

CRYPTOSPORIDIUM AND CRYPTOSPORIDIOSIS

by

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B.Sc (Hons)

Submitted in partial fulfilment of  
the requirements for the degree of

MASTER OF SCIENCE (MED SCIENCE)

in the

Department of Medical Microbiology

Faculty of Medicine

University of Natal

Durban

1990

**ABSTRACT**

Cryptosporidium parvum can cause debilitating disease in immunocompetent persons with cholera-like symptoms characterised by self-limiting, profuse diarrhoea; on the other hand asymptomatic infection with this organism frequently occurs. However, in immunocompromised patients, the disease is more severe and is life-threatening.

A pivotal aspect of the present survey was a comparative assessment of four commonly used staining techniques (viz. modified Ziehl-Neelsen, safranin-methylene blue, auramine phenol fluorescence and Sheather's sucrose flotation) for the detection and identification of Cryptosporidium oocysts. The Sheather's flotation method proved to be superior to the other three procedures which were not only less sensitive but also less specific. A modification of the Sheather's flotation technique was developed for use with diarrhoeal stools; this was found to be simple, reliable, cost-effective and the least time consuming of the above methods; this was used exclusively in a subsequent survey of the association of Cryptosporidium infection with diarrhoea in hospitalised children.

Although previous epidemiological surveys of cryptosporidiosis have been conducted in South Africa standardised methods have not been employed. This initial assessment of diagnostic techniques therefore provided a tool for accurately assessing the importance of Cryptosporidium as a causative organism of diarrhoea. In an

extensive study performed on children younger than 10 years old, who were hospitalised with a primary diagnosis of diarrhoea at King Edward VIII Hospital, it was found that 9,0% (111/1229) were passing Cryptosporidium oocysts; this was the second most common enteric pathogen. In 72% (80/111) of patients with Cryptosporidium infections it was the only pathogen. The prevalence of cryptosporidiosis was highest during the months of February, March, April and May; direct correlation between the rainfall in the Durban area and the prevalence of cryptosporidiosis was demonstrated ( $r = 0,6125$ ). Cryptosporidium infection was more prevalent in the 4-6 month age group ( $p = 0,001$ ).

The fact that Cryptosporidium infections may be symptomatic in some individuals and asymptomatic in others, suggests that strain differences in respect of pathogenic potential may occur. A prerequisite to the investigation of strain differences was to increase parasite numbers; both in vivo and in vitro culture techniques were employed. Culture in chicken embryos failed to increase the parasite population and only limited areas of the chorio-allantoic membranes showed a few developmental stages. Cell cultures proved to be more suitable for Cryptosporidium growth and parasite numbers increased proportionally with duration in culture. Attempts at infecting suckling Balb/c mice were unsuccessful; however experimental infection of immunosuppressed adult rats facilitated the examination of various developmental stages of the parasite.

Isoenzyme electrophoresis is an excellent method for demonstrating polymorphism in many species. Of the five enzyme systems that were tested, glucose phosphate isomerase, malic enzyme and phosphoglucose dehydrogenase proved to be the most promising. The electrophoresis of lysates, prepared from oocysts, in an agarose gel system was found to give adequate and reproducible resolution of isoenzyme patterns. Isoenzyme polymorphism could be demonstrated in oocysts harvested from the stools of four children. Such polymorphism has not been described previously and indicates a more extensive study to investigate strain differences, and to correlate these with the clinical histories of infected subjects. This approach may be invaluable in elucidating the pathogenesis of Cryptosporidium infections in man.

**SUPPORTING SERVICES**

In this research the statistical analyses have been done with the support of the Institute for Biostatistics of the Medical Research Council.

## PREFACE

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

The research described in this dissertation was carried out in the Research Institute for Diseases in a Tropical Environment, of the South African Medical Research Council under the supervision of Dr TFHG Jackson.

### ACKNOWLEDGEMENTS

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

Dr TFHG Jackson, supervisor, Senior Specialist Scientist at the Research Institute for Diseases in a Tropical Environment (RIDTE), Durban, for his expert guidance and constructive criticism.

Prof J van den Ende, Head of the Department of Medical Microbiology, University of Natal, and Dr V Gathiram, Department of Medicine, for their valuable advice and professional criticisms.

The Superintendent, King Edward VIII Hospital, Congella, for permission to collect stool specimens from patients at the hospital.

Dr D F Wittenberg, Senior Lecturer at the Department of Paediatrics and Child Health, Faculty of Medicine, for his professional advice.

The technical staff at the Electron Microscopy Unit, Faculty of Medicine, University of Natal, for their efficient assistance.

Messrs S Suparsad and A Saikoolal for their invaluable support and assistance in iso-enzyme electrophoresis and animal experiments respectively.

Finally, my profound gratitude to my husband, Colin, my Mum and late Dad for their constant encouragement and devotion.



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## CHAPTER 1

### INTRODUCTION

Cryptosporidiosis is a diarrhoeal disease on which increasing interest has been focused following the discovery of its association with diarrhoea in patients with the acquired immunodeficiency syndrome (AIDS). Unlike other opportunistic parasites such as Pneumocystis carinii and the unknown causative agent of Kaposi's Sarcoma, Cryptosporidium is known to also infect immunocompetent individuals in whom it can produce debilitating diarrhoea which may even be fatal (Holley and Dover, 1986; Stehr-Green et al, 1987; Macfarlane and Horner-Bryce, 1987). Although previously overlooked by the medical profession, cryptosporidiosis has now been established as an important infectious disease particularly as it is found in both developing and developed countries.

#### History

The genus Cryptosporidium, a zoonotic protozoan parasite, was first described by Tyzzer in 1907 (Tyzzer, 1907), and subsequently considered in greater detail in 1910 (Tyzzer, 1910). The association of Cryptosporidium with diarrhoea in domestic animals was only recognised in 1971 (Panciera et al, 1971), and the first case of cryptosporidiosis in man was reported in 1976 (Nime et al, 1976).



### Taxonomy and Morphology

Cryptosporidium, is considered to be related to the more widely recognised coccidian parasites, Toxoplasma, Isospora and Sarcocystis. It was previously thought to be comprised of twenty species named according to the various vertebrate hosts in which the parasite was found. However, more recently, Upton and Current (1985) concluded that only two species infecting mammals viz. C. muris and C. parvum were valid. The infective stage, or oocyst of C. muris was described both by Tyzzer (1907) and Upton and Current (1985) as being ovoid (7,4 x 5,6µm), containing four vermiform sporozoites and a residuum composed of numerous granules. The residuum, the remains of sporulation, is a refractile organelle from which the sporozoites have proliferated. C. parvum oocysts are smaller (5,0 x 4,5µm) but have a similar morphology.

### Clinical Features

C. parvum, the species known to be infective to man, has been observed in both immunocompromised (Jonas et al, 1983) and immunocompetent (Stehr-Green et al, 1987) individuals. In the former, it has been associated with profuse, usually chronic, diarrhoea which in the case of AIDS victims proved to be fatal. However in immunologically intact patients, the diarrhoea is usually self-limiting and of a short duration but has recently been found to give rise to life-threatening disease in a small (3,5%) proportion (Macfarlane and Horner-Bryce, 1987). On the contrary, certain individuals harbouring Cryptosporidium have also been

reported as being asymptomatic (Zar et al, 1985; Current et al, 1983; Berkowitz et al, 1988).

Ultrastructural studies have shown that intestinal infection is associated with loss or shortening of microvilli, resulting in impaired absorption. The mechanism by which the organism induces diarrhoea has not been established. However, with the use of light and electron microscopy (Bird and Smith, 1980), the exact location of host-parasite interaction was found to be the microvillous border of intestinal epithelial cells. Additionally the parasite is also responsible for limited superficial epithelial damage as a result of parasite penetration.

Cryptosporidium has also been reported to infect the oesophagus (Kazlow et al, 1986), and bronchi of humans (Miller et al, 1984; Kibbler et al, 1987) and uterus (Liebler et al, 1986) of experimentally infected animals. In severely infected patients fever, weight loss and malaise have been reported (Current et al, 1983; Wolfson et al, 1985). Asymptomatic infections have also been described in recent surveys (Holten-Anderson et al, 1984; Holley and Dover, 1986; Lahdevirta et al, 1987).

#### Transmission and Sources of Infection

Cryptosporidiosis was originally believed to be a zoonosis (Reese et al, 1982; Angus, 1983), but person-to-person transmission of Cryptosporidium oocysts has been reported. Cross infections in hospitals (Baxby et al, 1983) and day-care centres (Rahaman et al,

1985) have been reported. Since infection is transmitted via the faecal-oral route, personal hygiene and the behavioural characteristics of infected and susceptible persons play an important role in transmission. Direct person to person transmission has also been described on dairy farms (Ongerth and Stibbs, 1987) where the families of Cryptosporidium infected animal attendants were shown to harbour the parasite as well.

Water has recently been implicated as another source of cryptosporidial infection. The pollution of water by oocysts has been attributed to direct contamination by sewage, septic tank leakage and recreational bathing (Madore et al, 1987). Unhygienic food handling practices are another potential cause of Cryptosporidium infection (Hunt et al, 1984; Mann et al, 1986).

### Treatment

Various drugs have been appraised for the treatment of cryptosporidiosis; none has proved successful. The current recommended therapy is symptomatic and depends mainly on nutritional support and rehydration. Spiramycin, which has an antimicrobial spectrum similar to erythromycin, was shown to produce significant clinical improvement in some patients; however complete elimination of the parasite from the intestine was not achieved (Portnoy et al, 1984; Pilla et al, 1987). Somatostatin was recently reported to inhibit secretory diarrhoea in a Cryptosporidium infected AIDS patient. This is thought to be mediated by the drug's action on electrolyte absorption in the

small bowel (Cook et al, 1985). However the efficiency of somatostatin in long-term treatment has not been investigated and the cost will undoubtedly prohibit its widespread use.

#### STUDY OBJECTIVES

Recent reports of Cryptosporidium infections in Durban (Smith and van den Ende, 1986; Miller and van den Ende, 1987; Wittenberg et al, 1987) highlighted this as a newly recognised organism associated with diarrhoeal disease in young children. Subsequently other workers (Fripp and Bothma, 1987; Walters et al, 1988; Berkowitz et al, 1988) reported Cryptosporidium associated diarrhoea from other centres in South Africa.

The following objectives were identified in an attempt to extend the above-mentioned earlier work:

- (i) a reliable, rapid method was needed for parasite identification in stools to improve qualitative observations on this disease;
- (ii) a thorough hospital-based epidemiological study was needed to ascertain the importance of Cryptosporidium as a causative organism of diarrhoea;
- (iii) methods for maintenance of the parasite in culture by means of both in vitro and in vivo techniques were necessary for implementing biochemical and ultrastructural studies;
- (iv) as the parasite had been shown to occur in both symptomatic and asymptomatic subjects it was hypothesised that strain differences might be implicated; pilot studies, investigating

the feasibility of isoenzyme electrophoresis, as a potential technique for parasite characterisation, were undertaken.

## CHAPTER 2

## A COMPARATIVE ASSESSMENT OF COMMONLY EMPLOYED STAINING PROCEDURES FOR THE DIAGNOSIS OF CRYPTOSPORIDIOSIS

Since the description of Cryptosporidium as an aetiological agent in diarrhoeal illness in 1976 (Nime et al, 1976), the parasite has been described in both immunocompromised and immunocompetent individuals in most countries. The prevalence of cryptosporidiosis has been found to vary from 1% in Manitoba (USA) (Mann et al, 1986) to 18,4% in a recent survey carried out in the Republic of South Africa (Berkowitz et al, 1988). However it is difficult to compare different epidemiological results on cryptosporidiosis, since it has been pointed out that the rates of stool positivity may vary according to the diagnostic techniques employed (Bogaerts et al, 1984; Crawford and Vermund, 1988).

The more commonly employed procedures involve the demonstration of Cryptosporidium oocysts in stained faecal specimens and include the following techniques:- (i) acid-fast staining (Modified Ziehl-Neelsen (Henriksen and Pohlenz, 1981) and Kinyoun acid-fast (Garcia et al, 1983)); (ii) safranin-methylene blue (Malla et al, 1987); (iii) auramine-phenol fluorescence (Nichols and Thom, 1984) and direct immunofluorescence (Stibbs and Ongerth, 1986; Sterling and Arrowood, 1986); (iv) negative staining (Periodic acid-Schiff) (Horen, 1983); and (v) flotation (Sheather's sucrose flotation)

(Current et al, 1983). Cross et al (1985) used the Giemsa and the Ziehl-Neelsen stains on the same faecal specimens and found that the Giemsa stain failed to detect Cryptosporidium while 2,6% (19/735) of smears were positive using the modified Ziehl-Neelsen. Bogaerts et al (1984) compared the modified Ziehl-Neelsen method to the safranin-methylene blue method and found that they were equally specific with the safranin-methylene blue technique proving to be more sensitive. The latter technique was the first to be used for the diagnosis of cryptosporidiosis in Natal (Bush and Markus, 1986). Since Cryptosporidium oocysts have similar sizes (4-6µm in diameter), and shapes (round to spherical) to yeasts and other coccidia, diagnostic difficulties can be expected. Furthermore, Cryptosporidium oocysts have similar acid-fast properties to other coccidia, therefore making them indistinguishable when stained by the modified Ziehl-Neelsen method and other acid-fast staining techniques.

The four most commonly used procedures in South African clinical laboratories include the modified Ziehl-Neelsen (Henriksen and Pohlenz, 1981), safranin-methylene blue (Malla et al, 1987), auramine-phenol fluorescence (Nichols and Thom, 1984) and Sheather's flotation (Current et al, 1983) techniques. These were comparatively assessed for their relative efficacies in detecting Cryptosporidium oocysts in diarrhoeal stools with the aim of deciding on the most suitable technique for routine use locally.

## 2.1 MATERIALS AND METHODS

Stool specimens were collected from 93 children with diarrhoea admitted to King Edward VIII Hospital, Durban. Each specimen was examined for Cryptosporidium oocysts using the four above-mentioned techniques. A description of each of these is detailed below:

### 2.1.1 Modified Ziehl-Neelsen method

In this method thin faecal smears were first air-dried and heat-fixed by briefly passing through a flame and then stained with concentrated carbolfuchsin (E & O Reagents, Durban) for ten minutes at room temperature. This step was followed by decolourisation in 10% sulphuric acid until the background was free of excess stain and thereafter counterstained with 1% methylene blue for 1 minute. Stained preparations were examined by light microscopy at 400x magnification. This technique was completed in 15 minutes.

### 2.1.2 Safranin-methylene blue method

Heat-fixed thin faecal smears were stained in 1% aqueous safranin by heating over a flame to the point of simmering (approximately 5 minutes) followed by a brief wash in water to remove excess stain. Subsequently slides were counterstained in 1% methylene blue for 30 -60 seconds and rinsed quickly in tap water. Stained smears were air-dried and examined microscopically as above. Staining was completed in 8 minutes.



### 2.1.3 Auramine-phenol fluorescence method

Thin faecal smears were fixed in methanol for 5 minutes and stained in auramine-phenol (auramine-O 0,03g, phenol 3g and distilled water 100ml) for 10 minutes at room temperature. This was followed by quick rinses in tap water and decolourisation in a 3% hydrochloric acid-ethanol mixture for 5 minutes. The smears were again rinsed in water and counter-stained with 0,1% potassium permanganate for 30 seconds. Stained slides were air-dried and examined with a fluorescence microscope (Zeiss, West Germany) at 400x magnification. Total preparation time was 20 minutes.

### 2.1.4 Sheather's flotation method

A modification of Sheather's flotation method was developed for screening diarrhoeal stools. Previous personal experience with 40 Cryptosporidium positive diarrhoeal stools indicated that the original technique described by Current et al (1983) and a modification thereof (author's original modification) were equally effective in the detection of Cryptosporidium oocysts in diarrhoeal stools. Furthermore, it was also noted that the modified technique would only be equally effective if the mucoid portion of the stool is used. Since this modified Sheather's flotation method is directly comparable to the other three techniques in terms of volume of diarrhoeal stool screened, it was used in this study.

However, since the original technique involves concentration of the stool contents, both this and the modified techniques were compared on formed stools. This would define a more accurate method for screening formed stools.

Sheather's solution was prepared by dissolving 500g sucrose and 6,5g phenol in 320ml distilled water. The original Sheather's flotation method involved the concentration of oocysts by centrifugation of stools in Sheather's solution at 500 x g for 5 minutes; with the use of a wire-loop the buoyant oocysts are removed from the surface, placed on a slide, covered with a coverslip and examined microscopically at 400x magnification. In the author's modification a small quantity of faeces (roughly equivalent to that used to make a faecal smear) was first emulsified in a drop of Sheather's solution on a slide by use of a wooden applicator stick. The preparation was mounted directly on a slide, covered with coverslip and examined by bright field microscopy at 400x magnification. Total preparation time was 2 minutes.

