyman & Marchin, 1984; Phillips *et al.*, 1984; Diamond, 1995).

In his report on cryopreservation and storage of parasitic protozoa, Diamond (1995) advises that the best time to harvest many cell types, including protozoa, is during the transition phase between logarithmic and stationary growth as rapidly multiplying cells are more vulnerable. Other parameters such as the cryoprotective agent are also equally important in successfully cryopreserving cells. Various cryoprotective agents have been used to preserve cooled *Giardia* trophozoites. They include glycerol, dimethylsulphoxide (DMSO) (Meyer & Chadd, 1967; Lyman & Marchin, 1984; Phillips *et al.*, 1984), either glycerol or DMSO supplemented with glucose (Diamond, 1995) and DMSO supplemented with salts (Warhurst & Wright 1979). Lyman & Marchin, (1984) used a protocol which includes an ethanol bath. They recommended an ethanol cooling rate of minus 1,19°C/min for cryopreserving *G. lamblia* trophozoites.

Cryopreservation and successful retrieval of viable cultures has facilitated the establishment of cryobanks of axenic cultures. This helps to avoid the selection and variation of strains created by serial subcultures and minimises the cost of maintaining cultures indefinitely.

One of the aims of the present study was long term storage at cryogenic temperatures and establishment of a cryobank of locally isolated strains.

This section describes procedures undertaken to cryopreserve, store and retrieve into culture trophozoites that had been axenised.

## **6.2 MATERIALS AND METHODS**

### **6.2.1 Cooling of Cultured Trophozoites**

Two techniques of cooling were assessed for their efficacy in achieving long term preservation of trophozoites. Subsequent success in retrieval of viable cultures was also evaluated. In both, the same cryopreservant was used. A 9% (v/v) solution of Dimethyl sulphoxide (DMSO) (Saarchem, Durban, and SA.) was made in sterile TYI-S-33.

#### **6.2.1.1 Preparation of samples**

Tubes of cultures at early stationary phase were chilled on ice for 20 minutes to detach the trophozoites from the glass. After mixing by gentle inversion they were centrifuged at 400xg for 5 minutes at 4°C and supernatant medium was discarded. Pelletted trophozoites were resuspended in 1ml of fresh TYI-S-33 medium. The organisms in a 1ml aliquot were enumerated in a haemocytometer. One ml of suspensions containing  $1 \times 10^6$  trophozoites /ml were then transferred with a sterile Pasteur pipette to a Nunc cryotube (Nalge Nunc International, USA). Immediately before cooling, 1ml of 18% DMSO was gradually introduced (by dropwise addition) to the cryogenic vial to give a final concentration of 9% DMSO.

### **6.2.2 Cooling Techniques**

#### **6.2.2.1 Electronically controlled cryofreezing apparatus (modification of Phillips *et al.*, 1984 method)**

Vials containing cryopreservant and cells were promptly (within 5 minutes) transferred to a Liquid Nitrogen, programmable controlled freezer, Planer model Xb634 (Planer Products, Sanbury-On-Thames, England). The cooling rate was

adjusted to 5°C per minute from ambient temperature to 0°C; 1°C per minute from 0 to -25°C and then 5°C per minute from -25 to -135°C. At completion of the cooling cycle, the cryotubes were transferred to a liquid nitrogen (LN) storage Dewar flask (Taylor-Wharton, British Oxygen, UK) until required for retrieval.

***Modification: In the present study, cryopreservant was added at room temperature (22-25°C) whilst in the original method this was done at -4°C.***

#### **6.2.2.2 Mechanically insulated freezing container**

The procedure was carried out according to the manufacturer's instructions as follows. Isopropanol (250ml) (Merck, Darmstadt, Germany) was added into the Nalgene Cryo 1°C Freezing Polycarbonate Container (Nalge Nunc International, U.S.A, Cat.No.5100-0001). An insulating foam insert was immersed into the alcohol. Samples prepared as described in section 6. 2.1.1 were placed into the vial holder, the unit was promptly placed into a -70°C mechanical freezer and left undisturbed for at least 4 hours. This system allows a gradual rate of cooling at 1°C/min from room temperature to -70°C. The frozen vials were transferred for long-term storage into a liquid nitrogen Dewar flask until retrieval was necessary.

#### **6.2.3 Retrieval Of Frozen Cultures.**

Cryopreserved cultures were retrieved from liquid nitrogen and thawed quickly at 37°C for 2 minutes without agitating the tubes. Using a sterile Pasteur pipette, the contents were gently mixed and transferred to a sterile 8ml glass Kimax tube containing 7ml of TYI-S- 33 medium. The cultures were examined on an inverted microscope and the percentage of motile trophozoites (indicating viability) was

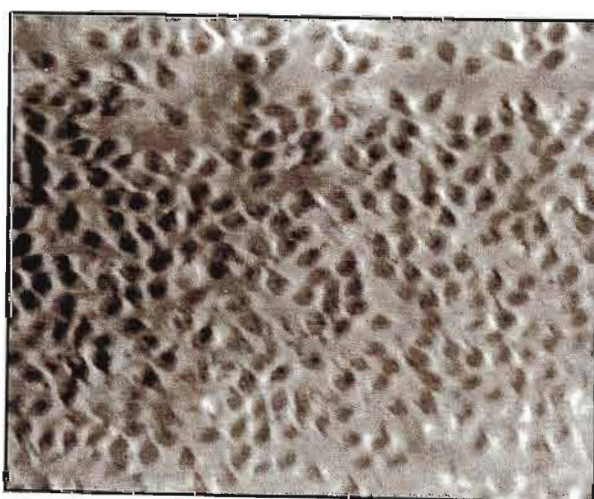
estimated. The tubes were then incubated at a 5° angle for 30 minutes at 35,5°C. Medium with cryopreservant and dead cells was gently poured off and replaced with fresh TYI-S-33.

The cultures were incubated and maintained as in 5.2.2.3 until adequate confluent growth was obtained.

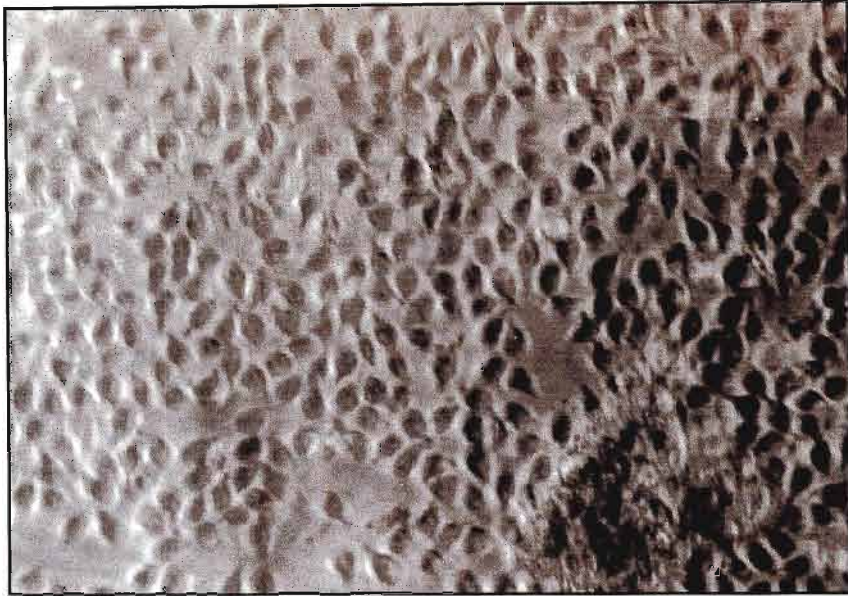
### 6.3 RESULTS

Trophozoites have been successfully preserved for periods varying from 23 days to approximately 2 years. Qualitative assessment upon retrieval revealed 70-80% viability of trophozoites. Within 30 minutes, more than 50% of the trophozoites were adhering to the inner surface of the glass culture tubes (Plate 6.1). Confluent cells were seen within 48h of culture (Plates 6.2 & 6.3). All retrieved samples have been re-established in culture: a 100% success rate has been achieved using both electronically controlled (6 times) and mechanical (15 times) cooling techniques. Table 6.1 presents the records of duration of preservation of the 6 local isolates and the two reference strains.

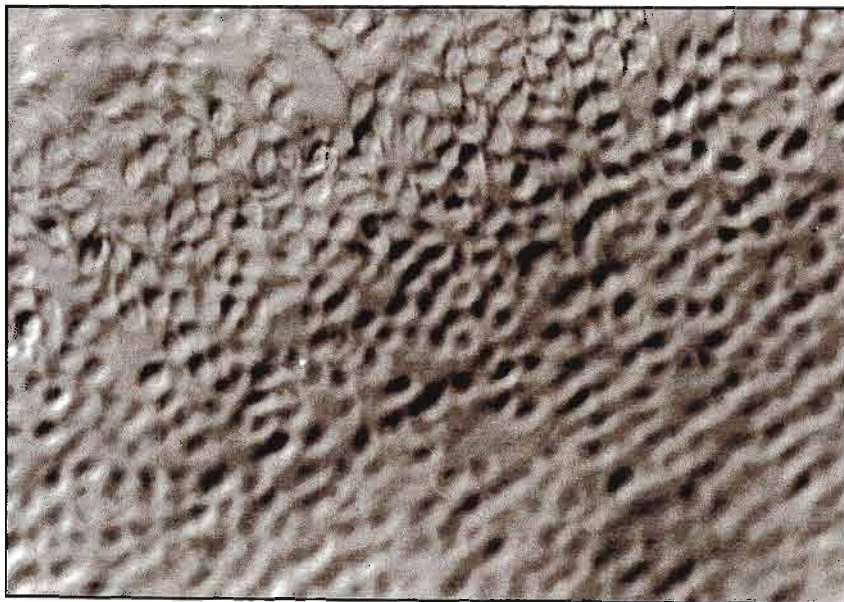
Viable cultures of the retrieved trophozoites have been maintained in culture for several months and cryopreserved again with success. A reserve supply (cryobank) has been established for the 6-axenised local strains.



**Plate 6.1** Trophozoites of *Giardia lamblia* that had been cryopreserved for 2 months 17 days. The organisms were retrieved from cryopreservation and incubated at 35,5°C for 30mins in TYI-S33 medium and residual DMSO. More than 50% of the organisms are adhering to the tube inner surface. (400x)



**Plate 6.2** Confluent growth of trophozoites that were cryopreserved for 2 months 17 days, retrieved from cryopreservation and incubated at 35,5°C for 48 hours in TYI-S33 medium. (400x magnification).



**Plate 6.3** A confluent culture of *G. lamblia* trophozoites 5 days after retrieval from cryopreservation in liquid nitrogen. (400x)



**Table 6.1.** A longevity record of samples of two reference isolates (WB & H7) and 6 axenic South African isolates cryopreserved in liquid nitrogen and retrieved into culture.

| Isolate | Date Preserved | Date Retrieved | Storage Period |
|---------|----------------|----------------|----------------|
| WB      | 03-01-97       | 17-3-97        | 2mnths 14d     |
| WB      | 03-01-97       | 18-2-97        | 1mnth 17d*     |
| WB      | 03-01-97       | 27-01-97       | 24d*           |
| H7      | 03-01-97       | 08-04-97       | 2mnths 5d*     |
| H7      | 03-01-97       | 27-01-97       | 24d*           |
| SA6     | 03-01-97       | 05-11-97       | 10mnths 2d     |
| SA6     | 03-01-97       | 19-08-97       | 7mnths 18d     |
| WB      | 16-04-97       | 05-11-97       | 6mnths 19d     |
| H7      | 30-04-97       | 23-05-97       | 23d            |
| H7      | 11-05-97       | 17-02-98       | 9mnths 6d      |
| WB      | 11-05-97       | 17-02-98       | 9mnths 6d      |
| SA18    | 05-09-97       | 06-02-98       | 5mths 1d       |
| SA29    | 05-09-97       | 17-02-98       | 5mnths 12d     |
| WB      | 14-04-97       | 05-11-97       | 6mnth 6d       |
| H7      | 03-01-97       | 23-05-97       | 4mths 20d *    |
| SA24    | 26-02-98       | 11-06-98       | 3mnths 15d     |
| SA29    | 25-09-97       | 22-09-98       | 11mnths 26d    |
| SA305   | 24-04-98       | 25-09-98       | 5mnths 1d      |
| SA7     | 03-01-97       | 28-08-98       | 1y 7mnths*     |
| WB      | 25-08-98       | 26-10-98       | 1mnth 1d       |
| SA6     | 25-08-98       | 26-10-98       | 1mnth 1d       |

Y = year  
 mnth/s = month/s  
 d = days  
 \* = electronically controlled cooling method

## 6.4 DISCUSSION

In the current work, effective cryopreservation (for approximately two years) has been achieved with the two different cooling techniques. These results concur with Diamond's report that "once a specimen is cryopreserved in liquid nitrogen or its' vapours it can be expected to remain viable for many years regardless of the technique employed to achieve preservation" (Diamond, 1995).

Although the two methods of cooling were found equally effective, the electronic cooling device is more expensive, requires the use of liquid nitrogen and is more time-consuming, whilst the Nunc cryopreservation system is simple in addition to being cost-and-time effective. The Nunc cryopreservation system therefore became the method of choice.

In our experience, cryopreserved *Giardia* cultures are more resilient than *Entamoeba*, which are documented to have increased fragility after cryopreservation. *Giardia* trophozoites remained viable even after exposure to cryopreservant (9% DMSO) for 30 minutes. Diamond (1995) stated that frozen *E.histolytica* lyse after recovery when kept longer than 10 minutes in the cryopreservant. Furthermore, Phillips *et al.*, (1984) stated that a washing step to remove cryopreservant, after thawing retrieved *Giardia* trophozoites, is essential for successful re-establishment of cultures. In the present work, trophozoites that were thawed and incubated in the presence of DMSO for 30 minutes could still be re-established as viable cultures.

Diamond (1995) stated that an important parameter in cryopreservation is the temperature at which the cryopreservant is added to the cells as osmotic equilibration takes place before freezing is initiated. He studied the effects of two temperatures (0°C and 24°C) on a monoxenic culture of *E.histolytica* using 5% DMSO as cryopreservant with 30 minutes equilibration. None of the amoebae survived cryopreservation when equilibrated at 0°C. Phillips *et al.*, (1984), maintained the cryopreservant and the cultures on ice prior to initiation of the freezing procedures, and reported more than 70% motile organisms after thawing. Warhurst & Wright (1979), added the 7,5% DMSO, equilibrated at room temperature, and reported more than 50% motility on thawing. In the current study, addition of DMSO and equilibration was allowed to take place at room temperature (22-25°C) and 70 -80% of the trophozoites were motile after thawing.

The protocol described in the present chapter has been found to be highly reproducible, as all cultures retrieved after cryopreservation have been successfully re-established in culture and repeatedly cryopreserved. It has also allowed us to establish the first cryobank for *Giardia* isolates in South Africa.

## CHAPTER 7

### APPLICATION OF ISOENZYME ELECTROPHORESIS TO *GIARDIA* LYSATES

#### 7.1 INTRODUCTION

Isoenzyme electrophoresis is a technique whereby separation of the multiple forms of a given enzyme in a single organism / individual, or in different members of the species, is achieved by electrophoresis. The characteristic banding patterns of enzymes then allow grouping into zymodemes (strains determined by isoenzyme patterns). Isoenzyme (and DNA) analyses have been proven to be excellent tools in determining the extent of inter- and intra-specific variation in protozoan, helminth and arthropod parasites (Heinz-1988). Isoenzyme electrophoresis distinguishes the distinct forms of enzymes that are genetically (or post-translationally) determined. Thus an isoenzyme profile is a direct reflection of the genetic make up of an organism.

The technique of isoenzyme electrophoresis has been successfully employed in characterisation of zymodemes of pathogenic and non-pathogenic strains of *Entamoeba histolytica* (Jackson *et al.*, 1982; Sargeant *et al.*, 1982; Matthews *et al.*, 1983). The technique has also been applied in an attempt to characterise strains of *Giardia duodenalis*, to address different aspects of giardiasis such as:

- **Epidemiological distribution**

Meloni, *et al.* (1988) compared thirty isolates from humans and felines by isoenzyme electrophoresis using ten of 25 enzyme systems that were assayed. They identified 13 different zymodemes, which could be grouped

into two categories. One group contained human isolates restricted to one geographic location (Western Australia), the other group comprised human and feline strains with world-wide distribution.

- **Determination of the role played by animals in transmission of *Giardia***

To determine if mammals such as beavers serve as reservoirs in waterborne outbreaks of giardiasis, Isaac-Renton *et al.*, (1993) employed isoenzyme electrophoresis to characterise outbreak and non-outbreak associated isolates. In this study they could group all outbreak-associated strains in one of eight zymodemes. Similarly, Meloni and colleagues (1988) characterised thirty isolates of *Giardia* (from humans and felines); they produced suggestive evidence for felines serving as a reservoir of infection to humans.

- **Characterisation of strains in relation to biological characteristics**

Cedillo-Rivera *et al.*(1989) performed isoenzyme electrophoresis of 19 *Giardia* isolates from symptomatic and asymptomatic patients from a single geographic locality. The study aimed at correlating the isoenzyme banding patterns to the behavioural characteristics (pathogenesis). In their findings, although the strains were genetically homogenous, there were no consistent zymodeme differences between isolates from symptomatic and asymptomatic groups. Similarly, Moss *et al.*, (1992) studied enzyme profiles of eleven *Giardia* strains from symptomatic (6) and asymptomatic (3) humans as well as animals (2), from different geographic locations. Although these strains were genetically different, they displayed some

common characteristics.

Several studies have showed that the isoenzyme characterisation technique has highly reputable performance characteristics. In these experiments the same sets of isolates were analysed by isoenzyme electrophoresis in conjunction with other molecular biological typing methods. Results of both pulsed field gel electrophoresis and isoenzyme analyses were similar when isolates retrieved from drinking water and from animal and human sources associated with a waterborne outbreak were subjected to both analytic methods (Isaac-Renton et al., 1993).

Morgan and colleagues (1993) analysed random amplified polymorphic deoxyribonucleic acid (RAPD) to characterise fourteen isolates of *Giardia* and grouped them into rapdenes (isolates with the same banding pattern). A comparison of the enzyme profiles of the same strains, which were previously obtained by isoenzyme electrophoresis, was also made. Both methods were significantly correlated, each resulting in 10 zymodemes (isoenzymes) and 10 rapdenes (RAPD).

The studies indicate that data obtained by other sensitive molecular techniques correlate with those obtained by isoenzyme electrophoresis. The feasibility of the latter method, compared to the other molecular typing methods that require specialised expertise, expensive and sophisticated equipment, particularly in the developing world makes isoenzyme analysis a practical choice.

Isoenzyme electrophoresis has been previously employed to characterise strains that were isolated from other countries. This section describes application of the

technique to the first local (South African) isolates of *Giardia*, in an attempt to investigate genetic differences among them. Modification of existing methods was undertaken to suit our local environment.

## **7.2 MATERIALS & METHODS**

### **7.2.1 Preparation of lysates**

Seventy two-hour confluent cultures were ice-chilled for 20 minutes and centrifuged at 400xg for 10 min at 4°C. Supernatant medium was gently discarded and the pelleted cells were transferred to Eppendorf microtubes using a sterile Pasteur pipette. The cells were treated with equal volumes of 1M each of: ethylene diamine tetra acetic acid di-sodium salt, (Polychem, Durban, SA); 6-capronic acid, (BDH, Poole, England); Dithiothreitol (Sigma, St. Louis, USA) to stabilise the target enzymes and inhibit proteolytic activity.