An ideal technique is one that is 100% sensitive and 100% specific. In practice this does not occur, however the best technique in terms of sensitivity and specificity may well be used as a gold standard. In this study the sensitivities and specificities of the modified Ziehl-Neelsen, auramine-phenol fluorescence and safranin-methylene blue techniques were compared to the modified Sheather's flotation method. The specificity and sensitivity was calculated as follows:-

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

## 2.2 RESULTS

Cryptosporidium oocysts measure 4 - 6µm in diameter and are spherical to slightly oval in shape.

### 2.2.1 Modified Ziehl-Neelsen method

The acid-fast Cryptosporidium oocysts stain red and the internal structures, which comprise four sporozoites and the residual bodies, are not clearly defined but stain more strongly than the oocyst wall (Plate 1). Yeasts, bacteria, faecal debris and other non-acidfast organisms fail to

retain carbolfuchsin in the decolourisation step and thus take up the counterstain and appear blue. However certain bacterial spores and other unidentified spherical organisms (Plate 2), which are also acid-fast, can easily be confused with Cryptosporidium oocysts.

#### 2.2.2 Safranin-methylene blue method

Cryptosporidium oocysts take up the safranin stain and appear reddish-orange while the faecal debris stains blue. However only a small proportion of oocysts stained uniformly while others stained weakly or not at all (Plate 3). Poor definition of structural details within the oocysts was noted with this technique.

#### 2.2.3 Auramine-phenol fluorescence method

With fluorescence microscopy Cryptosporidium oocysts were seen as bright yellow discs (Plate 4) surrounded by a pale halo; no characteristic structural features were visible. However non-specific staining of other organisms and faecal debris does occur and these could be mistaken for Cryptosporidium oocysts (Plate 5) by the inexperienced observer.

#### 2.2.4 Sheather's flotation method

When examined microscopically Cryptosporidium oocysts appear as pink, refractile, round to ovoid bodies containing one or two residual granules which appear green. Oocysts are

buoyant in Sheather's solution and are detected in higher planes of focus immediately beneath the coverslip, while non-refractile yeasts and other faecal debris are found in lower planes of focus (Plate 6). Although most coccidia are refractile in Sheather's solution (Plates 7 and 8), the characteristic morphology of Cryptosporidium oocysts (containing one or two residual bodies), shape (spherical) and size (4-6µm) allow them to be easily distinguished from other coccidia.

A comparative assessment indicated that after the modified Sheather's flotation technique, the Modified Ziehl-Neelsen technique was the next most sensitive technique in the detection of Cryptosporidium oocysts in diarrhoeal faeces, followed by safranin-methylene blue and auramine-phenol techniques respectively (Table I). However, the safranin-methylene blue technique proved to be more specific when compared to the other two.

#### **2.2.4.1 Comparative Assessment of the Original and Modified Sheather's Techniques in Screening Formed Stools**

On comparing both Sheather's techniques on 236 formed stools, 8 were positive for Cryptosporidium with the original method. However, the modified technique indicated Cryptosporidium oocysts in only 6 of the 8 stools. On multiple (9 x) random sampling of the 8 stools (3 heavy infestations, 5 light infestations) with the modified

technique, statistical analysis (Chi-square) (shown below) demonstrated that this technique would be just as accurate as the concentration technique in stools of heavy parasite infestation. However, in light infestations there would be a higher probability of reporting false negative results (p,0.001).

Statistical Analysis (Chi-square):

	Positive	Negative
Heavy Infestation	27	0
Light Infestation	14	31

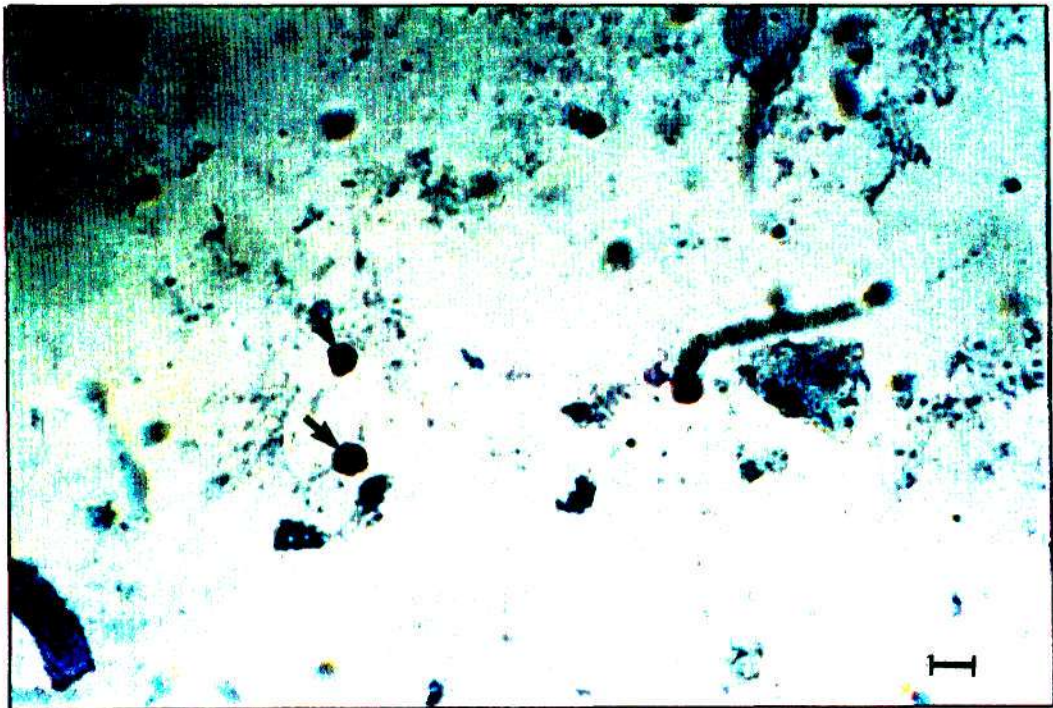
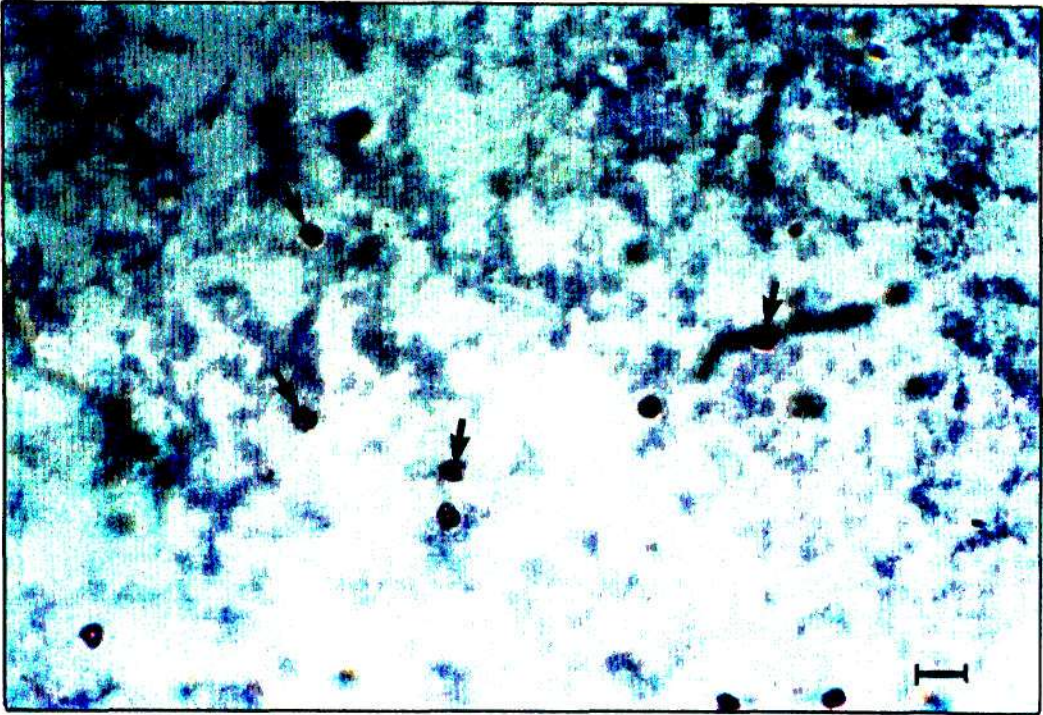
$$\begin{aligned}
 \chi^2 &= \frac{72 (837 - 0)^2}{27 \times 45 \times 41 \times 31} \\
 &= 32,66
 \end{aligned}$$

Therefore  $p < 0,001$ .

#### 2.2.5 General Observations

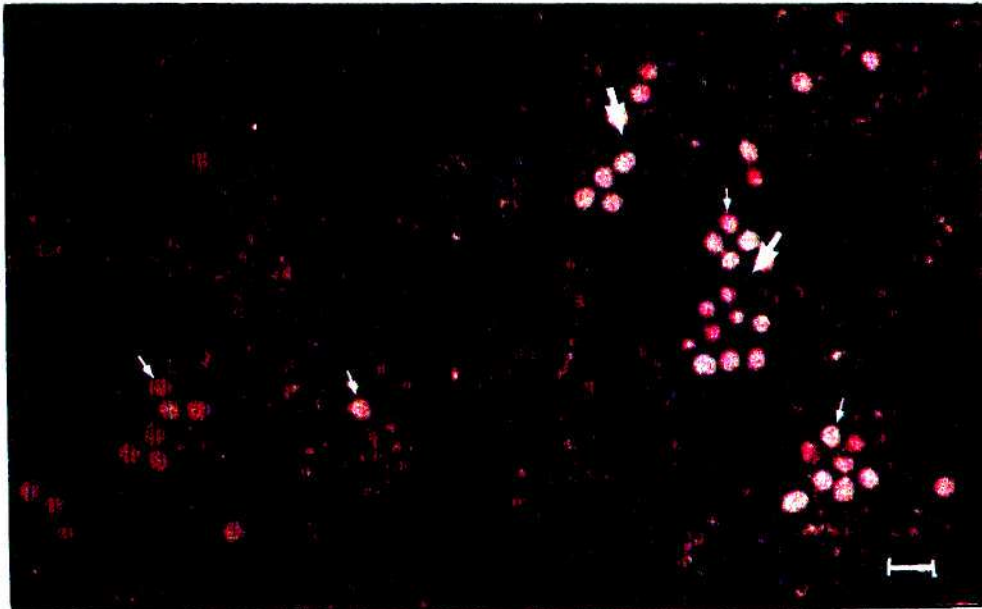
An observation made with all of the techniques employed was that a larger number of oocysts could be found in faecal smears made from the mucoid portion of diarrhoeal stool specimens. It is evident from Table I that the modification of the Sheather's flotation test detected more Cryptosporidium positive faecal smears and was the least time consuming.



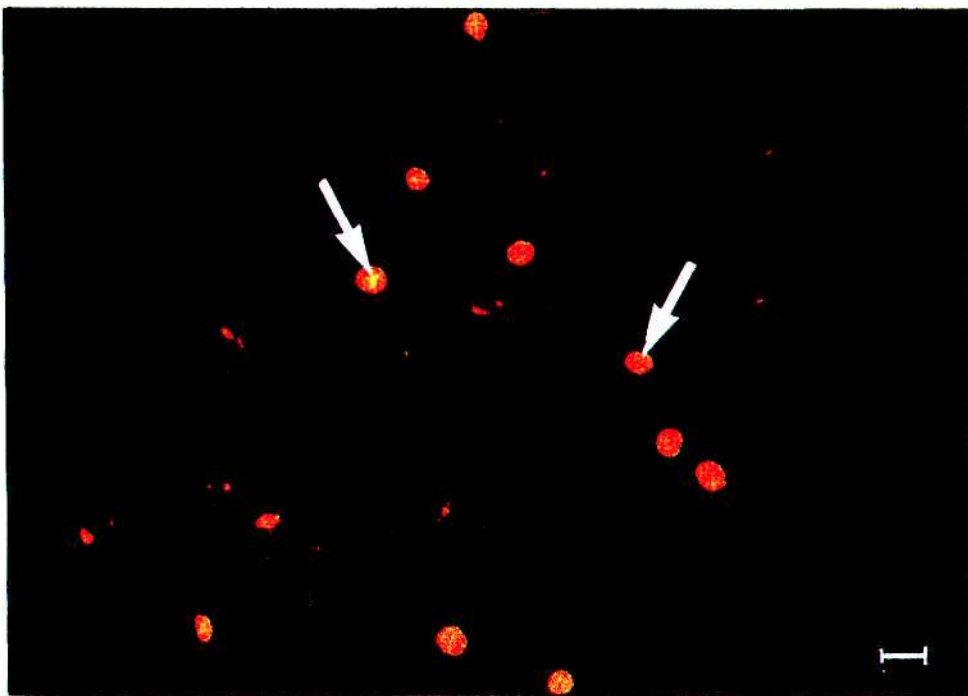
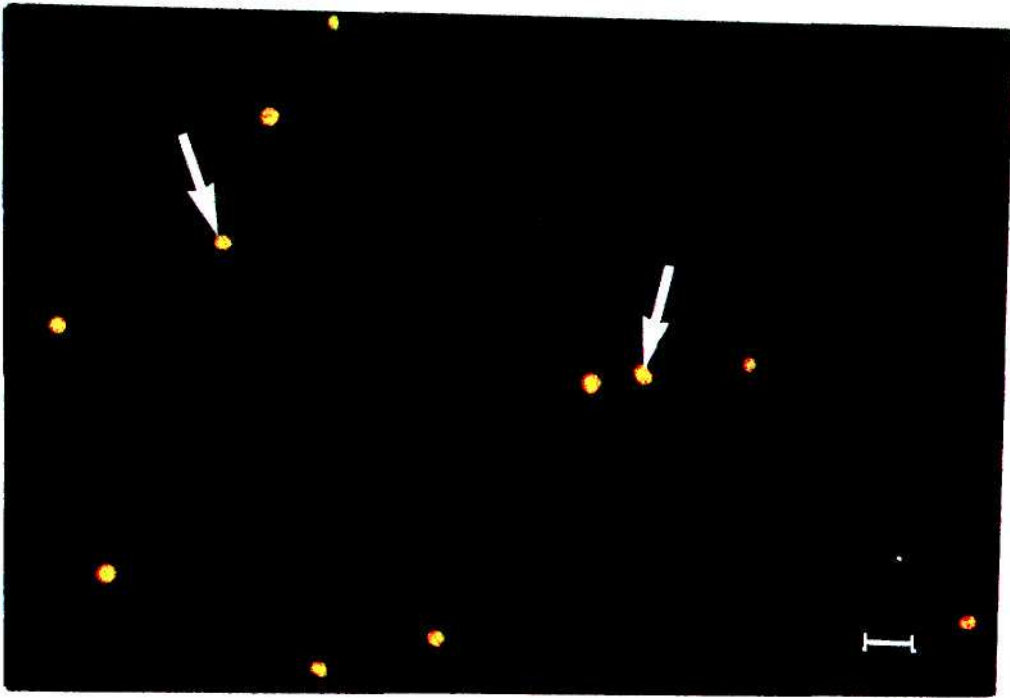