The mixture was centrifuged in a microfuge (Beckman Instruments) at 15000xg for 2.5 min and frozen at -20°C for 24hours. The samples were subsequently thawed at 22°C and re-centrifuged as before. Using a sterile Pasteur pipette, supernatant was collected and formed into beads by adding it dropwise into liquid nitrogen in a plastic beaker. The settled beads of lysate were collected and placed in Nunc cryogenic vials and stored in liquid nitrogen until electrophoresis was undertaken.

### **7.2.2 Electrophoresis**

#### **7.2.2.1 Enzyme systems**

The enzyme systems that were used are based on methods modified from Harris and Hopkinson (1976). Seven different enzymes were employed to determine the banding patterns of the local isolates. Their buffer systems are described in Appendix 8 and the preparation of buffers and quantities of substrates, co-factors and enzymes added are outlined in Appendices 9(b) & 10 respectively. Several preliminary runs were performed to determine optimum electrophoresis conditions



for *Giardia* lysates. These included variations in: duration of electrophoresis, buffer pH and concentration of some substrates and cofactors. The seven enzyme systems employed were:

- (i) Glucose phosphate isomerase (GPI) \*E.C.5.3.1.9
- (ii) Malic enzyme (ME) E.C.1.1.1.40
- (iii) Phosphoglucomutase (PGM) E.C 2.7.5.1
- (iv) Hexokinase (HK) E.C 2.7.1.1
- (v) Glucose-6- phosphate dehydrogenase (G6PD) E.C 1.1.1. 49
- (vi) 6-Phosphogluconate dehydrogenase (PDG) E.C. 1.1.1.44
- (vii) Glutamate oxaloacetate transaminase (GOT) E.C 2.6.1.1

**\*Note:** The numbers represent the Enzyme Commission's (EC) numbering according to the recommendations of the Commission on Biological Nomenclature(Harris & Hopkinson, 1976)

### **7.2.2.2 Electrophoresis procedure**

Twelve-percent starch (w/v) (Connaught Laboratories Ltd.) was dissolved in the appropriate gel buffers for each of the different enzymes as described in Appendix7.

The mixture was melted by boiling gently in a round bottom flask over a flame, degassed and poured onto framed glass plates (230x5x3 mm) and allowed to cool and gel. Using a metal edged cutter, transverse wells were cut onto the surface of the gelled starch in each plate. A paper template was used to ensure accurate alignment of the inoculation slots in a straight line. Beaded lysates (as prepared in 7.2.1) were retrieved from storage, thawed and absorbed onto crotchet cotton (5mm long and 0.5mm thick) and embedded into the slots in each gel. Appropriate

bridge buffers for each enzyme (Appendix 7) were introduced (in 75ml volumes) into each chamber of the electrophoresis apparatus (LKB, Biochrom, Model 2103). Inoculated plates were placed in their respective tanks and saturated wicks adjoining the anodal and cathodal compartments were laid onto each end of the inoculated plate. A cooling platform was incorporated into the system to maintain the temperature at 11°C during electrophoresis. The surface of the cooling plate was insulated with plastic sheeting and inoculated gels were electrophoresed for 2 hours in the electrophoresis apparatus at 240V. Following electrophoresis the gels were laid on a flat surface and a Perspex frame covering the whole migration zone was placed around the edges of the gel to retain the staining solution. Agar (as prepared in Appendix 8a) was melted by heating over a Bunsen burner and mixed with a staining solution specific for each enzyme. The agar/stain mixture was overlaid onto the gel and the plates were incubated at 37°C for 1 hour in the dark. The formazan reaction was used to visualise the banding patterns of the enzymes.

#### CONTROLS:

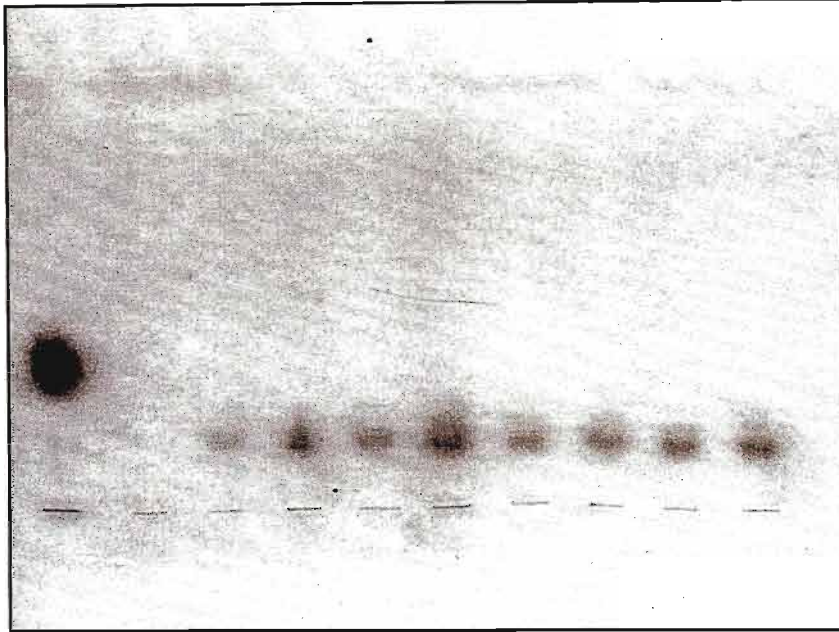
1. *Entamoeba histolytica* zymodeme II (strain HM1) was included as a control in every run. The H7 and WB reference strains were used as standard *Giardia* controls.
2. Uninoculated TYI-S-33 medium was included as a control in every electrophoresis run.
3. Lysates were prepared from different subcultures of the WB reference strain to assess the stability of isoenzymes during serial subculture of trophozoites. Lysates made from serial subcultures of the local isolates were also inoculated within runs.

4. To exclude the presence of bacterial bands, supernatant medium from (i) a WB culture that had been contaminated with *Xanthomonas spp.* and subsequently axenised through use of antibiotics, and (ii) a local isolate that was obtained from mice intestines and axenised, were also assessed by isoenzyme electrophoresis.

### 7.3 RESULTS

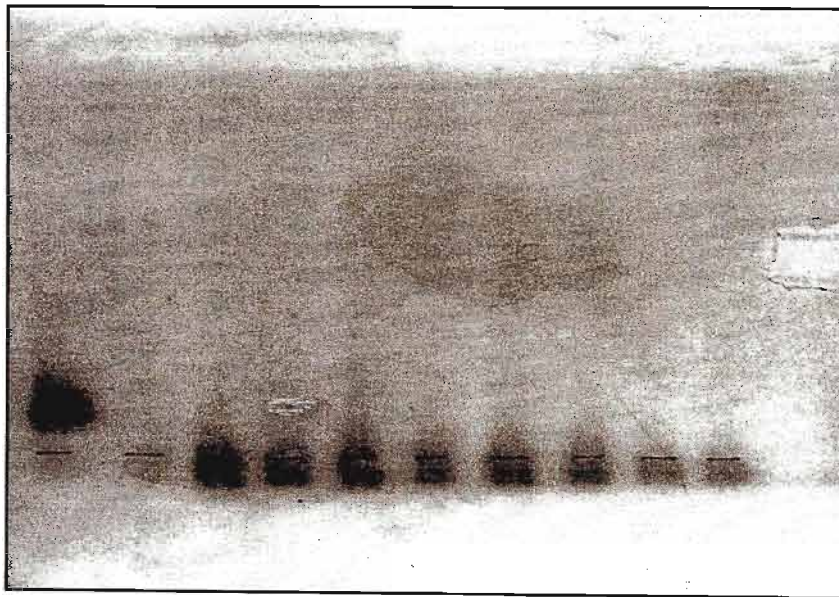
Although isoenzyme electrophoresis (ME, GPI, PGM and HK) of *Entamoeba spp.* is routinely performed in the Amoebiasis Research Lab (where this work was done) a series of preliminary runs were performed to establish the optimal conditions for *Giardia* isolates for these and 3 other additional enzyme systems set up for analysis of six local strains and two reference strains. The optimised electrophoretic conditions for *Giardia* resulted in atypical bands for the *E. histolytica* control sample; for example the shorter run time resulted in poorly separated bands for GPI, and a shorter than usual migration distance in HK.

Lysates of serial subcultures of the WB isolate consistently gave similar banding patterns in ME and GPI (Plates 7.1 and 7.2). Uninoculated TYI-S-33 medium demonstrated no bands for all the enzymes analysed. Differences in enzyme migration of the two protozoa (*E. histolytica* and *Giardia*) were distinguishable in the gels. Electrophoresis was replicated several times; of the seven enzyme systems, 6 gave reproducible results in terms of their banding patterns. These were GPI, PGM, ME, HK, G6PD, and 6PGD. Representative examples of their electrophoretic mobility patterns are shown in Plates 7.3-7.8.



**Plate 7.1** Banding pattern of 8 serially cultivated trophozoites of Giardia WB isolate in Malic enzyme. Consistent bands were observed for all 8 different subcultures.

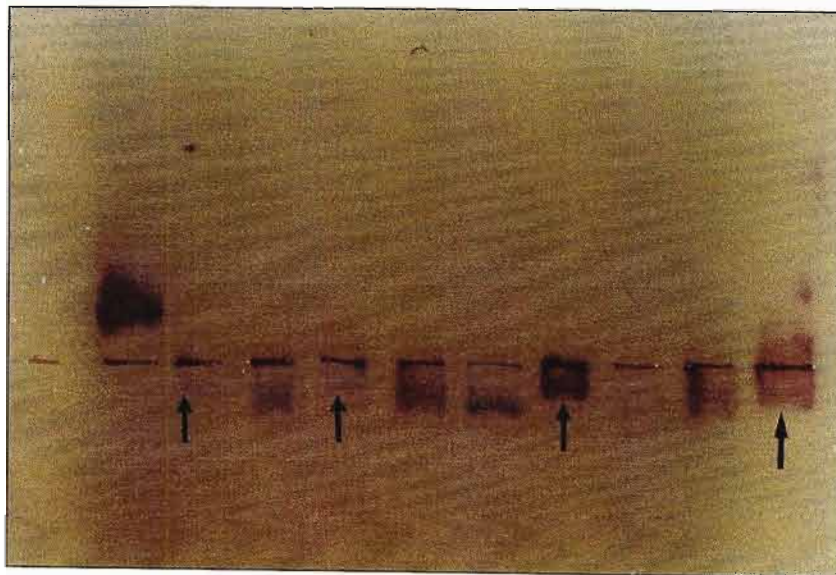
Lane 1. *E. histolytica* control. Lane 2. Uninoculated TYI -S-33 medium. Lanes 3-10. Serially subcultured WB isolate (12 –57 subcultures).