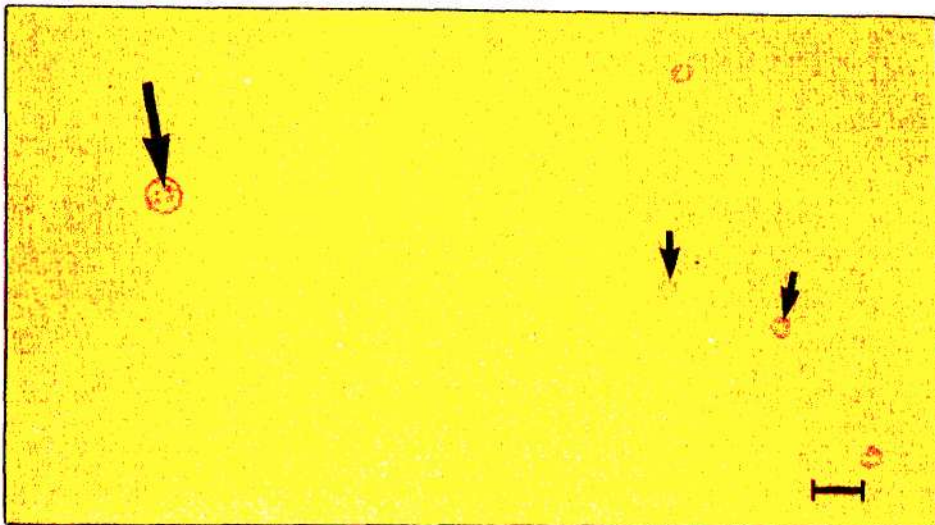
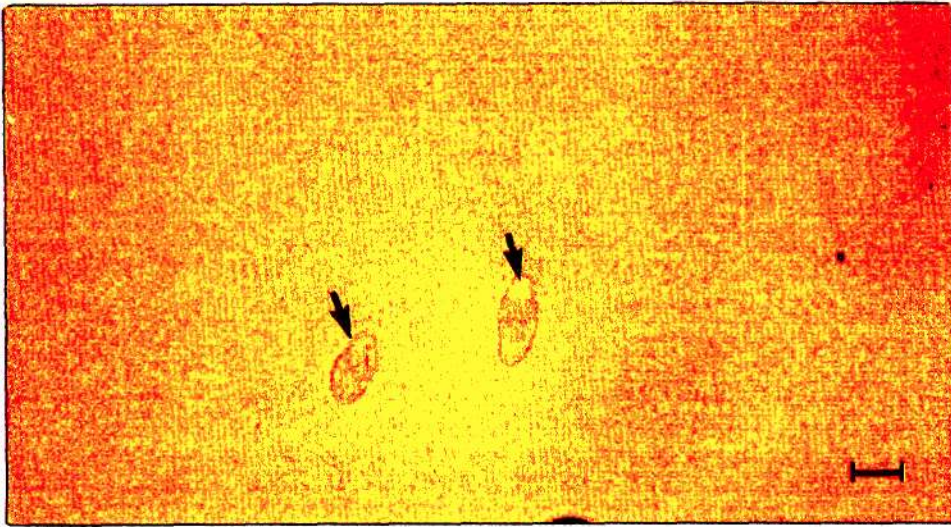
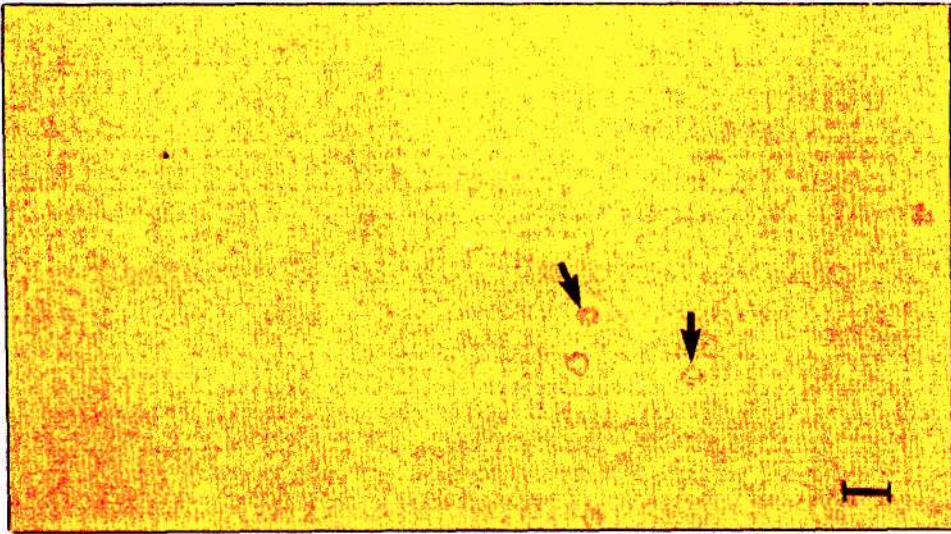


TABLE I: COMPARATIVE ANALYSIS OF THE FOUR STAINING TECHNIQUES FOR THE DETECTION OF CRYPTOSPORIDIUM IN 93 FAECAL SPECIMENS

	MOD.Z/N	SAFR/METH	AUR/PHENOL	MOD.SHEATH
No. positive	32	29	25	38
(%)	(34,4)	(31,2)	(26,9)	(40,9)
"False positive"	1	0	1	0
Preparation Time (min)	15	8	20	2
Sensitivity (%)	84.2	76.3	65.8	*
Specificity (%)	98.2	100	98.2	*

MOD.Z/N = MODIFIED ZIEHL NEELSEN TECHNIQUE

SAFR/METH = SAFRANIN METHYLENE BLUE TECHNIQUE

AUR/PHENOL = AURAMINE PHENOL FLUORESCENCE

MOD.SHEATH = MODIFIED SHEATHER'S SUCROSE FLOTATION

\* This technique was used as the "gold standard".

### 2.3 DISCUSSION

Using light microscopy, unstained Cryptosporidium oocysts are not readily detected and appear to be morphologically similar to the spores of yeasts and oocysts of other coccidia. Thus the differentiation of these organisms in standard or wet faecal smears is difficult. Since the original Sheather's flotation technique concentrates Cryptosporidium oocysts, this technique is more efficient, enabling oocysts to be more readily detected and identified which therefore makes it an appropriate gold standard. Only those techniques that approach this level of efficiency are worth employing. The screening test indicated that after the modified Sheather's method the modified Ziehl-Neelsen is the next most sensitive technique in the detection of Cryptosporidium oocysts in diarrhoeal faeces (Table I).

In this study the modified Sheather's flotation procedure proved to be superior to the other staining techniques assessed for the detection of Cryptosporidium oocysts (Table I). The advantages of the Sheather's flotation technique over the modified Ziehl-Neelsen, safranin-methylene blue and auramine-phenol fluorescence methods were, firstly, that it detected Cryptosporidium oocysts in the largest number of stool specimens, secondly, the reagents are cheap and easy to prepare and, lastly, the procedure is quick to perform. Thus, the modified Sheather's flotation technique is the most effective of the four smear-based methods described for screening



diarrhoeal stools. In studies which include asymptomatic (ie. non-diarrhoeic) persons, the use of the conventional Sheather's technique is strongly recommended as the oocysts are concentrated thereby amplifying the results and in addition definitive identification is possible. Furthermore it has been shown to be the best method for formed stools (Current et al, 1983).

Disadvantages of the modified Sheather's flotation technique are (i) that permanent preparations cannot be made, since oocysts are osmotically sensitive and tend to disintegrate in the sucrose solution; (ii) the refractile oocysts are best detected using 400x magnification and thus only a smaller field can be examined and (iii) although it is more effective than any of the other smear techniques described, it is not appropriate for screening formed stools.

Faecal specimens stored in 2,5% potassium dichromate solution for up to five months can be used with both the Sheather's techniques discussed when diagnostic difficulties arise (Current et al, 1986; author's personal experience).

Some authors (Anderson, 1981; Jokipii et al, 1983; Fripp and Bothma, 1987) report that smears stained by the modified Ziehl-Neelsen method are easy to interpret and therefore regard this method as reliable in their hands, because the acidfast Cryptosporidium oocysts stain strongly while yeasts and faecal

debris do not. Originally described by Henriksen and Pohlenz (1981), this is the most popularly used technique (Miller and van den Ende, 1987; Biggs et al, 1987; Reinthaler et al, 1987; Walters et al, 1988). However variability in carbolfuchsin uptake by Cryptosporidium oocysts and the loss of acidfast properties with time (Casemore et al, 1985; Collignon, 1987; Baxby et al, 1987) can lead to errors in the identification of the parasite. Non-specific staining of faecal debris and other micro-organisms may lead to diagnostic inaccuracy as has been reported with both the safranin-methylene blue (Bush and Markus, 1986; Jackson and Casemore, 1986; Cartensen et al, 1987) and auramine-phenol fluorescence (Angus, 1981; Ma and Soave, 1983) techniques. Furthermore the auramine-phenol fluorescence technique necessitates the use of a fluorescence microscope, a facility not present in many peripheral laboratories.

Direct fluorescent antibody tests (Stibbs and Ongerth, 1986; Sterling and Arrowood, 1986) have been developed that are reportedly sensitive as well as specific; the use of high quality monoclonal antibodies will undoubtedly further enhance the specificity and sensitivity of these tests. Unfortunately manufacturing costs are likely to make them too expensive for routine use.

With the increasing implication of Cryptosporidium in diarrhoeal diseases, it is expected that a greater burden will

be placed on microbiology laboratories for the detection and identification of this parasite. Consequently, as it is reliable, fast and cost-effective, the modified Sheather's flotation technique is recommended for screening loose stools. However, the conventional Sheather's flotation method should be used for community studies in which formed stools would also be examined.

## CHAPTER 3

SEASONAL VARIATION, AGE DISTRIBUTION AND DISEASE STATUS OF  
CRYPTOSPORIDIUM INFECTIONS IN CHILDREN IN DURBAN

Previously reported prevalences of Cryptosporidium infections at King Edward VIII Hospital (Durban) (Smith and Van den Ende, 1986; Miller and Van den Ende, 1987; Wittenberg et al, 1987) have varied between 3,2% and 15,4%. None of the reports nor any other study conducted in South Africa (Fripp and Bothma, 1987; Berkowitz et al, 1988; Walters et al, 1988) has fully appraised the occurrence of Cryptosporidium infections in relation to seasonal variation. Not many seasonal prevalence studies of Cryptosporidium have been published; and in those published infections were reported all year round with peak prevalence in the warmer, wetter months (Tzipori, et al, 1983; Current et al, 1983; Navin and Juranek, 1984; Rahaman et al, 1985).

Although studies of Cryptosporidium in other countries (Tzipori et al, 1983; Rahaman et al, 1985; Isaacs et al, 1985) have indicated the importance of the organism as a potential enteric pathogen in diarrhoeal illness in children, only one local study has addressed the importance of Cryptosporidium as an intestinal pathogen (Smith and Van den Ende, 1986).

The aims of the present study were therefore (a) to carry out a more detailed longitudinal investigation on the occurrence

of Cryptosporidium infections in children during a one year period with a view to monitoring any seasonal variations; (b) to determine whether or not this infection has any age-related prevalence and (c) to confirm the role of Cryptosporidium as a local causative agent of diarrhoeal illness.

### 3.1 PATIENTS AND METHODS

Observations were made over a one year period, from May 1987 to April 1988. The study population comprised 1229 children under the age of 10 years who were admitted to King Edward VIII Hospital with a primary diagnosis of gastroenteritis. A smaller in-patient group of 149 children within the same age group served as controls; none of these patients was suffering from diarrhoea. This hospital predominantly serves the Black community of the surrounding peri-urban and rural areas of Durban. A stool specimen was collected from each study patient within 24 hours of admission to hospital. Diarrhoeal specimens were screened for the presence of Cryptosporidium oocysts employing the author's modification of the Sheather's flotation technique (Chapter 2); this was previously established to be the most reliable method for detecting Cryptosporidium oocysts in diarrhoeal faeces (Chapter 2). Non-diarrhoeal stools were screened with the original Sheather's technique (Current *et al*, 1983) which was found to be more suitable in detecting Cryptosporidium oocysts since it involves a concentration step. All faecal specimens were also examined in the Department of

Medical Microbiology, King Edward VIII Hospital, for the presence of other enteric pathogens viz. pathogenic bacteria, protozoa, round worms and tapeworms with the exclusion of the viruses. The prevalence of cryptosporidiosis was then correlated with seasonal changes and age of infected individuals.

### 3.2 RESULTS

During the study period 9,0% (111/1229) of patients with diarrhoea were found to be passing Cryptosporidium oocysts as compared to only 3,4% (5/149) of the control patients. Table II summarises the number and range of recognised enteric pathogens detected in the study group. Cryptosporidium was the second most common enteric pathogen isolated. In the patients with Cryptosporidium infection, other potential enteric pathogens were detected in 31/111 (27,9%) (Table III) stools. Consequently, Cryptosporidium was the only potential pathogen identified in 80/1229 (6,5%) of patients. Other potential pathogens detected in the 124 controls, included enteropathogenic Escherichia coli (EPEC) (4), Shigella spp (5), Giardia lamblia (3) and Entamoeba histolytica (1).

Of the 111 children excreting Cryptosporidium oocysts in their stools, 96 (89,7%) were under 2 years of age; 65 (67,7%) were less than one year old (Table IV). Furthermore, the prevalence of Cryptosporidium infections in the latter group of children was found to be significantly ( $p = 0,001$ ; Fisher's Exact Test)

higher in the 4 - 6 months age group (Fig 1) than other age groups. The high prevalence in the 13 to 24 months age group in Fig 1 is not comparable to the other 3-month age groups since it covers a wider age range ie. 12 months. Other concomitant clinical diseases in patients with Cryptosporidium-associated diarrhoea included bronchopneumonia (10), kwashiorkor (8), marasmus (9), anaemia (2) and septicaemia (1).

Eleven (10%) of the 111 children infected with Cryptosporidium died. Autopsy findings indicated gastro-enteritis to be the cause of death in 7 of these patients. Cryptosporidium was the only enteric pathogen detected in 4. Other deaths were attributed to septicaemia, pneumonia and hypoxic encephalopathy.

Cryptosporidiosis occurred throughout the year and the monthly prevalence varied between 1,2% and 20,9% (Table V). Notably the prevalence of infection was higher during February, March, April and May (Fig 2). Rainfall data for the Durban area for the period January 1978 to December 1988 were obtained from the Weather Bureau, Pretoria and this was correlated with the monthly prevalence of cryptosporidiosis. Multiple linear regression analysis indicated a significant correlation between rainfall and prevalence of cryptosporidiosis ( $r = 0,6125$ ;  $p = 0,0342$ ). There was no correlation ( $r = 0,4305$ ;  $p = 0,1625$ ) between temperature and the incidence of Cryptosporidium infections.

TABLE II: FREQUENCY OF POTENTIAL INTESTINAL PATHOGENS IN DIARRHOEAL STOOLS FROM 1229 CHILDREN UNDER 10 YEARS OF AGE

<u>PATHOGENS</u>	<u>% FREQUENCY</u>	
<u>Salmonella</u> spp.	14,0	(172/1229)
<u>Shigella</u> spp.	2,7	(33/1229)
<u>Campylobacter</u> spp.	1,5	(18/1229)
<u>Escherichia coli</u> (Enteropathogenic)	3,0	(37/1229)
<u>Cryptosporidium</u>	9,0	(111/1229)
<u>Giardia lamblia</u>	1,4	(17/1229)
<u>Entamoeba histolytica</u>	0,2	(2/1229)

TABLE III : OTHER POTENTIAL ENTERIC PATHOGENS DETECTED IN STOOLS OF PATIENTS WITH CRYPTOSPORIDIUM-ASSOCIATED DIARRHOEA

<u>OTHER ORGANISMS DETECTED</u>	<u>NUMBER</u>
<u>BACTERIA</u>	
<u>Salmonella</u> spp.	22
<u>Shigella</u> spp.	4
<u>Escherichia coli</u> (EPEC)	2
<u>PROTOZOA</u>	
<u>Giardia lamblia</u>	2
<u>NO OTHER ENTERIC PATHOGENS</u>	80
<u>TOTAL</u>	111



TABLE IV: AGE DISTRIBUTION OF CRYPTOSPORIDIUM-POSITIVE CHILDREN UNDER 10 YEARS OF AGE WITH DIARRHOEA

AGE IN MONTHS	% POSITIVE
< 1	5,2 (3/58)
1-3	6,4 (10/157)
3-6	14,2 (31/218)
6-9	7,8 (12/154)
9-12	6,9 (9/130)
12-24	12,9 (31/241)
24-120	4,3 (15/255)
TOTAL	9 (111/1213)*

\* 16 subjects (none of whom were Cryptosporidium carriers) were excluded from this table as their exact ages were not known; they were all < 10 years of age.

TABLE V: RELATIONSHIP BETWEEN PREVALENCE OF CRYPTOSPORIDIOSIS IN CHILDREN UNDER 10 YEARS OF AGE AND AVERAGE RAINFALL AND TEMPERATURE FOR THE YEAR ENDING APRIL 1988

MONTH	RAINFALL (MM)	TEMPERATURE (oC)	% PREVALENCE
MAY	1215	21,8	20,9 (32/153)
JUN	544	18,6	8,2 (10/122)
JUL	620	18,8	1,8 (2/109)
AUG	272	19,6	6,1 (8/132)
SEPT	402	22,0	1,2 (1/83)
OCT	707	20,1	1,9 (1/53)
NOV	1139	21,7	2,3 (2/86)
DEC	1107	23,4	7,5 (5/67)
JAN	1148	26,6	6,0 (6/100)
FEB	997	27,1	11,9 (14/118)
MAR	1564	26,2	15,0 (21/140)
APR	1260	24,5	13,6 (9/66)
AVERAGE			9 % (111/1229)

Fig 1. PREVALENCE OF CRYPTOSPORIDIOSIS IN CHILDREN UNDER 10 YEARS OF AGE FOR THE PERIOD MAY 1987 TO APRIL 1988

% PREVALENCE

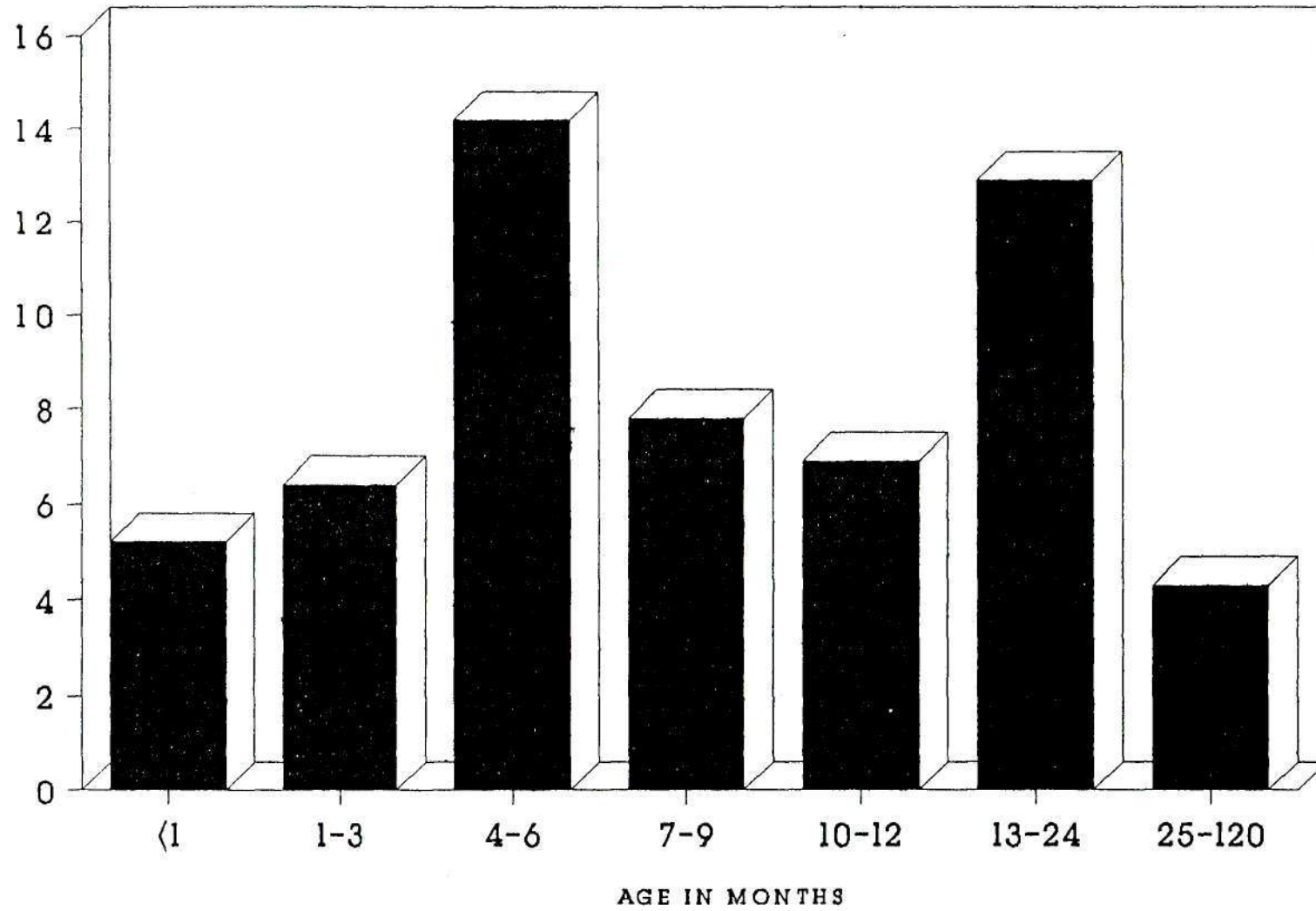
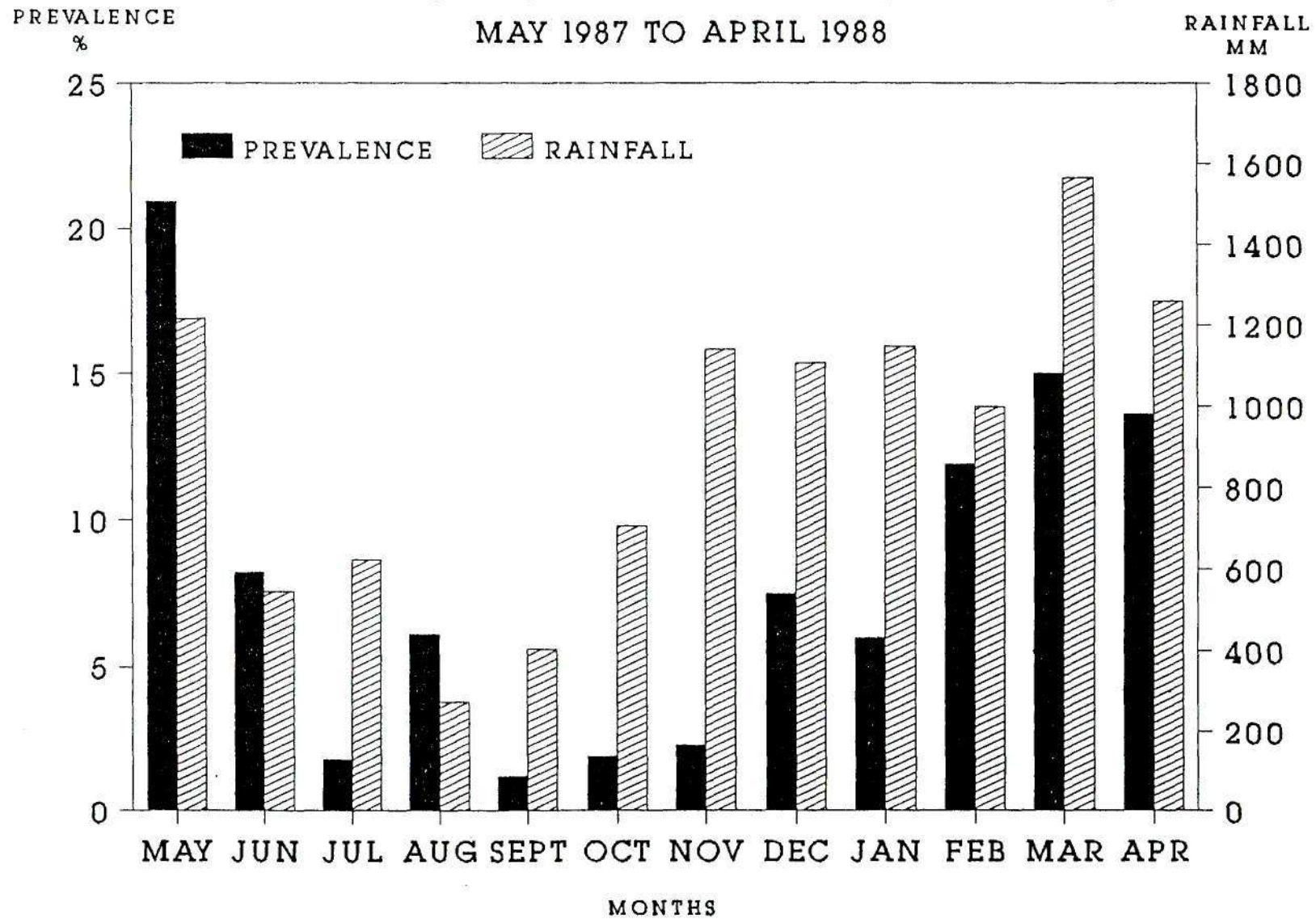


Fig 2. RELATIONSHIP BETWEEN PREVALENCE OF CRYPTOSPORIDIUM AND AVERAGE MONTHLY RAINFALL FOR THE PERIOD



### 3.3 DISCUSSION

Cryptosporidiosis was originally believed to be a zoonosis (Current *et al*, 1983; Rahaman *et al*, 1985) but recently transmission from person to person (Baxby *et al*, 1983) has been reported. The parasite is known to be primarily transmitted by the faecal-oral route; the poor sanitary conditions attributable to delayed urbanisation are therefore considered to contribute to the high frequency of Cryptosporidium infections documented for Third World countries (Crawford and Vermund, 1988) like Bangladesh (7,8%) (Rahaman *et al*, 1985), Liberia (7,8%) (Hojlyng *et al*, 1986), Sudan (6,1%) (Robinson *et al*, 1986) and Haiti (16,7%) (Pape *et al*, 1987) while the more developed countries such as Australia (2,5%) (Biggs *et al*, 1987), USA (0,1%) (Hamoudi *et al*, 1988) and Britain (3,2%) (Isaacs *et al*, 1985) have lower prevalences of the parasite. However, in South Africa the high prevalence of Cryptosporidium infections in Blacks (9,0% in the present study; 18,4% (Berkowitz *et al*, 1988); 11,9% (Wittenberg *et al*, 1987) was not unexpected since a large proportion of this population group lives in areas characteristic of Third World situations where proper sewage disposal systems and protected water supplies are deficient.

During a previous longitudinal survey of Cryptosporidium infections carried out at King Edward VIII Hospital (Miller and Van Den Ende, 1987), a lower overall prevalence of 3,2% was reported when compared to the results of the present

survey. We believe that the lower prevalence observed in the previous study was due to the following factors: 1) microscopical examination of faeces was done by several different observers during the study period whereas in the present investigation all specimens were examined personally by the author; 2) The Sheather's flotation technique was used throughout the present study; it has been shown to be superior to the modified Ziehl-Neelsen and auramine fluorescence methods used previously (Henriksen and Pohlenz, 1981; Nichols and Thom, 1984). Both of these methods produced non-specific staining, a feature which has also been reported by other workers (Angus, 1981; Ma and Soave, 1983; Casemore et al, 1985; Collignon, 1987; Baxby et al, 1987).

Seasonal variations in prevalence of Cryptosporidium infections have also been reported from Bangladesh (Shahid et al, 1987) and Australia (Tzipori et al, 1983). Like giardiasis, cryptosporidiosis is a waterborne disease (Ongerth and Stibbs, 1987) and in a recent study in Finland the parasite was associated with travellers diarrhoea due to the drinking of contaminated water (Jokipii et al, 1985). It is possible that contamination of rivers by storm-water run-off containing faeces of infected humans or animals resulted in the observed higher incidence of Cryptosporidium infections during the high rainfall months.

The observed high prevalence of Cryptosporidium infections in children under 2 years of age is consistent with the findings of other workers (Malla et al, 1987; Hojlyng et al, 1986; Robinson et al, 1986) including those of Smith and van den Ende (1986). This strongly implicates Cryptosporidium as an important cause of gastroenteritis in children, particularly those belonging to this age group. The lower prevalence of Cryptosporidium infections in neonates may be due to a decreased risk of exposure to infection since babies are carried on their mother's backs. Breast feeding is also known to protect infants against gastro-enteritis. Although concomitant infections with more than one enteric pathogen such as Salmonella spp and Shigella spp are likely to occur in patients with diarrhoea due to a common risk factor, a noteworthy observation was that Cryptosporidium was the only enteric pathogen isolated in 6,5% of children with diarrhoea. This strongly reinforces the view of others (Reinthaler et al, 1987; Heijbel et al, 1987) that Cryptosporidium is a causative agent of diarrhoea.

Interestingly, Salmonella and Shigella infections occurred more frequently in Cryptosporidium carriers (19,8%, 3,6% respectively) than in non-Cryptosporidium carriers (13,5%, 2,6% respectively). This has been anticipated since shigellosis and salmonellosis are also known to be strongly associated with poor sanitary practices.

A relatively high morbidity and mortality in patients with diarrhoea due to Cryptosporidium has been reported by Smith and van den Ende (1986) (22,6%) and Wittenberg et al (1987) (23%). The lower mortality (10%) associated with Cryptosporidium-related diarrhoea recorded in the present study is believed to reflect the recent overall improvement in mortality from gastroenteritis, resulting from improved management of these patients (Wittenberg - personal communication).

It is possible that the 3,4% prevalence of Cryptosporidium in the control subjects might reflect differences in virulence between strains; a situation which may be analogous to that reported in the case of Entamoeba histolytica where non-pathogenic zymodemes (strains determined by isoenzyme electrophoresis) can be isolated from individuals without symptoms of amoebiasis (Sargeant et al, 1982; Sargeant, 1985). Asymptomatic Cryptosporidium infections have also been documented previously in AIDS (Zar et al, 1985) as well as immunocompetent subjects (Current et al, 1983; Pohjola et al, 1986; Berkowitz et al, 1988; Walters et al, 1988). Although strain differences in Cryptosporidium have not yet been demonstrated by either western blotting (Mead et al, 1988a) or pulse-field gel electrophoresis of DNA (Mead et al, 1988b), an

isoenzyme study relating the different strains of the parasite to the clinical status of infected patients may provide information in this regard.



## CHAPTER 4

## IN VITRO AND IN VIVO DEVELOPMENT OF CRYPTOSPORIDIUM

Detailed studies of life cycle stages, antigenic structure, nutritional requirements, drug sensitivity and morphology of parasites can usually only be successfully accomplished after methods for culturing them have been established. Furthermore in vitro culture can be used to effectively amplify the available material, thereby providing adequate quantities for biochemical analysis. Successful in vitro culture of Cryptosporidium has recently been reported in human foetal lung cells (Current and Haynes, 1985), a human rectal cell line (Woodmansee and Pohlenz, 1983), as well as in chicken (Current and Long, 1985) and other avian embryos (Lindsay, 1988).

This chapter describes attempts to establish growth of Cryptosporidium in tissue culture using the techniques of Current and Long (1983) and Current and Haynes (1985). In addition, experimental infections of suckling Balb/c mice and immunosuppressed adult rats with human isolates of Cryptosporidium were also investigated to observe the ultrastructural features of the parasite in vivo.

## 4.1 MATERIALS AND METHODS

### 4.1.1 Preparation of Sporozoites from Oocysts

- (i) Faeces containing Cryptosporidium oocysts were collected from children with a primary diagnosis of diarrhoea, and stored in an equal volume of 2,5% potassium dichromate at 4°C for up to three months before use.
  
- (ii) Preserved faecal pellets were washed twice in phosphate buffered saline (PBS; 150mM sodium chloride, 2mM sodium dihydrogen phosphate-2-hydrate, 8mM di-sodium hydrogen orthophosphate; pH 7,2) by centrifugation at 500 x g for 10 minutes. As most of the faecal specimens received for culture were mucoid, they were suspended in 10% (wt/vol) potassium hydroxide in a 20ml centrifuge tube for 2 minutes and subsequently homogenised by vigorous shaking. The KOH-faecal suspension was then washed once in PBS by centrifugation at 650 x g for 5 minutes to sediment oocysts.
  
- (iii) Oocyst concentration:  
Cryptosporidium oocysts were resuspended in 5ml Sheather's solution (Chapter 2) in a 10 ml conical centrifuge tube and centrifuged at 450 x g for 5 minutes at 4°C. The oocysts were located in a film on the

surface of the resulting suspension from which they were harvested using a wire-loop. This step was carried out within ten minutes to avoid prolonged exposure to the hypertonic sucrose solution. They were resuspended in 2ml PBS and washed once by centrifugation at 650 x g for 5 minutes. As the sediment still contained faecal debris, it was resuspended in PBS and allowed to stand at room temperature for approximately 10 minutes to let the debris settle. The resulting supernatant containing oocysts was pretreated with 3% sodium hypochlorite in an ice-bath for 20 minutes followed by washing once in PBS. Overnight storage in 2ml PBS containing 5000 IU/ml penicillin, 5 mg/ml streptomycin and 50 ug/ml amphotericin at 37°C, eliminated any remaining bacterial or fungal contamination.

(iv) Excystment:

The purified Cryptosporidium oocysts were washed once in sterile PBS by centrifugation at 500 x g for 5 minutes in sterile 5ml test tubes and resuspended in 5 ml sterile PBS containing 0,5% trypsin and 1,5% sodium taurocholate. The solution was adjusted to pH 7.5 with 5 to 10 drops of 0.1N NaOH. Induction of excystment was accomplished by incubation in a 37°C water bath for 1 hour. Following excystment the sporozoites were washed

once in sterile PBS by centrifugation at 800 x g for 5 minutes at 4°C.

The following two in vitro culture techniques were assessed for their ability to produce adequate quantities of the various Cryptosporidium life-cycle stages for use in electrophoresis. Each culture system was used twice to confirm the reproducibility of observations.

#### 4.1.2 Culture in Chicken Embryos

##### (i) Infection:

Twenty-four 6-8 day old chicken embryos (Rainbow Chicken Farm, Hammarsdale) were incubated at 30°C. Each embryo was inoculated with 80 000 - 100 000 sporozoites via the chorio-allantoic route using a 1ml syringe fitted with an 18 gauge needle. Inoculated embryos were incubated at 37°C for eight days.

##### (ii) Harvest:

Eight days after inoculation, the chorio-allantoic membrane from each of the embryos was removed and preserved in formalin; these were subsequently examined by means of phase contrast microscopy. Nomarski interference microscopy apparently provides better results (Current and Haynes, 1985) than phase contrast but unfortunately interference optics were not available. The allantoic fluid was extracted and

centrifuged at 800 x g for 5 minutes. The sediment was subsequently examined for oocysts using Sheather's technique (Chapter 2).

#### 4.1.3 Culture in Human Foetal Lung Cells

##### (i) Infection and Maintenance:

Human foetal lung cells (Flow 2002 - Highveld Biological, Sandton, Republic of South Africa) of finite life (cells capable of being passaged up to 15 to 18 times) were grown on coverslips in 2ml of Eagle's Minimum Essential Medium (MEM) with Earle's Salts in 10ml Leighton tubes. Each tube contained two such coverslips. The growth medium was supplemented with 10% foetal calf serum, penicillin (100IU/ml), streptomycin (100ug/ml), and amphotericin (0,25ug/ml). Approximately 200 000 motile Cryptosporidium sporozoites were suspended in 1ml Growth Medium (MEM with 10% foetal calf serum) and inoculated into each of ten tubes of confluent fibroblasts under sterile conditions. Inoculated cultures were gassed with 5% CO<sub>2</sub> (95% air) and incubated at 37°C for 4 hours to allow for sporozoite penetration. Growth medium was removed 4 hours after inoculation and cells were rinsed vigorously in Maintenance Medium (MEM with 2% foetal calf serum). Infected cells were maintained in Maintenance Medium at 37°C in a 5% CO<sub>2</sub> atmosphere.