**Plate 7.2.** Glucosephosphate isomerase enzyme pattern of 8 serially cultivated trophozoites of Giardia WB isolate. Consistency of bands for all subcultures was noted.

Lane 1. *E. histolytica* control. Lane 2. Uninoculated TYI -S-33 medium. Lanes 3-10. WB subcultures. The number of subcultures ranged between 12 & 57).

For GPI activity, different subcultures of the WB isolate showed consistent bands (Plate 7.2). Poor separation of *E. histolytica* (resulting from a shorter run time) was observed. The *Giardia* enzymes migrated cathodally at pH 7.0. *E. histolytica* enzyme migrated anodally. Attempts to alter the direction of migration by changing the pH of the bridge buffer to 8.6 were unsuccessful as no bands were obtained at this pH. Single bands were displayed by all isolates. There were 2 patterns wherein the reference strain-WB and local isolates SA6, SA24 and SA305 showed a shorter migration distance than the reference strain H7 and local isolates SA7, SA18 and SA29 (Plate 7.3)

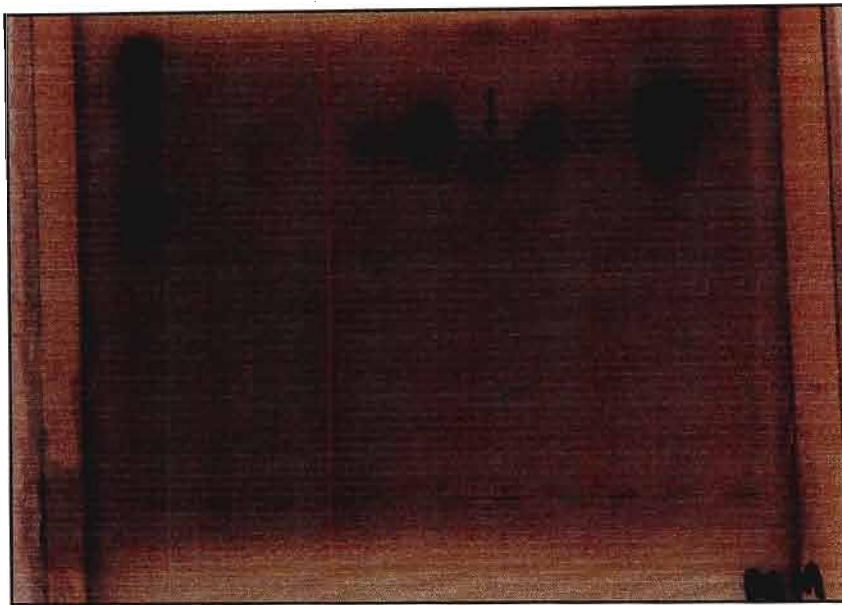


**Plate 7.3.** Glucosephosphate isomerase bands.

Lane 1. Uninoculated TYI medium. Lane 2. *E. histolytica* control. Lanes 3-11 *G. lamblia* trophozoites H7, WB, SA6, SA7, SA18, SA24, SA29a, SA29b and SA305. (SA29a =27 subcultures; SA29b =54 subcultures)

Two patterns are noted: Similar migration was obtained for reference strain WB, SA6 and SA24 (arrows) while the ref. strain H7 showed similar migration with local strains SA7, SA18, SA29 and SA305. Consistent bands were seen for the serial subcultures of the same isolate (SA29).

PGM activity was displayed by *Giardia* isolates and two banding patterns were seen. Five local isolates showed similar migration with both reference strains but one isolate (SA6) showed a different, slightly slower migration. Two isolates, WB and SA29 exhibited intense bands while SA7 and SA305 consistently showed very diffuse bands (Plate7.4)

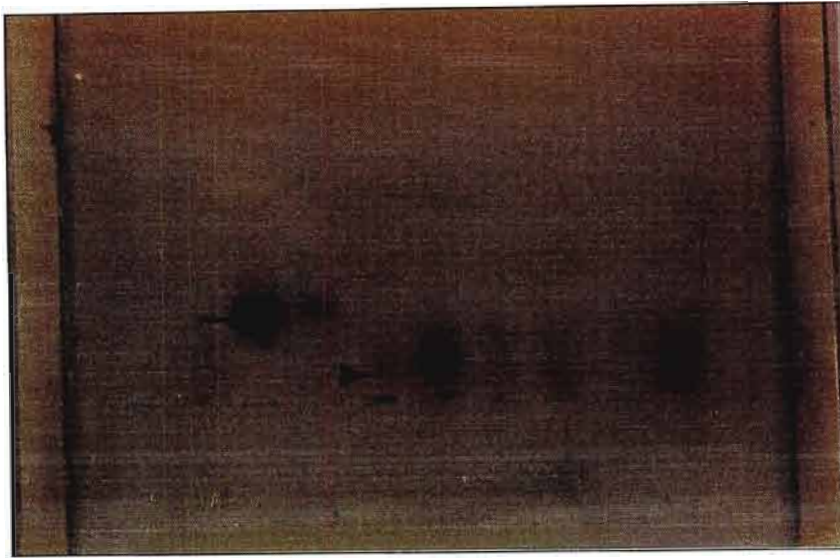


**Plate 7.4.** Phosphoglucomutase.

Lane1. *E. histolytica* control. Lane 2, Uninoculated TYI medium. Lanes 3-10.SA7, SA305, H7, WB, SA6, SA18, SA24 and SA29.

Two patterns are seen in which both reference strains WB and H7 had similar migration with local isolates SA18, SA24, SA29 while local isolate SA6 showed a different, slightly shorter migration zone (arrow). WB and SA29 exhibited intense bands while SA7and SA305 consistently showed very diffuse bands.

In Malic enzyme, a single band with similar electrophoretic pattern common to all isolates was observed in all the runs (Plate 7,5). This is also a typical pattern seen in *Entamoeba* ME activity. Two isolates, the reference strain WB and SA29 displayed intense bands while the rest of the other isolates exhibited diffuse bands.

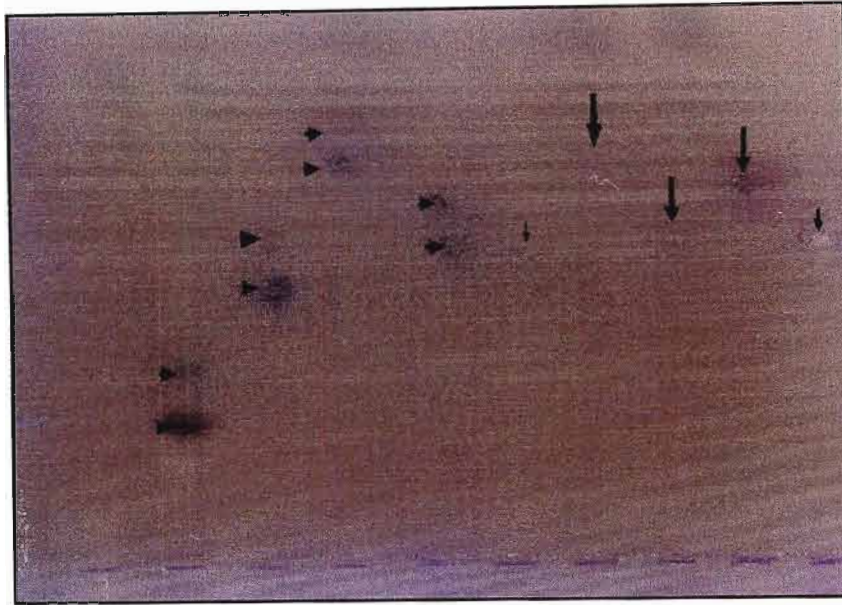


**Plate 7.5. Malic enzyme**

Lane 1. Uninoculated TYI. Lane 2. SA305. Lanes 3-4. *E. histolytica* group ii and xi  
Lanes 5-11. H7, WB, SA6, 7, 18, 24 and 29. Similar *Giardia* enzymes (arrowhead)  
migrated slower than those of the *Entamoeba* controls (arrow).

*Entamoeba histolytica* demonstrated typical HK activity. Marked heterogeneity  
was observed in *Giardia* isolates in which 3 isolates (WB, H7 and SA6) showed  
double bands of different mobilities for each isolate and 3 isolates displayed  
indistinct single bands of at least 2 different mobilities (i) (SA7, SA24 and SA305);  
(ii) (SA18 and SA29). The isoenzymes of all *Giardia* isolates migrated farther  
than those of *E. histolytica* (Plate 7.6).



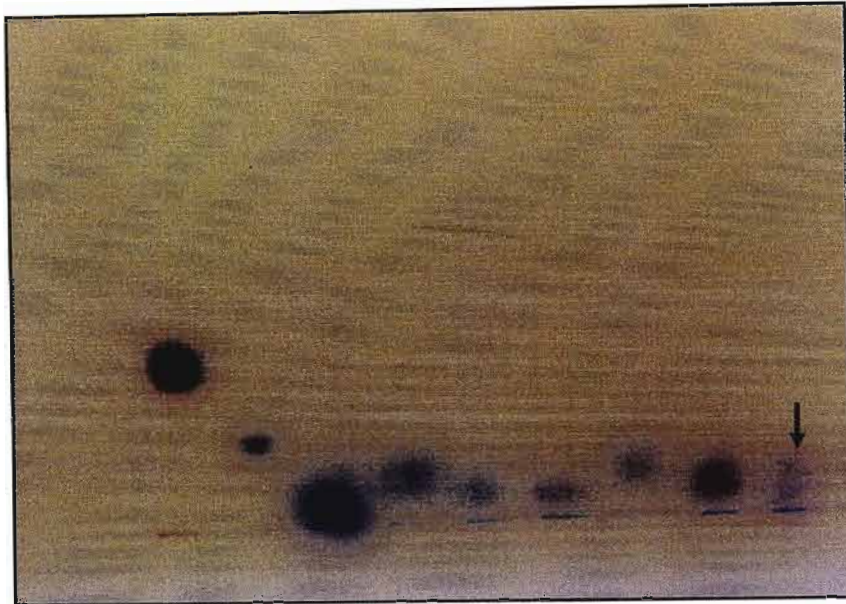


**Plate 7.6. Hexokinase**

Lane 1. Uninoculated TYI. Lane 2. *E. histolytica* control. Lanes 3-11. WB, H7, SA6, SA7, SA18, SA24, SA29 SA305a and SA305b, SA305c. 305a =54 x subcultured; 306b =111 subcultures, SA305c=216 subcultures. Consistent bands in all 3 lanes.