(ii) Phase Contrast Microscopy:

On the first, fourth and eight days following inoculation a coverslip was removed from the culture tube, rinsed twice in PBS and fixed in Bouin's fixative for at least 1 hour. After removal of the coverslips the tubes were retained for eventual parasite harvesting as infected cells continued growing on the remaining coverslip and the sidewalls of culture vessels. Coverslips containing fixed monolayers of infected cells were mounted on clean microscope slides and examined by means of phase contrast microscopy.

(iii) Harvest:

Eight days after inoculation, the culture medium in each tube was removed and centrifuged at 600 x g for 5 minutes. The pellet was examined for Cryptosporidium oocysts. Cells adhering to sidewalls of the culture vessels were rinsed once with PBS and dislodged with 0.25% trypsin in PBS to dislodge them. The freed cells were then rinsed in PBS by centrifugation at 550 x g for 5 minutes. Infected fibroblasts were incubated in 2.5% potassium dichromate at 4°C for 3 hours to kill all developmental stages besides oocysts.

#### 4.1.4 In Vivo Study of Cryptosporidium in Experimental Animals

(i) Infection of Balb/c Mice:

Three groups of four 4-day old suckling Balb/c mice were housed with their mothers in separate cages. They were caged in isolation in a room outside the animal facility. Using a 90 gauge catheter, two of the three groups were inoculated per os with approximately 150000 viable Cryptosporidium oocysts. The remaining group served as controls. Six, seven and eight days after inoculation, faecal pellets were examined for Cryptosporidium oocysts, using the original Sheather's flotation technique.

On the eighth day after inoculation all the mice were sacrificed and the caecal contents and mucosal scrapings of the small intestine were removed at autopsy and examined for Cryptosporidium oocysts using the modified Sheathers technique.

(ii) Immunosuppressive Therapy:

Ten adult male Wistar rats weighing between 200g and 260g were obtained from the breeding unit at the Natal Institute of Immunology, Pinetown. They were caged in pairs in isolation from the other animals in the animal facility to prevent cross-contamination. Prior to starting the study their faeces were checked for

parasites including Cryptosporidium. The daily water consumption of each rat was monitored for 3 days prior to the immunosuppressive therapy as drug levels are calculated on this basis (Rehg *et al*, 1987). Each animal was administered cyclophosphamide (Endoxan, Bristol - Meyers, UK) in its drinking water at a dose of 50 mg/kg body weight per day, for 14 days prior to Cryptosporidium inoculation. This immunosuppressive therapy was followed by infecting each rat orally with 150 000 - 200 000 Cryptosporidium oocysts suspended in 1 ml PBS.

(iii) Faecal Examination:

Faecal pellets from all the animals were examined for Cryptosporidium oocysts daily after inoculation, using the original Sheather's flotation technique. To prevent contamination of the previous day's faecal deposits, the bedding was renewed each morning. On the seventh day following inoculation, each rat was killed under anaesthesia and autopsied. Fine segments of the posterior 2 cm of the ileum were removed at autopsy and processed for transmission electron microscopy using standard procedures recommended by Weakley (1981).



## 4.2 RESULTS

### 4.2.1 In Vitro Culture of Cryptosporidium

#### Oocyst Purification

The number of oocysts (Plate 9) isolated from human stools ranged from 500 000 to 1,3 million per ml; this provided sufficient material for in vitro and in vivo culture. It was also found that prolonged storage in potassium dichromate inhibited bacterial growth, while not apparently affecting excystment.

#### Excystment

Incubation in the bile salt-trypsin solution always resulted in more than 70 % excystment of Cryptosporidium oocysts. Sporozoites, which exhibit twisting and flexing movements, are comma-shaped; they are 5-6 um in length which tapers anteriorly and has a characteristic rounded posterior end (Plate 10). The following sequential stages were observed during the excystment process.

1. Fully sporulated oocyst (Plate 11).
2. Sporozoite escaping through a single opening in the oocyst wall (Plate 12).
3. After the release of all four sporozoites, oocyst with remaining residual body (Plate 13).
4. Oocyst wall remaining after the expulsion of the residuum (Plate 14).

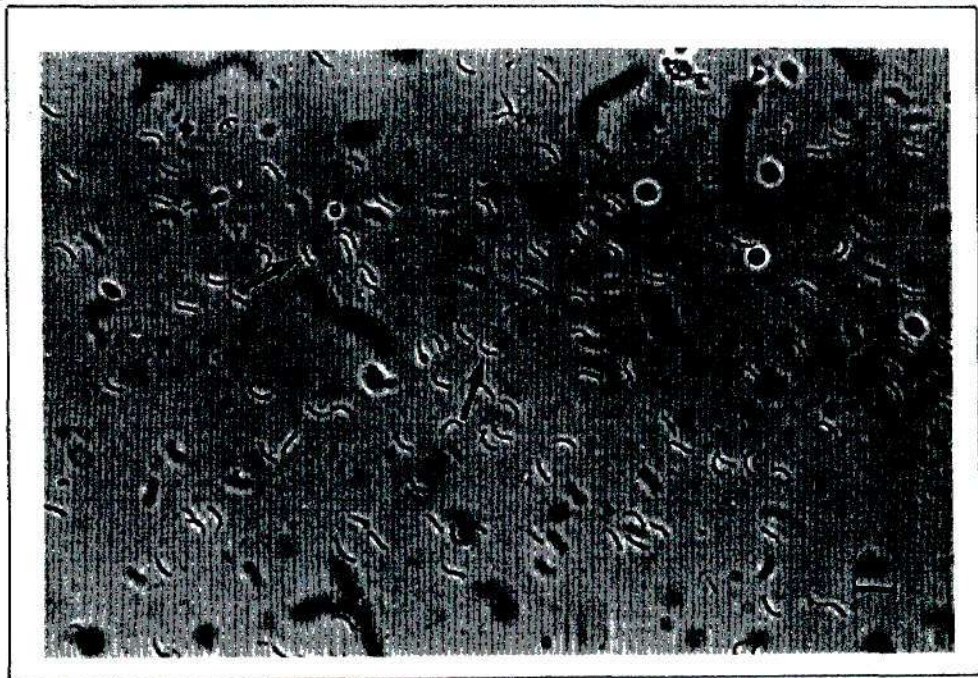
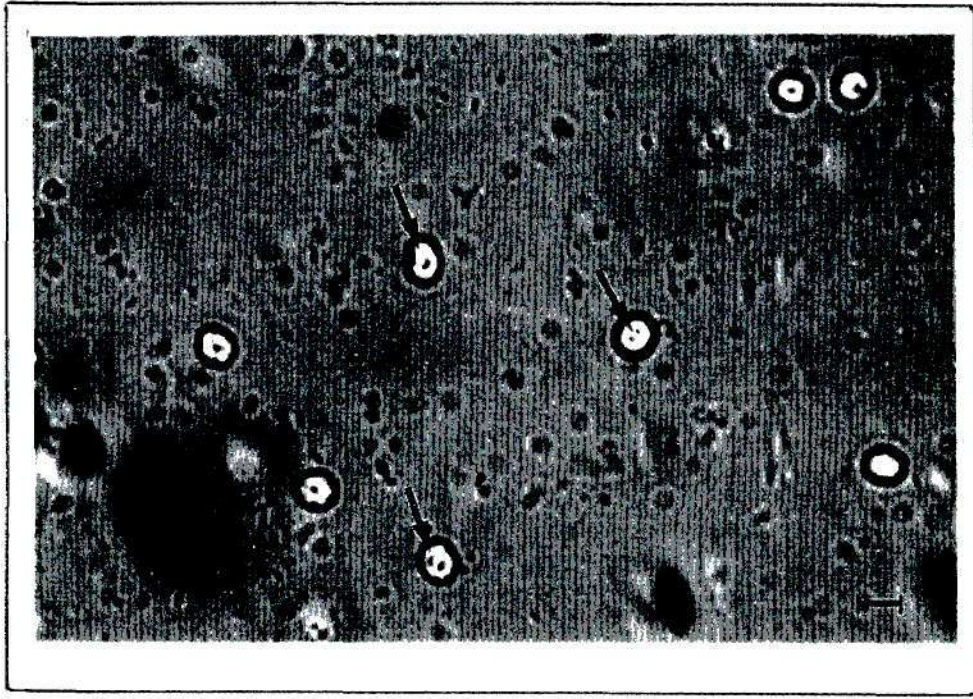
#### 4.2.2 Culture in Chicken Embryos

Infection: Of the 24 embryos inoculated with Cryptosporidium only 5 were sparsely infected.

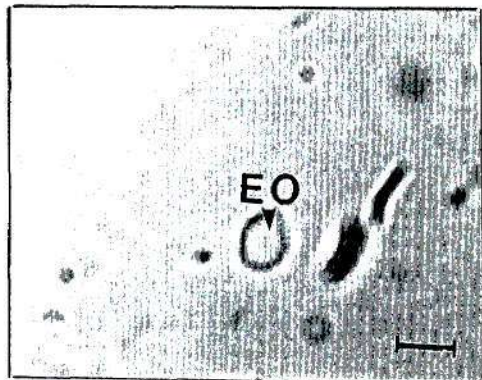
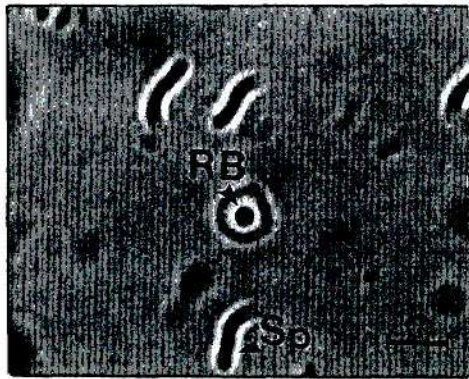
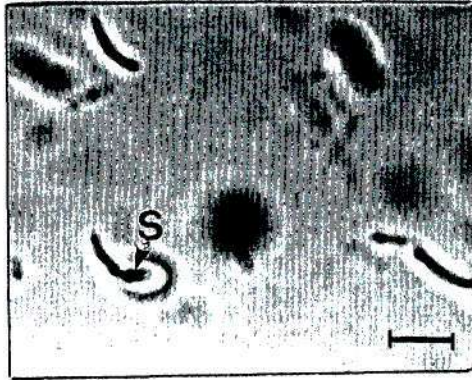
Microscopy: Examination of sedimented allantoic fluid showed very few oocysts and no other intermediate stages. However microscopy performed on the chorio-allantoic membrane demonstrated a moderate Cryptosporidium infection in certain areas of the membrane. Due to the thickness of the membrane, and the non-availability of Nomarsky interference optics, the developmental stages could not be differentiated. Most oocysts were found clustered in groups of 6 or 8 (Plate 15). Developmental stages ranged in size from 3 - 6um in diameter.

Harvest: The drawback to this method of cultivation is that it is not suitable for harvesting a functional (ie. capable of producing an infection) concentration of Cryptosporidium oocysts from the membrane or the allantoic fluid. Only a small proportion of oocysts are shed into the allantoic cavity. Treatment with trypsin failed to disintegrate the membrane sufficiently to release Cryptosporidium oocysts.

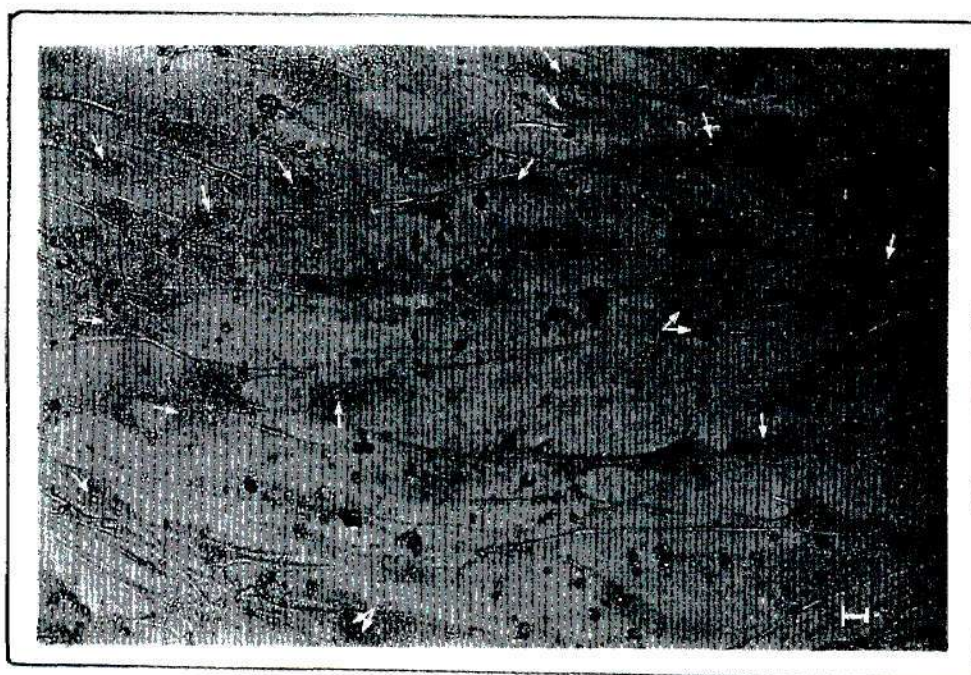
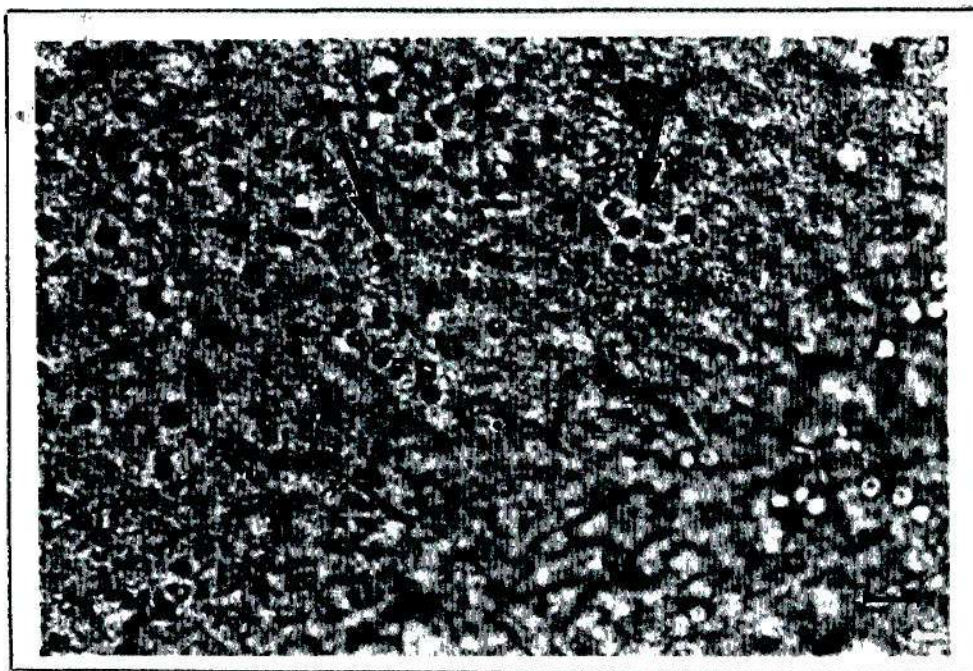














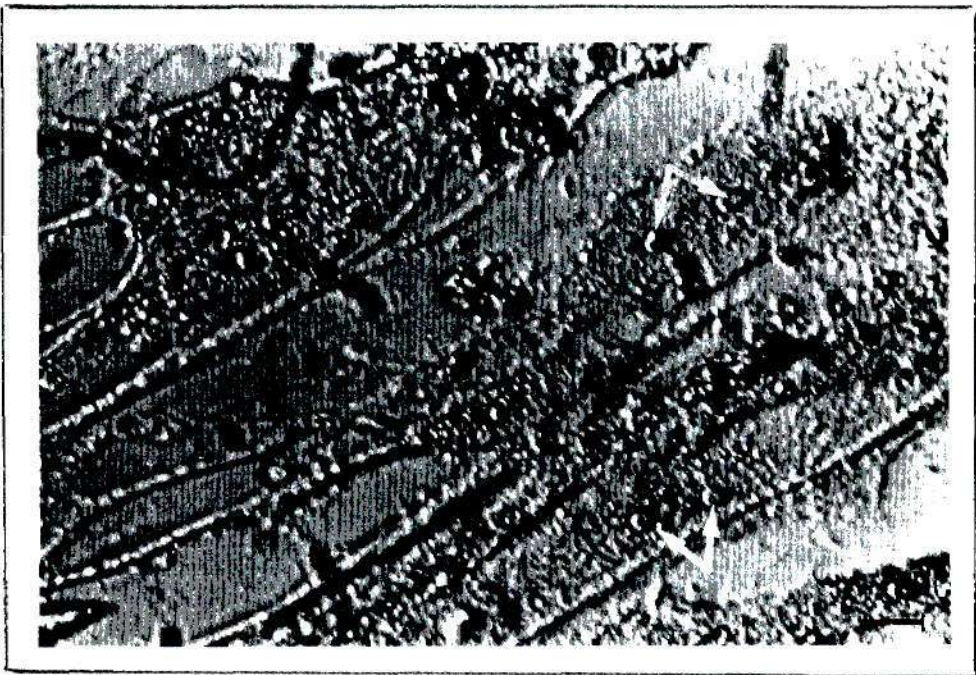
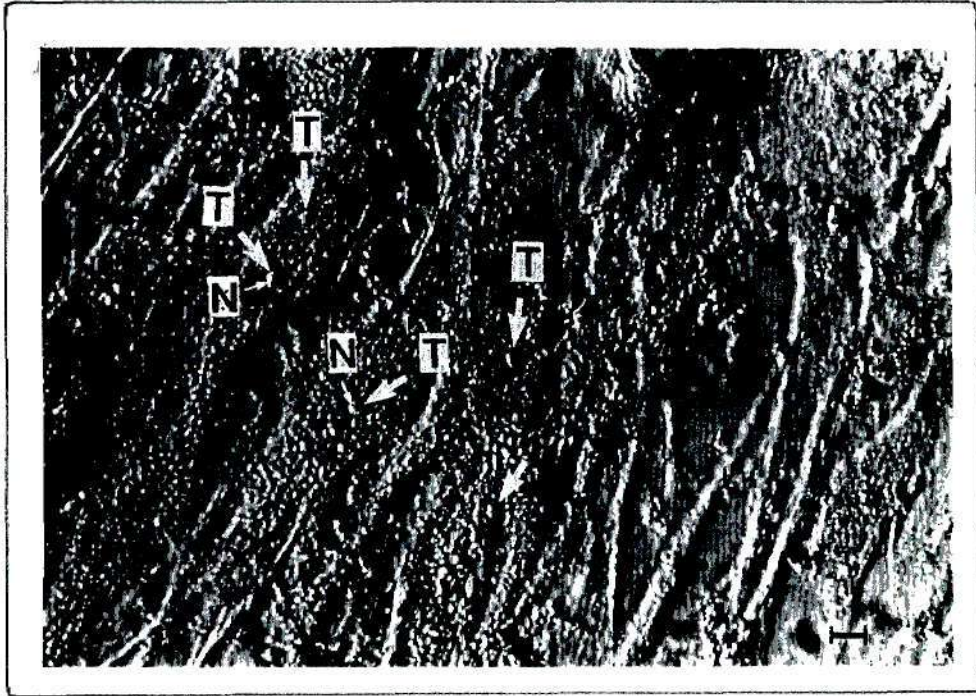
These initial culture attempts were made in chicken embryos as this seemed to be a convenient, bacteria free host that is easily maintained. However as adequate yields of cultured Cryptosporidium oocysts were unobtainable, human foetal lung cells were subsequently assessed as an alternative substrate.

#### 4.2.3 Development in Human Foetal Lung Cells

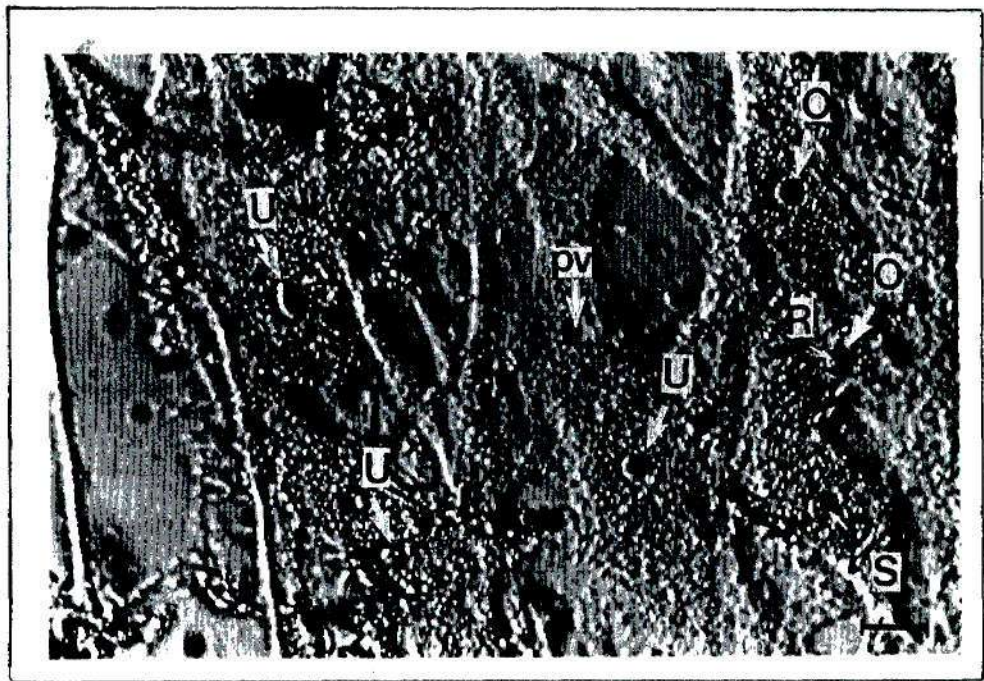
Phase Contrast Microscopy: Twenty-four hours after inoculation more than 50% of the fibroblasts (Plate 16) were infected with developmental stages of Cryptosporidium. At a higher magnification (400x) (Plate 17) these developmental stages had the characteristic morphology of trophozoites; these were spherical, 3-4 um in diameter and had an eccentric nucleus. On examination four days after inoculation the number of developmental stages infecting the fibroblasts had almost doubled (Table VI) and two or three organisms could be seen infecting each fibroblast (Plate 18). Eight days after inoculation most of the developmental stages were clearly oocysts with prominent residual bodies. Only a small proportion of them were released into the culture medium leaving behind crater-like depressions viz. parasitophorous vacuoles (Plate 19).











## .2.4 Experimental Animal Cryptosporidiosis

### Infection of Balb/c Mice

None of the inoculated mice shed Cryptosporidium oocysts in their faeces. Examination of mucosal scrapings of selected areas of the small intestine ruled out any possibility of an infection. After repeated attempts to infect suckling mice, immunosuppressed adult rats were used as alternative experimental hosts.

### Infection of Immunosuppressed Rats

Of the ten rats inoculated, three did not survive the cyclophosphamide treatment; one died on the 4th day of treatment while the others died on the 5th day. Post-mortem examination of caecal contents and ileal surface showed no signs of Cryptosporidium infection. However the mucosal lining of the small bowel exhibited marked surface destruction with villous distortion presumably due to cyclophosphamide treatment.

On the 7th day after inoculation four (4/7) of the remaining immunosuppressed rats were found to be excreting Cryptosporidium oocysts in their faeces; at autopsy, in addition to villous atrophy in the ileum, oocysts were observed in the caecal contents of these four animals. None of the rats developed diarrhoea stools.

### Transmission Electron Microscopy

Using conventional light microscopy (x 400 magnification) ten 1 um thick sections of intact ileal mucosa were selected from the four infected animals; these were used for transmission electron microscopy. Four of the ten ileal sections examined, contained various developmental stages of Cryptosporidium. Ultrastructural features described by Bird and Smith (1980), Angus (1983), Liebler et al (1986), Current and Reese (1986) and Uni et al (1987) were used to confirm the findings described in this study.

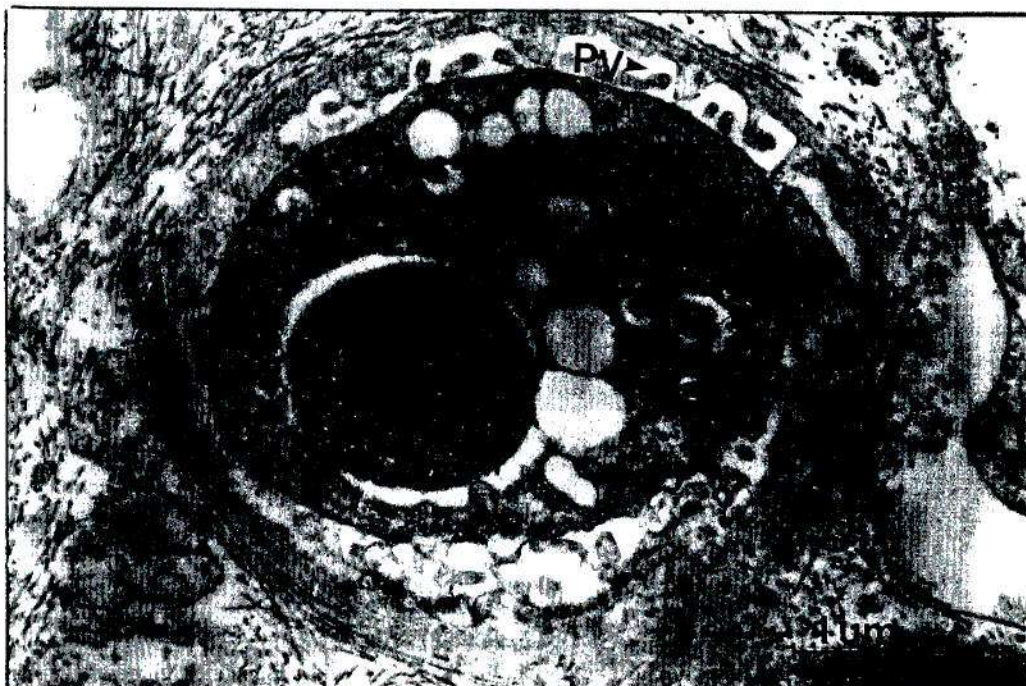
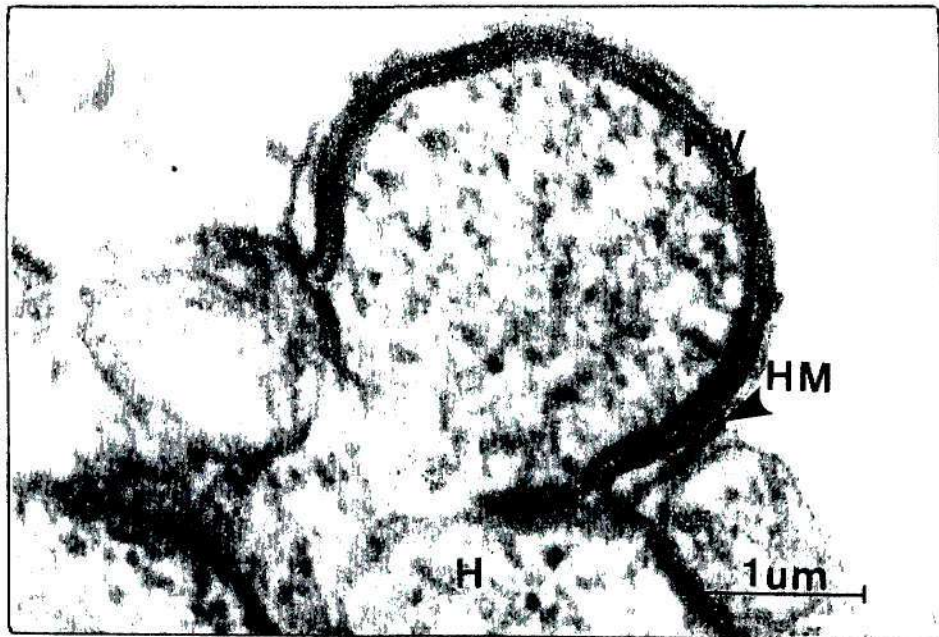
### Trophozoite

Following penetration of the sporozoite into the villous epithelium, it rounds up to form a trophozoite (Plate 20). A double membrane boundary envelops the entire trophozoite; the outer membrane being that of the host.

Cryptosporidium subsequently assumes an intracellular position, being enveloped by the host membrane, yet the parasite is extra-cytoplasmic since it is located within a parasitophorous vacuole (Plate 21). The sheath-like capsule around the parasite has not been described in the past. Other reports (Bird and Smith, 1980; Angus, 1983; Liebler et al, 1986; Current and Reese, 1986; Uni et al, 1987) were used to describe certain features observed in this study.







### Mature Meront

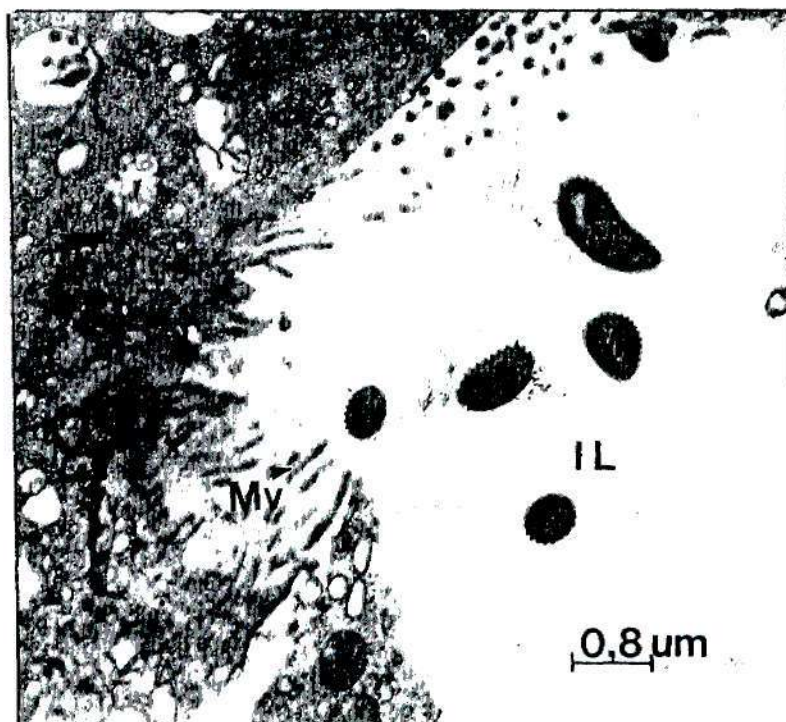
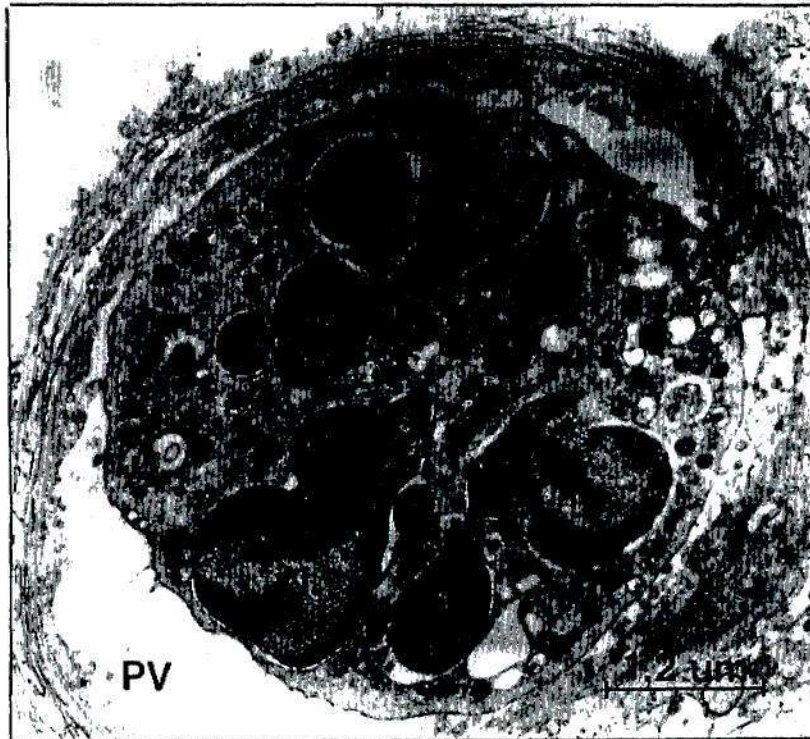
According to Vetterling et al (1971) and Bird and Smith (1980) further differentiation of the cytoplasm and multiple nuclear divisions of the trophozoite results in a mature meront. Two types (Type I and Type II) of this lifecycle stage have been described by these authors. The structure of the organism presented in Plate 22 is characteristic of only the Type I meront with more than four dividing nuclei. Each of the nuclei (usually 6 to 8) would supposedly be incorporated into a budding merozoite. Type II meronts which characteristically have four nuclei were not observed in this study.