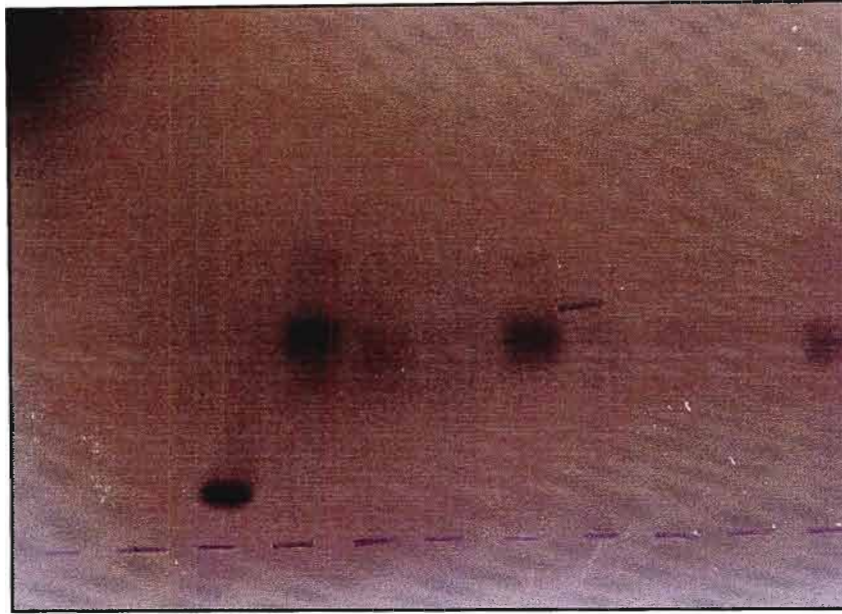
Distinct double bands(3 different patterns) were consistently exhibited by 3 *Giardia* isolates WB, H7 and SA6 (arrow-heads), indistinct single bands were seen in isolates SA18, 24 and 29 (big arrows) while very poor band resolution was seen for isolates SA7 and 305 (small arrows). All 3 isolates with single bands had a different pattern. Shorter migration for *E. histolytica* resulted from the shorter run time and *Giardia* enzymes migrated farther.

InG6PD 4 electrophoretic patterns were observed; in the first pattern, WB isolate migrated farther than the rest of the isolates, in the second, SA6SA18, SA24 and SA305 showed similar migration while in the third, H7 and SA7 migrated slowest. One isolate (SA29) showed double banding in the fourth pattern. Intense bands were observed in all but one isolate (SA305), seen in Plate7.7.



**Plate 7.7.** Glucose-6-phosphate dehydrogenase  
 Lane1. Uninoculated TYI. Lane 2. *E. histolytica* control. Lanes 3-11. WB, H7, SA6, SA7a, SA7b, SA18, SA24, SA29, SA305. SA7a=33 and b=108 subcultures. Similar bands in both lanes.  
 In G6PD, Single bands were observed for all *Giardia* isolates while isolate SA29 had double bands (arrow).

No bands were detected in 6PDG for *E. histolytica*. 2 different patterns were displayed by *Giardia* isolates; WB migrated slower while H7&SA6, SA7, SA18, SA24, SA29 and SA305 showed a similar migration distance. However, distinct bands were seen in WB, H7, SA18 and SA305 while the other isolates consistently displayed diffuse bands Plate 7.8.



**Plate 7.8.** Phosphogluconate dehydrogenase

Lane1. Uninoculated TYI-S-33. Lane 2. *E. histolytica* control. Lanes 3-11 WB, H7, SA6, SA7, SA18, SA24, SA 29a, SA29b, SA305.

Distinct bands were exhibited by the WB, H7, SA7, S18, and SA305 isolates.

Double bands were also detected from the latter isolate (lane 11). Poor band

resolution was obtained for the rest of the other isolates. WB migrated slower than

the all the isolates. No bands were detected for *E. histolytica* in this enzyme.

No bands were detected in GOT on repeated attempts.

## 7.4 DISCUSSION

As *Giardia lamblia* infections may have an asymptomatic course or they may produce acute or chronic diarrhoea, a mechanism of establishing if the different clinical outcome of these infections could be due to genetic strain differences would be useful. Isoenzyme analysis has proved to be an invaluable biochemical tool in differentiating pathogenic and non-pathogenic strains of *Entamoeba histolytica* (Jackson *et al.*, 1982; Sargeant *et al.*, 1982). Genetic variation in *Giardia* has been shown by isoenzyme analysis (Cedillo-Rivera *et al.*, 1989; Isaac-Renton *et al.*, 1993) however none of the investigations have demonstrated genetic variations that are correlated to pathogenicity. In the present study, it was not possible to characterise and group strains from symptomatic and asymptomatic subjects owing to limited numbers of isolates. Five isolates were obtained from "presumably" healthy children and 1 from a patient with diarrhoea. However, in the latter concomitant infection with *Salmonella spp* was present; therefore, symptomatology could not be unequivocally assigned to giardiasis. Furthermore, there was marked heterogeneity among the few isolates that were analysed by the enzyme systems described in the current work. An exhaustive genetic interpretation of the banding patterns of these isolates was therefore not made. Nonetheless, some polymorphism was demonstrated in this limited study. This suggests that the different isolates analysed in the present study possess enzyme variants, and therefore heterogeneous.

In this study, it is shown that serial cultivation of trophozoites does not alter the isoenzymes as shown by consistent banding patterns of serially cultivated isolates. This could indicate that the genes encoding the respective enzymes are

stably expressed between different subcultures; or that the culture conditions induce expression of stable genotypes. Therefore isoenzyme analysis of cultured trophozoites seems to be a reliable method for characterisation of different strains.

The findings reported in this work are comparable to results obtained by other investigators. For example, in ME, a homogenous pattern of single bands were exhibited by all isolates analysed in this study and Cedillo-Rivera *et al.*, (1989) reported strong homogeneity of electrophoretic patterns (all comprising of a single band) for ME when they analysed 19 strains isolated from symptomatic and asymptomatic patients in Mexico. However, other investigators reported different isoenzyme patterns when they analysed strains obtained from (i) widely-distributed geographic locations and (ii) both human and animal strains. Proctor *et al.*, (1989) found single ME bands with 3 different patterns when they analysed 32 *G. duodenalis* isolates from humans and animals of various geographic locations. Similar findings (2 different patterns of single bands) were reported by Moss *et al.*, (1992) for 11 *G.lambliia* strains from various geographic locations; Isaac-Renton *et al.*, 1993, reported similar findings for this enzyme. Meloni *et al.*, (1988) obtained single and multiple banding of various patterns for 30 isolates from humans and felines with worldwide distribution. From these studies it would appear therefore that the ME homogeneity is restricted to human isolates from a defined locality.

In the present study, no bands were obtained in the GOT enzyme system despite replicated attempts. Similarly, Proctor *et al.*, 1989 reported unsatisfactory results

for GOT while Moss *et al.*, 1992 reported that GOT did not develop bands when they analysed the isoenzymes of 11 axenic *G. lamblia* strains. However, Meloni *et al.*, 1988 were able to detect some activity with this enzyme.

In the current work, for the other enzymes, the number of different banding patterns demonstrated by each enzyme system ranged from 2 (GPI, PGM & 6PDG) or 4 (G6PD) to 6 (HK). These results indicate heterogeneity among the few isolates analysed in this work. This finding is contrary to a previous report by Cedillo-Rivera *et al.*, (1989) which suggested that isolates of *G. lamblia* from a common geographical locality show little heterogeneity. In the present work, homogeneity was demonstrated in ME only. It would appear that this suggestion holds for Malic enzyme (as discussed above).

Although *Giardia* display marked heterogeneity among different isolates, some similarities exist in the banding patterns of the various enzyme systems exist, for example the commonly poor or undetectable GOT bands and the common single bands in ME reported in this discussion.

Isoenzyme electrophoresis requires a relatively large number of trophozoites to ensure that the lysate has adequate enzyme activity. This in turn necessitates good growth of the organisms in culture. This is labour-intensive, time-consuming and unreliable, as it depends on successful excystation of cysts or invasive procedures of retrieving trophozoites from the gut. Another limiting factor is that establishment of viable cultures is not always guaranteed. It would appear therefore that other methods of differentiation should be sought. Molecular typing

techniques, which would allow differentiation of strains directly from cysts, and that require small amounts of material, would be ideal. The polymerase chain reaction could potentially meet these specifications.

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## APPENDICES

### APPENDIX 1

#### Information to participants and Consent Form

The aim of the study is to establish if strain variation that correlates with pathogenicity occurs within the species of the parasite, *Giardia lamblia*.

The parasite will be isolated from faeces. If you/your child participate in the study, faeces will be collected from you/your child and screened for the presence of *Giardia lamblia* cysts in the laboratory. If cysts are found in your specimen, you or your doctor will be notified so that you may seek treatment, or if already hospitalised, it is availed to you from the hospital.

The results from this study will be used solely for research purposes and your identity will not be released to any person for any other reason.

Participation in this study will, in no way interfere with all your hospital needs as deemed necessary by your doctor. You/your child may not participate in, or withdraw from, the study at will without suffering any prejudice as far as your medical care is concerned.

Knowledge gained from this study will help in future management of *G. lamblia* infections and hence the health of the community appropriately cared for.

I..... understand the aims of the study and what is expected of me should I agree to participate.

## APPENDICES

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Participation in this study will, in no way interfere with all your hospital needs as deemed necessary by your doctor. You/your child may not participate in, or withdraw from, the study at will without suffering any prejudice as far as your medical care is concerned.

Knowledge gained from this study will help in future management of *G. lamblia* infections and hence the health of the community appropriately cared for.

I..... understand the aims of the study and what is expected of me should I agree to participate.

I agree to participate in the study and will donate my/ my child's faecal sample when requested to.

Signature of participant/parent:..... Date.....

Witness:..... Date.....

### **Zulu version**

Inhloso yalolucwaningo ukuthola ukuba isilwayana esibizwa ngokuthi yi- *Giardia lamblia* esitholakala emathunjini abanye abantu sihlukahlukene yini. Ingabe kukhona uhlobo olugulisayo kanye nolungagulisi na.

Lesisilwanyana sizokhishwa emakakeni. Uma wena (nomaingane yakho) uvuma ukungenela lolucwaningo, azothathwa amakaka ayocwaningwa eLabhorethiukuthi awanawo amaqanda alesilwanyana. Uma lamaqanda etholakala emakakeni akho/omntwana wakho, wena noma udokotela wakho uyokwaziswa khona uzofuna umuthi wokuzelapha, noma uma vele ususesibhedlela, lomuthi uzonikwa khona.



Unayo imvume yokunqaba ukungenela lolucwaningo. Uma wenqaba, ukwlashwa kwakho esibhedlela angeke kuphazamiseke noma ngaluphi uhlobo.

Imiphumela iyosetshenziselwa kuphela ucwaningo, namagama akho ngeke lidalulwe kunoma imuphi umuntu. Ulwazi oluyotholakala kulolucwaningo luzosiza ekwnzeni ngcono ukwelapha izifo ezibangwa yilesisilwanyana, ngalokhom impilo yompohakathi inakekelwe kangcono.

Mina..... Ngiyaziqonda izinjongo zalolucwaningo. Ngiyaqonda futhi ukuthi uma ngivuma ukulungenela ucwaningo yini ekumele ngiyenze.

Ngiyavuma ukungenela ucwaningo. Ngiyonikela ngamakaka ami/omntwana wami uma ngicelwa.

Isandla songelela

ucwaningo/umzali:.....Date.....

Ufakazi.....Date.....

## Appendix 2

### Preparation of Beemer's stain

#### Solution A

|   |       |
|---|-------|
| Methylene blue (Polychem, Durban South Africa)          | 0.2g  |
| Sodium citrate (Polychem, Durban, SA)                   | 1.1g  |
| Sodium chloride (Polychem, Durban, SA)                  | 0.55g |
| Saturated mercuric chloride (Merck, Darmstadt, Germany) | 0.1ml |
| Distilled water   | 100ml |

#### Solution B

|  |       |
|--|-------|
| Eosin (BDH)                            | 1g    |
| 0.15 M Sodium Chloride (normal saline) | 100ml |

The working solution is prepared by mixing equal volumes of solutions A and B.

### Appendix 3

Excystation and culture of cysts from symptomatic and asymptomatic patients stools using the modified Rice and Schaefer method (Hamilton & Jackson, 1990). Total cyst counts and percent viabilities are indicated. Some cultures were contaminated while others died without evident contamination (indicated as sterile)

| Source | cyst count (x10 <sup>5</sup> ) | % Viability | % Excystation | Duration in Culture | Final Culture Status |
|--------|--------------------------------|-------------|---------------|---------------------|----------------------|
| Asympt | 12                             | 56          | 5             | 24hrs               | contaminated         |
| ?      | 34                             | 30          | Nil           | -                   |                      |
| Sympt  | 10                             | 70          | 10            | 24hrs               | contaminated         |
| Sympt  | 79                             | 44          | *8            | 24hrs               | contaminated         |
| Asympt | 11                             | 82          | 5             | 72hrs               | contaminated         |
| Asympt | 8                              | 100         | nil           | -                   | -                    |
| Sympt  | 110                            | 27          | *2            | 6hrs                | contaminated         |
| Asympt | 18                             | 75          | 8             | 6hrs                | contaminated         |
| Asympt | 10                             | 88.2        | 10            | 24hrs               | Sterile              |
| Asympt | 5                              | 97          | nil           | -                   | -                    |
| Asympt | 8                              | 87.5        | 2             | 12hrs               | contaminated         |
| Sympt  | 103                            | 90.9        | *36           | 72hrs               | contaminated         |
| Sympt  | 14                             | 63          | 3             | < 12hrs             | contaminated         |
| Sympt  | 11                             | 71          | 12            | < 12hrs             | contaminated         |
| Asympt | 3                              | 94          | 5             | 24hrs               | Sterile              |
| Asympt | 13                             | 87          | nil           | -                   | -                    |
| Asympt | 21                             | 64          | 33            | 72hrs               | Sterile              |
| Asympt | 2                              | 41          | nil           | -                   | -                    |
| Asympt | 7                              | 70          | 10            | 24hrs               | contaminated         |
| Asympt | 11                             | 55          | 2             | < 12hrs             | contaminated         |
| Asympt | 23                             | 94          | 20            | 72hrs               | Sterile              |
| Saympt | 9                              | 43          | nil           | -                   | -                    |
| Asympt | 8                              | 36          | nil           | -                   | -                    |
| Asympt | 17                             | 33          | 2             | -                   | -                    |

| Source | cyst count (x10 <sup>5</sup> ) | % Viability | % Excystation | Duration in Culture | Final Culture Status |
|--------|--------------------------------|-------------|---------------|---------------------|----------------------|
| Asympt | 14                             | 83          | nil           | -                   | -                    |
| Asympt | 23                             | 86          | *41           | 24hrs               | contaminated         |
| Asympt | 5                              | 41          | 7             | 48hrs               | sterile              |
| ?      | 7                              | 79          | 10            | 24hrs               | sterile              |
| Asympt | 11                             | 87          | 20            | < 12hrs             | contaminated         |
| Asympt | 13                             | 56          | 20            | < 12hrs             | contaminated         |
| Asympt | 2                              | 81          | 10            | 72hrs               | sterile              |
| Asympt | 14                             | 96          | 22            | < 12hrs             | contaminated         |
| Sympt  | 10                             | 81.3        | Nil           | -                   | -                    |
| Sympt  | 13                             | 75.4        | 5             | 24hrs               | sterile              |

\*Result represents average of duplicated attempts to excyst.

## Appendix 4

Results of excystation and culture using the modified Bingham & Meyer (1979) acid pepsin method. Total cyst counts and percent viabilities are indicated. Some cultures were contaminated while others died without evident contamination (indicated as sterile)

| Source | cyst count( x10 <sup>5</sup> ) | %Viability | % Excystation | Duration in Culture | Final Culture Status |
|--------|--------------------------------|------------|---------------|---------------------|----------------------|
| Asympt | 33                             | 96         | 20            | 96hrs               | Contaminated         |
| Asympt | 39                             | 85         | 20            | 72hrs               | Contaminated         |
| Asympt | 49                             | 90         | 20            | 96hrs               | Contaminated         |
| ?      | 15                             | 45         | 2             | <12hrs              | Contaminated         |
| ?      | 8                              | 55         | nil           | -                   | -                    |
| Sympt  | 25                             | 70         | 6             | <12hrs              | contaminated         |
| Asympt | 11                             | 84         | nil           | -                   | -                    |
| Asympt | 2                              | 79         | 2             | 72hrs               | sterile              |
| Asympt | 16                             | 95         | 15            | 24hrs               | contaminated         |
| Asympt | 4                              | 100        | 8             | <12hrs              | contaminated         |
| Asympt | 2                              | 33         | nil           | -                   | -                    |
| Asympt | 2                              | 87.5       | nil           | -                   | -                    |
| Asympt | 4                              | 22.5       | nil           | -                   | -                    |
| Asympt | 9                              | 77.7       | nil           | -                   | -                    |
| ?      | 154                            | 52         | 15            | <12hrs              | Contaminated         |
| Asympt | 10                             | 40         | 20            | 24hrs               | sterile              |
| Asympt | 23                             | 85.7       | nil           | -                   | -                    |
| Asympt | 14                             | 25         | nil           | -                   | -                    |
| Sympt  | 2                              | 73         | nil           | -                   | -                    |
| Sympt  | 3                              | 66         | nil           | -                   | -                    |
| Sympt  | 13                             | 87         | nil           | -                   | -                    |
| Sympt  | 13                             | 50         | nil           | -                   | -                    |
| Sympt  | 67                             | 58.2       | 22            | 72hrs               | sterile              |
| ?      | 15                             | 84         | nil           | -                   | -                    |
|        |                                |            |               |                     | -                    |

| Source   | cyst count( x10 <sup>5</sup> ) | %Viability | % Excystation | Duration in Culture | Final Culture Status |
|----------|--------------------------------|------------|---------------|---------------------|----------------------|
| Sympt    | 33                             | 15         | nil           | -                   |                      |
| Sympt    | 55                             | 16         | 10            | 96hrs               | contaminated         |
| Sympt    | 12                             | 33         | nil           | -                   | -                    |
| Sympt    | 46                             | 79         | nil           | -                   | -                    |
| Sympt    | 23                             | 57         | nil           | -                   | -                    |
| Sympt    | 54                             | 33         | nil           | -                   | -                    |
| Asympt H | 12                             | 26         | nil           | -                   | -                    |
| Asympt H | 4                              | 18         | nil           | -                   | -                    |
| Asympt   | 6                              | 66         | nil           | -                   | -                    |
| Asympt   | 87                             | 82.8       | 10            | 72hrs               | contaminated         |
| Asympt   | 31                             | 67.6       | 15            | 72hrs               | contaminated         |
| Asympt   | 66                             | 66         | 5             | 72hrs               | contaminated         |
| Asympt   | 43                             | 81.4       | 10            | 7dys                | sterile              |
| Sympt    | 25                             | 60         | nil           | -                   | -                    |
| Asympt   | 4                              | 50         | nil           | -                   | -                    |
| Asympt   | 32                             | 65.6       | 10            | 24hrs               | contaminated         |
| Asympt   | 144                            | 88.9       | 25            | <12hrs              | contaminated         |
| Asympt   | 11                             | 81.8       | 20            | 48hrs               | sterile              |
| Asympt   | 55                             | 78.2       | 20            | 7dys                | sterile              |
| Asympt H | 185                            | 68.6       | 30            | 48hrs               | contaminated         |
| Asympt H | 13                             | 100        | nil           | -                   | -                    |
| Sympt    | 52                             | 46         | 5             | 48hrs               | sterile              |
| Sympt    | 40                             | 58         | nil           | -                   | -                    |
| Asympt H | 13                             | 49         | nil           | -                   | -                    |
| Asympt H | 10                             | 32         | nil           | -                   | -                    |
| Sympt    | 87                             | 16.4       | 10            | 48hrs               | contaminated         |
| Sympt    | 12                             | 33.3       | Nil           | -                   | -                    |