### Merozoites

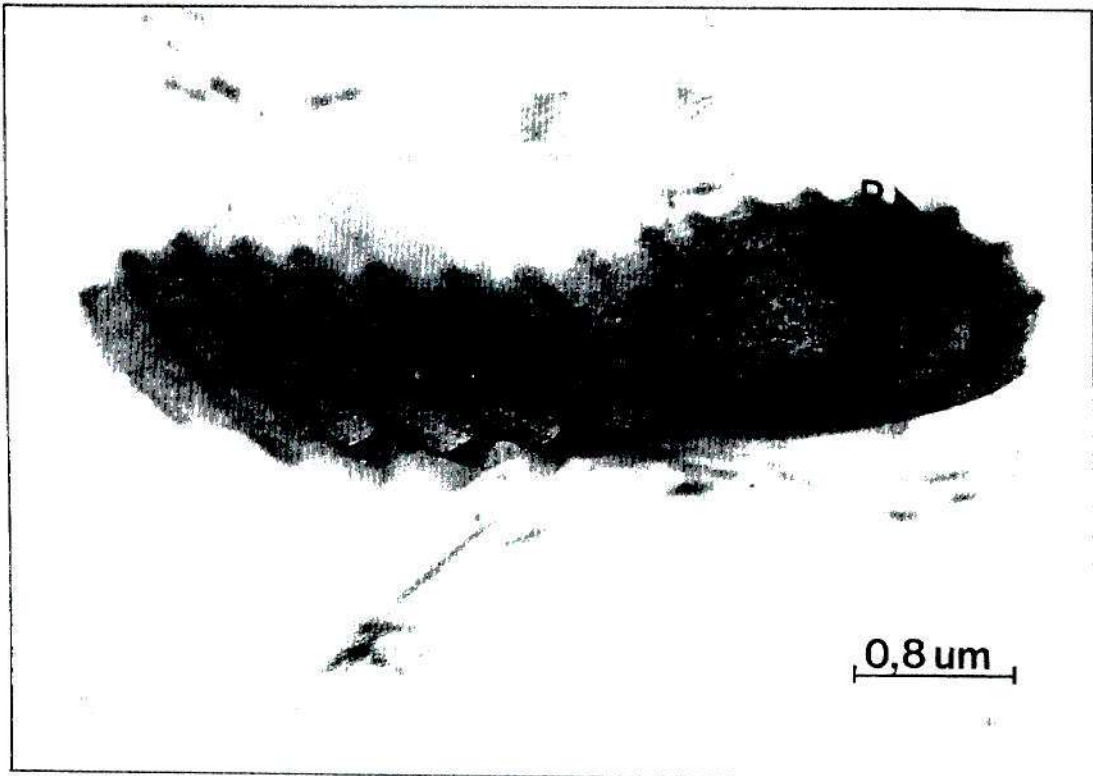
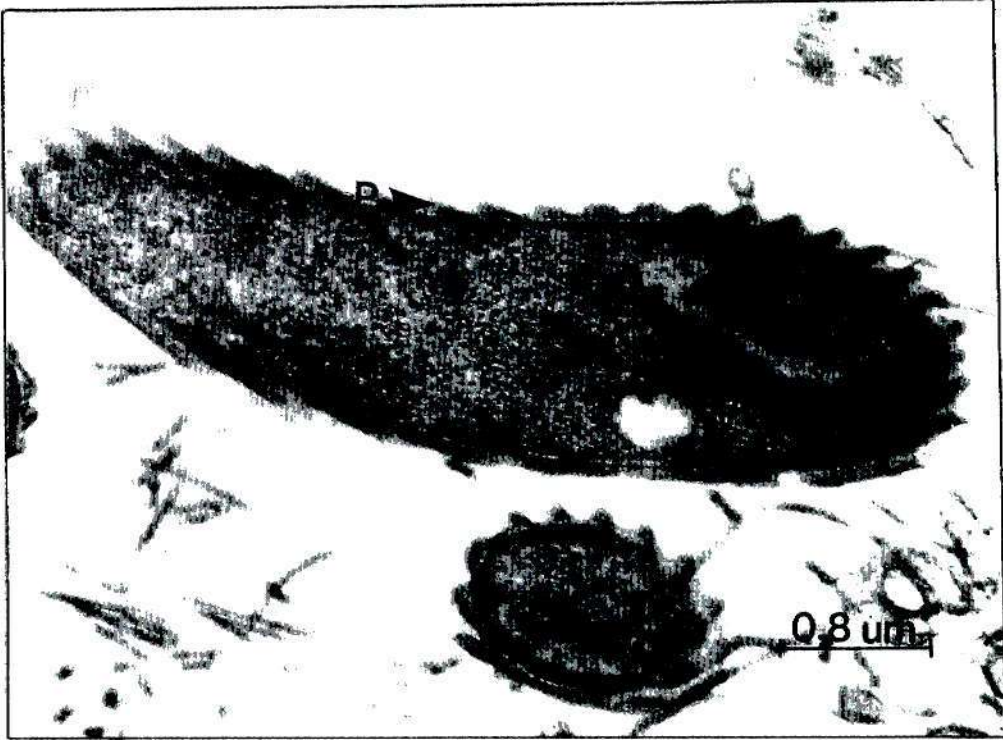
Sections of infected ileum showed numerous merozoites in close proximity to the brush border (Plate 23). Although two types of merozoites have previously been described (Current et al, 1986; Current and Reese, 1986), in the present study only Type I was observed.

The characteristic structure of the merozoite includes a prominent nucleus at the posterior end which is usually the blunt end and a granular cytoplasm with 2 to 6 vesicles adjacent to the nucleus (Plate 24). The pellicle or wall of the merozoite is modified into spiral ridges that run across the entire organism (Plate 25).









### Oocyst

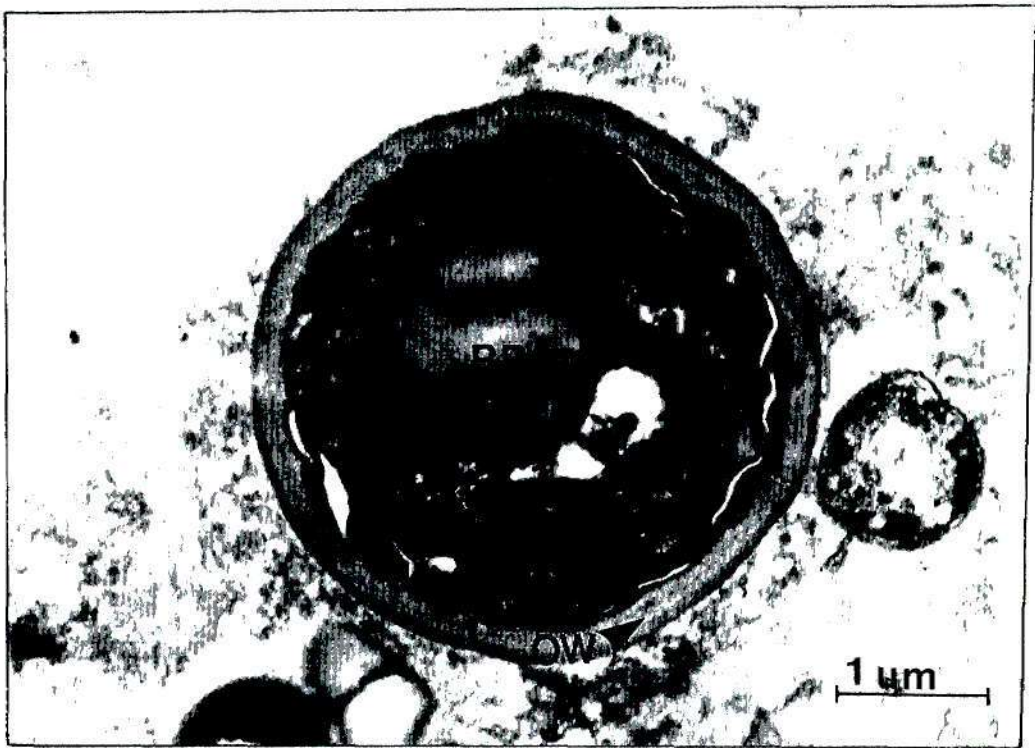
The sexual phase of the life cycle culminates in the development of the oocyst (zygote); this is the infective stage of Cryptosporidium. The zygote is enveloped by a thick wall (Plate 26), and undergoes sporulation to contain four comma-shaped sporozoites. Sporozoites are curled within the oocyst and lie over the residual body, from which they have originated. No other organelles could be seen.

The present study has highlighted three structural variations namely:

- i) Location:- On comparing the location of the trophozoite and the meront on the ileal surface of the immunosuppressed rat to that of man and other experimental hosts, trophozoites and meronts in the rat appear to be more deeply embedded in the mucosal lining.
- ii) Capsule:- Meronts in this study are encased in a sheath-like capsule, which has not been described in the past. This capsule separates the parasite from the host tissue. The parasite lies within a cavity which has been referred to







by some authors (Current and Reese, 1986; Upton and Current, 1985) as a parasitophorous vacuole, part of which can be seen in Plates 21 and 22.

iii) Merozoites:- Although this stage in the life cycle had the characteristic shape, size and internal structure to those described by most other authors, only one other study (Uni *et al*, 1987) has described the spiral ridges of the pellicle observed in this study.

#### 4.3 DISCUSSION

##### 4.3.1 Purification of Oocysts

Although isolation of Cryptosporidium oocysts from faeces using Sheather's flotation technique yields oocysts with bacteria and other debris, the method used for further purification in this study served to concentrate and decontaminate oocysts. The resultant inoculum was successfully used for culture of Cryptosporidium under aseptic conditions. Techniques using Percoll (Pharmacia, Sweden) (Current and Reese, 1986; Kilani and Sekla, 1987) are both time consuming and expensive.

#### 4.3.2 Oocyst Excystment

Oocyst excystment and sporozoite survival are two major factors for achieving successful in vitro cultivation of Cryptosporidium. Several authors have recommended two basic excystment stimulants viz. sodium taurocholate (bile salt) and trypsin (Fayer and Leek, 1984; Speer and Reduker, 1985; Woodmansee, 1986; Sundermann et al, 1987) but concentrations and incubation times vary. The process of excystment of Cryptosporidium baileyi has previously been described by Sundermann et al (1987) and does not differ much from that for Cryptosporidium parvum in the present study. Sporozoites demonstrated twisting and flexing movements similar to those reported by Reduker and Speer (1985). A further interesting observation was that storage of oocysts in potassium dichromate did not affect their viability; an observation also made by Current et al (1983). Their viability is most likely due to the robust sheath-like capsule.

#### 4.3.3 Development in Chicken Embryos

Culture of Cryptosporidium in chicken embryos failed to produce an amplification of the parasite population. Similar problems were encountered by Current and Long (1985) who reported finding only a few oocysts in the allantoic fluid. Therefore they recommended the development of techniques for the transfer of merozoites from the allantoic fluid since

merozoites could also be used to amplify parasite population.

Lindsay (1988) successfully cultured C. baileyi in different avian embryos and several passages of oocysts were possible. He indicated that oocysts excyst in allantoic fluid. However this did not occur in C. parvum in the present study; whether this observation is related to host-specificity is not known. The natural hosts of C. parvum are mammals and rodents but not birds.

#### 4.3.4 Cryptosporidium Development in Human Foetal Lung Cells

Human and calf isolates of Cryptosporidium parvum have previously been grown in foetal lung cells by Current and Haynes (1985). The present study has yielded similar results, except that fewer numbers of developmental stages of Cryptosporidium were observed in a given microscopic field at a magnification of 400x.

#### 4.3.5 Experimental Animal Cryptosporidiosis

Cryptosporidium infections have been previously described in a variety of hosts such as hamsters (Kim, 1987), mice (Liebler et al, 1986; Current and Reese, 1986), rats (Rehg et al, 1987), monkeys (Blanchard et al, 1987), chickens

(Current et al, 1986), and ferrets (Rehg et al, 1988) (natural infection). In this study, mice were chosen as experimental hosts because of their easy availability. The unsuccessful attempts in infecting suckling mice was not only unexpected but also inexplicable as laboratory mice have been reported to be susceptible hosts for Cryptosporidium (Ernest et al, 1986; Uni et al, 1987). However Bird and Smith (1980) reported similar results when they attempted to infect laboratory mice with a human isolate of Cryptosporidium. One possible explanation is that there are differences in the pathogenic capabilities of different strains isolated from various sources. Such possible strain differences could be researched further using biochemical markers such as iso-enzymes. Human isolates of Cryptosporidium oocysts reported in this study descriptively resemble C. parvum (Upton and Current, 1985; Anderson, 1987). Suckling mice, the natural hosts of C muris, may not be susceptible to this strain of C parvum; this may be an alternate explanation for the observed results. The host-specificities of the different species and strains of the parasite also needs further investigation.

It has been repeatedly documented that experimental cryptosporidial infections in small laboratory animals (rodents) do not induce diarrhoea and are not fatal

(Tzipori, 1983; Angus, 1983; Moon et al, 1985). The present study supports the above findings. However the severe villous atrophy and mucosal damage described in this study is thought to be primarily due to the cyclophosphamide treatment. In humans such epithelial damage induced by Cryptosporidium has been reported to be minimal (Bird and Smith, 1980).

While other reports have described a more superficial location of the parasite in ileal enterocytes (Vetterling et al, 1971; Moon et al, 1985; Current and Reese, 1986), in the present study Cryptosporidium was found to be more deeply embedded. The robust sheath-like capsule, enveloping the trophozoites and meronts of Cryptosporidium may be responsible for the difficulties encountered in clearing humans of this parasite. The significance of these structural variations of Cryptosporidium from those previously reported needs to be further investigated.

In the present study macro- and micro-gametes were not observed. There have been reports that early macro-gametes are indistinguishable from trophozoites, and that micro-gametes are similar to meronts; it is therefore possible that in this study these sexual stages were missed during microscopy (Vetterling et al, 1971; Upton and Current, 1985). Nevertheless, the presence of oocysts in the

intestinal lumen and faeces seven days after inoculation indicates that Cryptosporidium completed its entire life-cycle in the ileum of the immunosuppressed adult rat.



## CHAPTER 5

ISO-ENZYME ELECTROPHORESIS: A BIOCHEMICAL INVESTIGATION INTO THE POSSIBLE EXISTENCE OF STRAIN DIFFERENCES IN CRYPTOSPORIDIUM

In recent years iso-enzyme electrophoresis has proved to be an invaluable technique for distinguishing between strains of parasites such as Trypanosoma cruzi (Lanham et al, 1981) and Besnoitia besnoiti (Le Blancq et al, 1986), on the basis that their characteristic iso-enzyme patterns are effectively genetic markers. Sargeant et al (1985) have described 21 distinct zymodemes (strains characterised by their iso-enzyme pattern) of Entamoeba histolytica; in addition they could also distinguish between pathogenic (isolates from cases of clinical amoebiasis) and non-pathogenic strains (isolated from asymptomatic carriers) using this technique (Sargeant et al, 1982).

The reported findings of both the current study (Chapter 3) and of other workers (Rahaman et al, 1985; Smith and van den Ende, 1986) have confirmed that Cryptosporidium is an important pathogen in diarrhoeal disease. It is presently understood that the parasite may produce debilitating symptoms and even death in certain hosts, while an asymptomatic carrier state has frequently been observed in others (Current et al, 1983; Zar et al, 1985; Berkowitz et al, 1988). This led to the hypothesis that strain differences in terms of the pathogenicity of the parasite occur in this species.

Only one study (Mead et al, 1988b) attempted to establish whether biochemical markers could be used to identify strain differences in Cryptosporidium. Using pulse-field gel electrophoresis of DNA Mead et al (1988b) showed differences in chromosomal DNA between isolates of C. parvum and C. baileyi; however, no differences were found among different isolates of C. parvum. Detailed studies are currently being carried out by the same authors to detect antigen structure differences resulting from variations in DNA sequences.

In the current study iso-enzyme electrophoresis (a technique not previously employed in Cryptosporidium research) was employed to determine if this technique could be used for studying strain differentiation in Cryptosporidium.

## 5.1 MATERIALS AND METHODS

Faeces containing a heavy infestation of Cryptosporidium oocysts were collected from 4 children aged less than two years, who were hospitalised at the King Edward VIII Hospital with a primary diagnosis of diarrhoea.

### 5.1.1 Oocyst Purification

To ensure optimum activity of the oocysts, faeces containing them were processed for electrophoresis on the day of collection. The faeces were homogenised by vigorous shaking in an equal volume of 10% potassium hydroxide to loosen mucus

and release oocysts into the resulting suspension. This was centrifuged at 800 x g for 5 minutes and the sediment was washed once in phosphate buffered saline (PBS) (pH 7,2; 0,25M) by centrifugation at 800 x g for 5 minutes at 4°C. Oocysts were separated from bacteria and other faecal debris using a discontinuous sucrose gradient (Mead et al, 1988a). Briefly, 5ml of oocyst suspension was layered over a gradient of two 10ml sucrose layers (1,064g/ml and 1,103g/ml) made up in 0,025M PBS (pH 7,2) and centrifuged at 1000 x g for 15 minutes. The layer of oocysts from the interface was removed and washed twice in PBS by centrifugation at 800 x g for 5 minutes.

### 5.1.2 Lysate Preparation

Two methods of lysate preparation were tested to assess their suitability for use in iso-enzyme electrophoresis. These were i) sonication of oocysts; and (ii) excystment followed by repeated freeze-thawing of sporozoites.