## Appendix 5

Results of *in vitro* (acid-pepsin) and *in vivo* (mice inoculations) excystation and subsequent cultivation *in vitro*. Cysts were obtained from symptomatic and asymptomatic subjects. Percent viability was determined by the eosin exclusion method. Litters of two mouse strains (C57BL/6 and Mastomys) were inoculated and the percentage of mice infected per litter is indicated. Survival time for each culture (derived from both excystation methods) is indicated.

| Source    | Total Cyst Count | % Viability | Excystation (acid pepsin) | Culture Result (acid pepsin) | Excystation (mice inoculation) | Mouse strain | Culture Result |
|-----------|------------------|-------------|---------------------------|------------------------------|--------------------------------|--------------|----------------|
| Asympt    | 33               | 96          | 20                        | 2 days (c)                   | 1 of 9(10%)                    | C57BL/6      | 2dys(s)        |
| Asympt    | 39               | 85          | 20                        | 4 days (c)                   | 4 of 4 (100%)                  | C57BL/6      | Axenised       |
| Asympt    | 49               | 90          | 20                        | 4days (c)                    | 7 of 7(100%)                   | C57Bl        | Axenised       |
| Asympt    | 3                | 33          | Nil                       |                              | Nil                            | C57BL/6      | -              |
| Sympt     | 53               | 64          | 30                        | 2 days (s)                   | Nil                            | C57BL/6      | -              |
| Asympt    | 282*             | 52          | nil                       | -                            | Nil                            | C57BL/6      | -              |
| Sympt     | 13               | 53.8        | nil                       | -                            | Nil                            | C57BL/6      | -              |
| Asympt    | 282*             | 52          | nil                       | -                            | Nil                            | C57BL/6      | -              |
| Asympt H  | 5                | 45          | nil                       | -                            | Nil                            | C57BL/6      | -              |
| Asympt    | 3                | 52          | nil                       | -                            | Nil                            | C57BL/6      | -              |
| Asympt    | 144*             | 88.9        | 20                        | 7days (s)                    | Nil                            | C57BL/6      | -              |
| Asympt    | 43               | 81.4        | 42                        | 1day (c)                     | Nil                            | C57BL/6      | -              |
| Asympt    | 55               | 78.2        | 20                        | 3 days (c)                   | 5 of 5(100%)                   | Mastomys     | Axenised       |
| Asympt    | 43               | 81.4        | 42                        | 5days (s)                    | 3 of 3(100%)                   | Mastomys     | Axenised       |
| Asympt    | 144*             | 88.9        | 20                        | 4days (c)                    | 7 of 7(100%)                   | Mastomys     | Axenised       |
| H7 Trophs | -                | -           | -                         |                              | Nil                            | C57BL/6      | -              |
| Asympt H  | 185*             | 68.6        | 30                        | 1 day (c)                    | Nil                            | C57BL/6      | -              |
| Sympt H   | 22               | 100         | nil                       | -                            | Nil                            | C57BL/6      | -              |
| AsymptH   | 27               | 49          | 1                         | 1day (c)                     | 3 of 3 (100%)                  | C57BL/6      | 48hrs (s)      |
| Asympt    | 87*              | 82.8        | 10                        | 3days (c)                    | Nil                            | C57BL/6      | -              |
| Asympt H  | 11               | 32          | nil                       | -                            | 2 of 8 (25%)                   | C57BL/6      | 28hrs(s)       |
| Asympt    | 31               | 67.7        | 10                        | 1day (s)                     | Nil                            | C57BL/6      | -              |
| Asympt H  | 20               | 42          | nil                       | -                            | Nil                            | C57BL/6      | -              |

| Source | Total Cyst Count | % Viabilit | Excystation (acid pepsin) | Culture Result (acid pepsin) | Excystation (mice inoculation) | Mouse strain | Culture Result  |
|--------|------------------|------------|---------------------------|------------------------------|--------------------------------|--------------|-----------------|
| Asympt | 6                | 66         | nil                       |                              | pups eaten                     | C57BL/6      | -               |
| Asympt | 31               | 67.7       | cont                      |                              | pups eaten                     | Mastomys     | -               |
| Asympt | 6                | 66         | nil                       |                              | pups eaten                     | Mastomys     | -               |
| Sympt  | 25               | 60         | nil                       | -                            | nil                            | C57BL/6      | -               |
| Asympt | 4                | 20         | nil                       | -                            | 1 of 4 (25%)                   | C57BL/6      | 24hrs(c)        |
| Asympt | 32               | 65.6       | 10                        | 3 days (s)                   | Nil                            | Mast         | -               |
| Asympt | 11               | 81.8       | 20                        | 1day (c)                     | 3 of 6 (50%)                   | Mast         | 72hrs(c)        |
| Asympt | 355*             | 66.2       | nil                       | -                            | Nil                            | C57BL/6      | -               |
| Sympt  | 14               | 75         | nil                       | -                            | pups eaten                     | Mastomys     | -               |
| Sympt  | 123*             | 78.9       | 1                         | 1day (s)                     | 4 of 8 (50%)                   | C57BL/6      | 48hrs(s)        |
| Sympt  | 86               | 25.7       | 1                         | 2 days (s)                   | Nil                            | Mastomys     | -               |
| Sympt  | 18               | 30         | nil                       | -                            | Nil                            | C57BL/6      | -               |
| Sympt  | 123*             | 66         | 10                        | 3days (c)                    | 4 of 8 (50%)                   | C57BI        | 8hrs(c)         |
| Sympt  | 16               | 59         | nil                       | -                            | Nil                            | C57BL/6      | -               |
| Sympt  | 16               | 59         | nil                       | -                            | Nil                            | C57BI        | -               |
| Sympt  | 123*             | 78.9       | 5                         | 4days (s)                    | ?±                             | Mastomys     | <i>H. muris</i> |
| Sympt  | 8                | 37         | nil                       | -                            | Nil                            | Mastomys     | -               |
| Asympt | 20               | 50         | nil                       | -                            | nil                            | Mastomys     | -               |
| Sympt  | 203*             | 39         | nil                       | -                            | Nil                            | C57BL/6      | -               |
| Sympt  | 18               | 65         | nil                       | -                            | 3 of 6 (50%)                   | C57BL/6      | 5days (s)       |
| Sympt  | 16               | 58         | nil                       | -                            | 3 of 4(75%).                   | C57BL/6      | 4days (s)       |
| Sympt  | 11               | 36         | nil                       | -                            | 3 of 5 (60%)                   | C57BL/6      | 3days (s)       |
| Sympt  | 18               | 65         | nil                       | -                            | 4 of 7(57%)                    | C57BL/6      | 4days (c)       |
| Sympt  | 11               | 40         | nil                       | -                            | Pups eaten                     | Mastomys     | -               |
| Sympt  | 11               | 36         | nil                       | -                            | 2 of 7(29%)                    | C57BL/6      | 5 days (s)      |
| Sympt  | 59               | 97         | nil                       | -                            | 5 of 7(71%)                    | C57BL/6      | 3days (c)       |
| Sympt  | 2836*            | 33         | nil                       | -                            | 6 of 6(100%)                   | C57BL/6      | Axenised        |
| Sympt  | 2836*            | 33         | nil                       | -                            | 9 of 9(100%)                   | Mastomys     | <i>H. muris</i> |
| Sympt  | 12               | 42%        | nil                       | -                            | nil                            | C57BL/6      | -               |



| Source | Total Cyst Count | % Viability | Excystation (acid pepsin) | Culture Result (acid pepsin) | Excystation (mice inoculation) | Mouse strain | Culture Result |
|--------|------------------|-------------|---------------------------|------------------------------|--------------------------------|--------------|----------------|
| Sympt  | 112*             | 64,2%       | nil                       | -                            | nil                            | C57Bl        | -              |

**Key to Appendix 5**

\*Replicated inoculation of mice.

± The mice were heavily infested with *Hexamita muris* such that the presence or absence of *Giardia* trophozoites could not be confirmed.

There was no difference in infectivity of cysts from symptomatic (40%) and asymptomatic (39%) hosts.

## **APPENDIX 6**

### **Preparation Of Excystation Media (Hamilton & Jackson, 1990)**

#### **Appendix 6a**

##### **Induction Medium**

Twenty-five ml of Hanks salt solution was made up to 100 ml with distilled water, autoclaved for 10 min at 103 kPa, cooled to 37°C and 0.40g L-cysteine hydrochloride (Polychem, Durban, South Africa) and 0.42 g sodium bicarbonate (Associated Chemicals Enterprise cc, Gauteng, South Africa) added. The pH was adjusted to 2,0 with 10M HCl (Polychem, Durban, and SA).