#### (i) Sonication of Oocysts

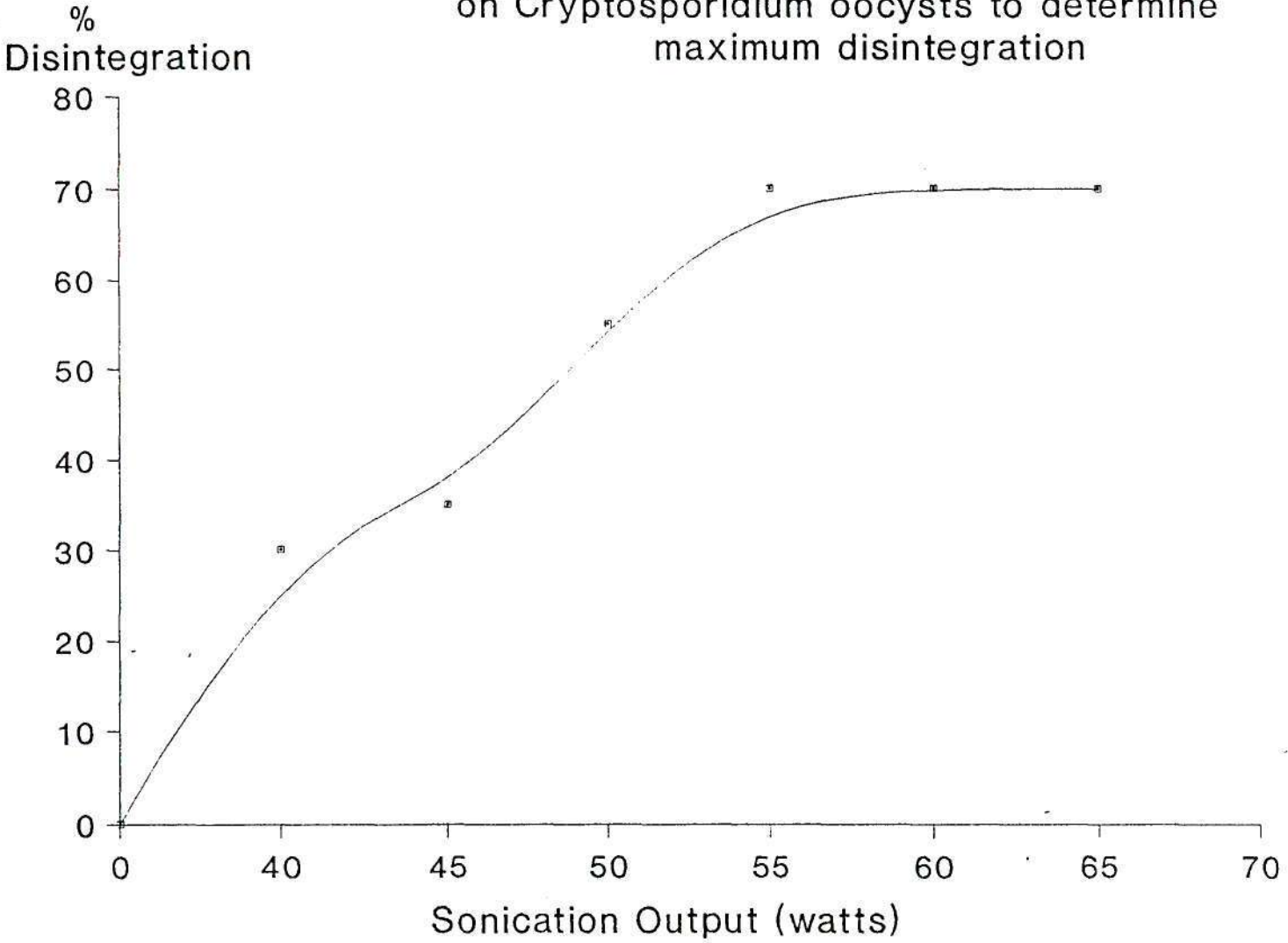
Between 500 000 and 600 000 purified oocysts were suspended in 50 ul of 1% Triton-X100 in Eppendorf tubes and maintained at 4°C on ice. In order to ascertain the optimum conditions for lysate preparation oocysts were sonicated in an Ultrasonics Disintegrator (Rapidis 350)

at various power outputs (40, 45, 50, 55, 60 and 65 watts) for a period of 30 minutes ie. total of five 2-minute pulses for each ultrasonic output with 5 minutes rest between each pulse. The sonicated oocyst suspension was then centrifuged in a microfuge at 1000 x g for 5 minutes. The resulting supernatant was examined microscopically to determine the ultrasonic output producing optimum disruption of oocysts (Fig 3). This lysate was stored at -20°C for subsequent use in electrophoresis.

(ii) Excystation and Freeze-thawing of Sporozoites

Between 500 000 and 600 000 purified oocysts were suspended in a bile salt-trypsin mixture (as described in Chapter 4) and excysted at 37°C for 1 hour. Viable sporozoites were washed once in PBS by centrifugation at 900 x g for 5 minutes at 4°C, resuspended in 50 ul of 1% Triton-X 100 in Eppendorf tubes and repeatedly freeze-thawed (at least 5 times) using liquid nitrogen until 60% disintegration of sporozoites resulted. The extent of disintegration of the sporozoites was assessed by viewing a drop of the suspension under a microscope at 100 x magnification after each step in the freeze-thaw cycle.

Fig 3. Assessment of various sonication outputs on *Cryptosporidium* oocysts to determine maximum disintegration



### 5.1.3 Electrophoresis

Electrophoresis was carried out on a system essentially similar to that described by Wraxall and Culliford (1968).

- (i) 1% agarose gel replaced starch gel and was prepared by melting agarose (Type L, Behring), in a round bottom flask over a flame, in 0,08M Tris phosphate, 0,002M EDTA (TPE) buffer (pH 8,0).
- (ii) The molten gel was poured onto framed glass plates (135 x 210 x 2 mm) and allowed to cool.
- (iii) Lysates were absorbed into 5mm lengths of crochet cotton and embedded into slots that had been pre-cut into the gel.
- (iv) The following enzyme systems were assessed: malic enzyme (ME, E.C. 1.1.1.40), glucosephosphate isomerase (GPI, E.C. 5.3.1.9), phospho-glucose dehydrogenase (PGD, E.C. 1.1.1.44), hexokinase (HK, E.C. 2.7.1.1) and lactate dehydrogenase (LDH, E.C. 1.1.1.27).

(v) Precooled TPE buffer was used as electrode buffers for all five enzymes and it was maintained at 11°C. A total voltage of 4,1 V/cm was applied for 90 minutes.

Iso-enzyme electrophoretic patterns were developed by means of formazan reaction. The following staining solutions were employed:-

GPI = 14ml Tris-chloride buffer (pH 8,0) + 1ml NADP + 0,4ml Fructose-6-phosphate (F<sub>6</sub>P) + 1ml Glucose-6-phosphate dehydrogenase (G<sub>6</sub>PD) + 0,4ml MgCl<sub>2</sub> + 0,01g/ml 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium Bromide (MTT) + 0,01g/ml Phenazine methosulfate (PMS)

ME = 14ml Tris-chloride buffer (pH 7,4) + 1ml NADP + 0,2ml malate + 0,4ml MgCl<sub>2</sub> + 0,01g/ml MTT + 0,01g/ml PMS

PGD = 50ml Tris-chloride buffer (pH 8,0) + 0.01g glucose-6-phosphate + 0,2ml MgCl<sub>2</sub> + 1ml NADP + 0,01g/ml MTT + 0,01g/ml PMS

LDH = 25ml Tris-chloride buffer (pH 7,4) + 1ml NADP + 0,02g lactose-6-phosphate + 0,4ml MgCl<sub>2</sub> + 0,01g/ml MTT + 0,01g/ml PMS.

HK =14ml Tris-chloride buffer (pH 8,0) + 1ml NADP + 0,4mg MgCl<sub>2</sub> + 0,01g/ml MTT + 0,01g/ml PMS.

## 5.2 RESULTS

A series of pilot studies were performed to establish the optimised conditions described above. Microscopy of lysates was considered in conjunction with electrophoresis and these observations indicated that the sonicated oocyst preparation was superior to the lysates made by freeze-thawing. Although the latter method resulted in 60% disintegration of sporozoites, no isoenzyme bands developed after electrophoresis.

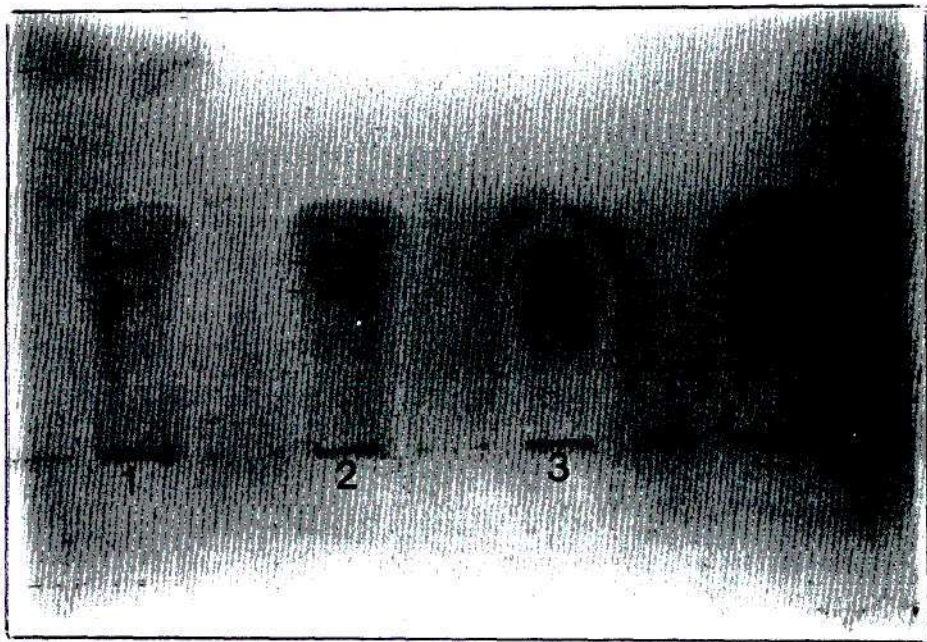
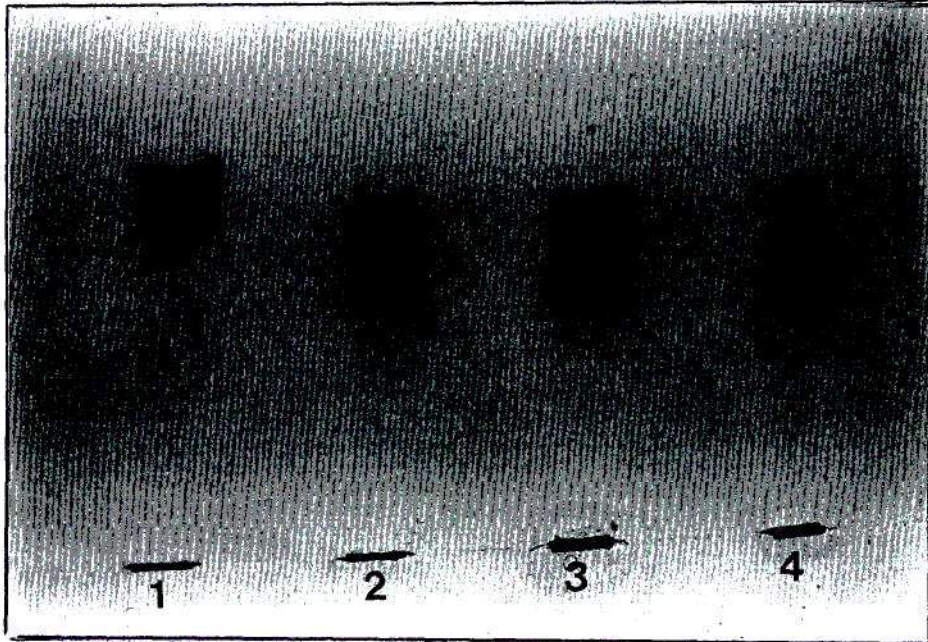
Furthermore, the running conditions for the electrophoresis were also varied in terms of voltage applied, temperature and duration of electrophoresis. The most satisfactory conditions for all five enzyme systems studied was voltage 4,1 V/cm, temperature 11°C and time 90 minutes.

Of the five enzyme systems, electrophoretic bands of the four lysates tested (see materials and methods) were only reproducibly observed for GPI, PGD and ME. Despite repeated attempts LDH and HK bands could not be developed in these experiments even though control material (eg. *E histolytica*) proved that the staining techniques were fully operational.

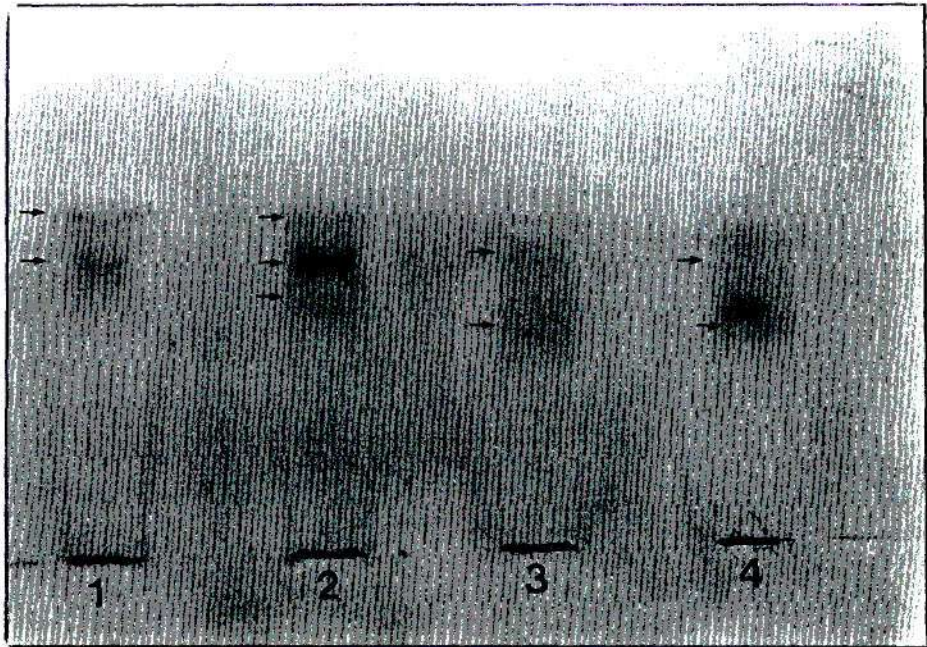
Two diffuse GPI bands were common to all except one of the four isolates (Sample 1, Plate 27). Samples 3 and 4 were identical for all three enzymes investigated while Samples 1 and 2











differed from each other and also from Samples 3 and 4 (Plates 27, 28 and 29).

### 3. DISCUSSION

The method of lysate preparation was found to be important. From this study, it is apparent that temperature changes during lysate preparation need to be carefully controlled. Increases in temperature produced during sonication can result in total protein destruction. Therefore sonication at low temperature (4°C) using a medium power output (55 watts) together with ample resting time (5 minutes) between pulses was found to be essential for lysate preparation.

Cultured Cryptosporidium may be a better source of material for experimental investigations such as electrophoresis as there is no bacterial contamination. Further work aimed at improving the culture techniques for the production of appropriate quantities of pure parasite material for lysate preparation is indicated as this should enhance the quality of the iso-enzyme electrophoresis.

Although iso-enzyme electrophoresis is a commonly used technique and is employed in the Institute (RIDTE), in which this work was performed, for studying E histolytica and mosquitoes, it was

necessary to establish modified methods for the isoenzyme electrophoresis of Cryptosporidium. Efforts were targetted at developing reproducible methods for isoenzyme electrophoresis of Cryptosporidium rather than a study of strain differences based on isoenzyme staining patterns; such a study is considered to be potentially valuable but was beyond the scope of the present work.

In this limited study, using agarose-gel electrophoresis, isoenzyme polymorphism was demonstrated in GPI, ME and PGD, and 3 different polymorphic forms of Cryptosporidium were observed. It is likely that many polymorphic forms of Cryptosporidium would be identified in a more extensive study particularly if other enzyme systems were included in the analysis. An assessment of the relationship between these electrophoretic patterns and pathogenic potential should be undertaken using isolates of Cryptosporidium derived both from asymptomatic carriers and subjects with a clinical disease.

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## APPENDIX

## 1. Sheather's Solution

500g sucrose  
6,5g phenol  
320ml distilled water

## 2. Phosphate Buffered saline(PBS) (pH 7,2; 0,25M)

85g NaCl  
1,07g Na<sub>2</sub>HPO<sub>4</sub> (anhydrous)  
0,39g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O  
1l distilled water

## 3. Excystment Medium (pH 7,5)

5ml PBS  
0,5% Trypsin  
1,5% Sodium Taurocholate

## 4. Eagles' Minimum Essential Medium

Salts  
Essential Amino acids  
Vitamins



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