#### **Appendix 6b**

##### **Excystment medium**

Forty millilitres of Hanks solution was made up to 100 ml with distilled water. One gram of Trypticase (Merck, Darmstadt, Germany) and 0,5g glucose (Polychem, Durban, SA) were added. The solution was autoclaved at 103 kPa for 10 min. Following cooling to 37°C, the following were added: 0,4 g L-cysteine hydrochloride (Polychem, Durban, SA.); 0,25 g Trypsin (Merck, Darmstadt, Germany) and 15 ml decomplexed horse serum \*(Highveld Biological, JHB, SA.) The pH was adjusted to 7,5 with 1M NaOH (Polychem. Durban, South Africa) and the medium distributed in 3ml aliquots in glass tubes, overlaid with 0.5ml liquid paraffin and incubated at 37°C. Excess medium was used for washing cysts following the induction step.

*\*Later, fetal calf serum (Sigma, St Louis, USA) was used in place of the Highveld brand of horse serum.*

## Appendix 7

### Preparation of culture medium – Trypticase-Yeast-Iron-Serum -33 (TYI-S-33)

modified from Keister (1983). Bile and an increased amount of L-cysteine were the essential modifications added by Keister to the original preparation.

Biosate Peptone (BBL, Cockeysville, USA) 30g/L

Sodium chloride (Polychem, Durban, SA) 2g/L

Di-potassium hydrogen phosphate (Polychem, Durban, SA) 1g/L

Potassium dihydrogen phosphate (BDH Chemicals) 0.6g/L

Ascorbic acid (Polychem, SA) 0.2g/L

Ferric ammonium citrate, brown granules (Merck, Germany) 0.023g/L

D-Glucose (Polychem, SA) 10g/L

L-cysteine monohydrochloride (Polychem, SA) 2g/L

Decomplemented fetal bovine serum (Sigma, St.Louis, USA) 100ml

Bovine bile (Sigma, St Louis, USA) 0.5g/L

The pH is adjusted to 7.0-7.2 with 10M NaOH (Polychem, Durban, SA).

The following antibiotics were added to the medium:

Penicillin (Biowhittaker, Walkersville, MD) 100 000 u/ml

Streptomycin (Biowhittaker, Walkersville, MD) 1000 ug/ml

Amphotericin B (Biowhittaker, Walkersville, MD) 5 ug/ml

Amikacin 10ug/ml (Bristol Myers, Squibb, New Jersey, USA)

Occasionally, Gentamycin (Bayer) 40 ug/ml was also used.

The medium is sterilised by filtration through a 0.22 $\mu$  filter (Millipore, Bedford, MA) and stored at 4°C.

Modification (Diamond, 1995; Nash TE and Conrad JT, personal communication)

A broth concentrate (five times concentrated by increasing the concentration of the ingredients listed on p182 five times) containing Biosate, glucose and sodium chloride was prepared and filter sterilised. This medium can be stored at 4°C for up to 1 year. A buffer containing 5x the concentration of the buffer salts was also made. A 6,5% bile solution and 2,28mg/ml Ferric ammonium citrate were prepared separately. All stock solutions were stored at 4°C. The complete medium was made by diluting the broth concentrate to 500 millilitres of distilled water, 10% serum, antibiotics, and a 5x concentrated, freshly prepared reducing solution of L-cysteine and Ascorbic acid. The complete medium was used within 1 week of preparation.

Our modification involved use of the other aminoglycosides because of expected widespread penicillin/streptomycin resistance among local bacterial strains.

## Appendix 8

### List of enzyme systems used for isoenzyme electrophoresis.

The numbering recommended by the Commission on Biological Nomenclature is used. This number (preceded by the letters E.C. for Enzyme Commission) is indicated after each enzyme.

(i) Glucose Phosphate Isomerase (GPI) E.C.5.3.1.9

Buffers: bridge 0.2M PO<sub>4</sub> pH 7.0

Gel 1 in 10 dilution of bridge buffer

(ii) Malic Enzyme (ME) E.C.1.1.1.40

Buffers: bridge 0.2M PO<sub>4</sub> / 0.2M citrate buffer pH 7.0

Gel 1 in 5 dilution of bridge buffer

(iii) Phosphoglucomutase (PGM) E.C 2.7.5.1

Bridge buffer: 0.1M Tris maleate pH 7.4

Gel buffer is 1 in 10 dilution of bridge buffer

(iv) Hexokinase (HK) E.C 2.7.1.1

Bridge buffer 0.2 M Tris maleate pH 7.4

Gel buffer is 1 in 10 dilution of Tris maleate

(v) Glucose- 6- Phosphate dehydrogenase (G6PD) E.C 1.1.1. 49

Buffers:

Bridge: 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0.

NADP (0.22ml of 5mg/ml) was added to 75ml of the cathodal end compartment.

Gel: 1 in 40 dilution of bridge buffer. NADP (0.48 ml of 5mg/ml) was added to heated gel mixture just before degassing.

(vi) 6-Phosphogluconate Dehydrogenase (PDG) E.C. 1.1.1.44

Buffers:

Bridge: 0.1M PO<sub>4</sub> buffer at pH 7.0 and 1.5mg of NADP was added to 75 ml of the cathodal compartment buffer.

Gel: A 1 in 10 dilution of bridge buffer and starch to which 2.4ml of NADP was added just before degassing.

(vii) Glutamate Oxaloacetate (GOT) E.C 2.6.1.1

Buffers:

Bridge: 0.2M di-Sodium Hydrogen Phosphate/ 0.03M Boric acid, pH 7,5

Gel: 1 in 10 dilution of bridge buffer

## **Appendix 9**

### **Appendix (9a)**

#### **Preparation of Agar for Overlay**

Agar Noble (Difco Labs., Detroit, USA) was prepared by dissolving 12g in one liter of distilled water, melted and allowed to cool to 37°C. Ten millilitre volumes were aliquoted in 50 ml bijou bottles and stored at -20°C until required.

### **Appendix (9b)**

#### **List of Electrophoresis Buffers and Their Preparation**

(1) 0.2 M Phosphate ( $\text{PO}_4$ ) Buffer.

17.4g/L di-Sodium Hydrogen orthophosphate (Polychem. Durban, SA.)

11.5g/L Sodium dihydrogen orthophosphate (BDH)

pH 7.0

(2) 0.1M Phosphate buffer was prepared similarly with the proportionate adjustments of quantities.

(3) 0.1 M Tris maleate Buffer

12.14 g/L Tris (Polychem. Durban, SA.)

11.16g/L Maleic acid (Saarchem. Krugersdorp, SA)

2.03 g/L Magnesium Chloride (Saarchem. SA.)

3.72g/L EDTA (Polychem, Durban, SA)

The pH was adjusted to 7.4 with 10 M Sodium Hydroxide.

(4) 0.2 and 0.3M Tris maleate buffers were prepared similarly, with the appropriate adjustments in quantities.

(5) 0.3 M Tris Buffer

37.34g/L Tris buffer (Associated Chemical Enterprises cc., Reuven, SA.)

(6) 0,3M Tris-Hydrochloride buffer, pH adjusted to 8,0 with 10M NaOH

(7) 0,3M Tris-Hydrochloride buffer, pH 7,4

(8) 0,1M Sodium di-Hydrogen phosphate, pH adjusted to 7,0 with di-Sodium Hydrogen phosphate

(9) 0,2M Sodium hydrogen phosphate / 0,03M Boric Acid

(10) 0,5M Tris Hydrochloride pH adjusted to 8.0 with 10M NaOH.

(11) L-Aspartic acid (30mM) is dissolved in 0.1M Tris/HCl buffer and pH adjusted to 8.0 with KOH

(12) 2-Oxoglutaric acid (15mM) is dissolved in 0.1M Tris/HCl buffer and pH adjusted to 8.0 with KOH

(13) 0, 2M Citrate buffer, pH 7,0



## Appendix 10.

Quantitative description of substrates, cofactors and activators of each enzyme system.

| Reagent                       | GPI   | ME    | PGM    | HK    | G6PD  | PDG   | GOT    |
|-------------------------------|-------|-------|--------|-------|-------|-------|--------|
| 0.3M Tris-Hydrochloride pH7.4 | 14mls |       | 14mls  |       |       |       |        |
| 0.3M Tris Hydrochloride pH8.0 |       | 14mls |        | 14mls | 14ml  | 14mls | 35 mls |
| NADP (10 m/gml)               | 1ml   | 1ml   | 1.5 ml | 1ml   | 1ml   | 1ml   |        |
| 1M Magnesium Chloride         | 0.4ml | 0.4ml | 0.5ml  | 0.4ml | 0.5ml | 0.5ml |        |
| G6PD (140 u/ml)               | 0.5ml |       | 0.2ml  | 0.2ml |       |       |        |
| Fructose6-Phosphate(Na3 salt) | 0.2ml |       |        |       |       |       |        |
| 1M Maleate                    |       | 0.4ml |        |       |       |       |        |
| Glucose-1Phoshate(Na2 salt)   |       |       | 25mg   |       |       |       |        |
| Glucose                       |       |       |        | 20mg  |       |       |        |
| ATP (Na <sub>2</sub> salt)    |       |       |        | 40mg  |       |       |        |
| G6P(0.6mM )Na2 salt           |       |       |        |       | 10mg  |       |        |
| 6Phosphogluconate0,8mM (Na3 ) |       |       |        |       |       | 10mg  |        |
| L-Aspartic acid(30mM)         |       |       |        |       |       |       | 7,5ml  |
| 2-Oxoglutaric acid (15mM)     |       |       |        |       |       |       | 7.5ml  |
| Fast-blue                     |       |       |        |       |       |       | 250mg  |
| *MTT( 0.05g/ml)               | 1ml   | 1ml   | 1ml    | 1ml   | 1ml   | 1ml   |        |
| †PMS (0.01g/ml)               | 1ml   | 1ml   | 1ml    | 1ml   | 1ml   |       |        |
| Agar (1.2%)                   | 10ml  | 10ml  | 10ml   | 10ml  | 10ml  | 10ml  | 10ml   |

\*3-(4,5Dimethylthiazolyl-2)-2,5 diphenyltetrazolium Bromide (MTT)

† Phenazinemethosulphate (PMS)